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Critical evaluation of CROSERF test methods for oil dispersant toxicity testing under subarctic conditions

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Abstract

The aquatic organism toxicity testing protocols developed by the Chemical Response to Oil Spills: Ecological Research Forum (CROSERF) were evaluated for applicability to assessing chemical dispersant toxicity under subarctic conditions. CROSERF participants developed aquatic toxicity testing protocols with the foremost objective of standardizing test methods and reducing inter-laboratory variability. A number of refinements are recommended to adapt the CROSERF protocols for testing with subarctic species under conditions of expected longer oil persistence. Recommended refinements of the CROSERF protocols include testing fresh and moderately weathered oil under conditions of moderate mixing energy, preparing toxicity test solutions using variable dilutions rather than variable loading, performing tests with subarctic species using static exposures in open chambers, increasing the duration of tests from 4 to 7 days, quantifying approximately 40 PAHs and their alkyl homologs, assessing the potential for photoenhanced toxicity, and incorporating a bioaccumulation endpoint by measuring tissue concentrations of PAHs. Refinements in the preparation of oil dosing solutions, exposure and light regimes, and analytical chemistry should increase the utility of the test results for interpreting the toxicity of chemically dispersed oil and making risk management decisions regarding dispersant use under subarctic conditions.
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1. Introduction

Research performed to date has not revealed a consistent trend in the relative toxicity of chemically dispersed and physically dispersed oil, with toxicity dependent on the specific dispersant and oil evaluated, the test organism, and the oil exposure regime (e.g., Anderson, 1985; Fucik et al., 1995; Singer et al., 1999; Clark et al., 2001; Fingas, 2002). It is well established that chemically dispersed oil is substantially more toxic than dispersant treatment alone for current generation chemical dispersants such as Corexit 9500 and 9527 (ExxonMobil, Houston, TX). There is no clear trend in the toxicity of chemically dispersed fresh oil compared to chemically dispersed weathered oil (Anderson, 1985; Singer et al., 1999; Clark et al., 2001; Rhoton et al., 2001). With only a few exceptions (Anderson, 1985; Rhoton et al., 2001; Barron et al., 2003), however, comparisons of the toxicity of chemical dispersions of fresh and weathered oil have not been performed with species and test conditions that occur in subarctic marine environments. As discussed below, dispersant efficacy and toxicity is dependent on temperature, salinity, mixing energy, dispersant application, and test species/toxicity endpoint (Fingas, 2002). Thus any laboratory toxicity test results must be viewed relative to the conditions used to prepare and expose aquatic organisms to WAFs of dispersed oil. Currently, the Unified Plan for Alaska (ARRT, 1999) allows dispersant use only when conventional response activities (e.g., mechanical recovery) are not feasible or are likely to be inadequate in containing an oil spill. Decisions concerning dispersant use must be based on an evaluation of the potential impacts of the chemically dispersed oil versus not dispersing the oil (ARRT, 1999), including the toxicity to aquatic organisms.

The CROSERF is a working group that was established to coordinate and disseminate research on

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Nomenclature

Acronyms

ANS	Alaska North Slope crude oil
CROSERF	Chemical Response to Oil Spills: Ecological Research Forum
LC50	median lethal concentration
MAHs	monoaromatic hydrocarbons
PAHs	polycyclic aromatic hydrocarbons
TPH	total petroleum hydrocarbons
UV	ultraviolet radiation
WAF	water accommodated fraction of oil

chemical oil spill dispersants. CROSERF participants included researchers at several universities, petroleum companies, and government agencies. The CROSERF working group developed aquatic toxicity testing protocols during 1994–2000 with the foremost objective of standardizing test methods and reducing inter-laboratory variability. CROSERF toxicity test methods and protocols have been published in various scientific and technical reports, with the most recent compilation presented in the proceedings of the 2001 International Oil Spill Symposium (e.g., Clark et al., 2001; Singer et al., 2001a,b; Rhoton et al., 2001). The protocols include standardized methods for preparing WAF of physically dispersed and chemically dispersed oil products and weathered oil. The test protocols also include methods for exposing early life stages of aquatic organisms to dispersed oil, with the objective of standardizing as many test parameters as possible.

This paper critically evaluates CROSERF toxicity test methods for their applicability to assessing the toxicity of chemically dispersed oil under subarctic conditions. Whereas CROSERF participants have reported extensively on the toxicity test methods and results, an independent critical assessment of the test protocols has not been presented. Refinements in the CROSERF toxicity testing protocols are recommended, rather than developing a subarctic dispersed oil testing program ffrom scratchff This recommendation is made with the recognition of the considerable effort and thought invested in the existing CROSERF protocols. Recommendations were focused on providing toxicity test data most relevant to risk management decisions regarding dispersant use in subarctic environments, rather than the primary CROSERF objective of standardizing procedures. While focused on Alaska relevant test conditions and species, the recommendations for altering the CROSERF WAF preparation and testing protocols should be considered for chemical dispersant testing in general.

2. Overview of CROSERF procedures

The CROSERF toxicity testing protocols were established to standardize WAF preparation, laboratory exposures to aquatic organisms, and analytical chemistry measurements used to determine the acute toxicity of physically dispersed and chemically dispersed oil. The procedures were adapted from early fslow stirff WAF preparation methods (e.g., Anderson, 1985) and standardized toxicity testing protocols from the US Environmental Protection Agency and American Society for Testing and Materials. The typical CROSERF WAF preparation includes an initial 0.45 lm seawater filtration, a geometric series of increasing oil to water loading rates, and 18 h of slow stirring of each water–oil mixture in a sealed carboy with a 20–25% headspace. The oil–water mixture is allowed to settle for 6 h then the aqueous phase WAF is used in 4 days acute toxicity tests. The WAF of chemically dispersed oil is prepared at a moderate mixing energy using a 1:10 dispersant to oil ratio.

CROSERF participants have standardized methods for conducting static renewal exposures with aquatic organisms, but recommend a short duration flow-through testing regime in airtight test vessels. The CROSERF objective of the flow-through test regime was to characterize toxicity from exposure to a decreasing concentration that was considered most representative of environmental mixing and dilution during an oil spill. Airtight test chambers were developed to reduce losses of volatile organic compounds such as MAHs. CROSERF test endpoints have included mortality or impaired mobility of a variety of standard Gulf of Mexico and Pacific Coast species after 4 days from initiation of oil exposure. CROSERF recommendations also include analytical verification of toxicity tests solutions by measuring specific aqueous phase volatile organic compounds and TPHs.

3. Recommended refinements to CROSERF procedures

The toxicity of chemically dispersed oil to aquatic organisms is a critical consideration in the evaluation of chemical dispersant use in oil spill response. An independent critical assessment of the CROSERF toxicity test protocols was performed because the data generated from these methods may be used by risk managers in assessing the potential impacts of chemically dispersed

oil. Also, the applicability of CROSERF test methods to subarctic conditions was evaluated because of the limited available toxicity data on chemically dispersed oil to subarctic species, and the likelihood of additional testing in the future. A number of refinements are recommended to adapt the protocols for testing with subarctic species under conditions of expected longer oil persistence. Refinements are recommended in WAF preparation, exposure and light regimes, and analytical chemistry to increase the utility of the test results for making risk management decisions regarding the toxicity of chemically dispersed oil and dispersant use under subarctic conditions. Table 1 summarizes CROSERF WAF preparation and testing procedures and recommended changes based on this evaluation.

3.1. Oil weathering

The most important determinant of dispersant efficacy is oil composition, which is directly affected by weathering (Fingas, 2002), and can occur at a substantially slower rate under subarctic conditions than in temperate environments (Payne et al., 1991). Based on the method for quantifying petroleum exposure, weathered oil has been reported as similar, more toxic, or less toxic than fresh oil in CROSERF reports (e.g., Clark et al., 2001; Rhoton et al., 2001; Singer et al., 2001a). Testing of both fresh and a moderately weathered ANS is recommended to evaluate the range of possible oil weathering that may be encountered under possible dispersant use scenarios. Singer et al. (2001a) used an oil weathering procedure of distillation (topping) to a vapor phase temperature of 200 ffC under controlled conditions in the laboratory to simulate evaporative weathering. This topping procedure is equivalent to 0.5–1 day under temperate conditions (Neff et al., 2000), and thus represents a reasonable ffworst caseffscenario for the degree of oil weathering that may occur prior to dispersant use under subarctic conditions.

3.2. Chemical dispersant application and mixing energy

Dispersant efficacy can be dependent on the type of chemical dispersant used, and efficacy generally increases with increasing dispersant application and increasing energy used to mix the oil, dispersant, and water (Fingas, 2002). Dispersants are known to increase the entrainment and dissolution of oil components into the water column, and increase the volume of water that is impacted during a spill (French McCay and Payne, 2001). The reported CROSERF tests have used a 1:10 dispersant to oil ratio (e.g., Clark et al., 2001; Rhoton et al., 2001; Singer et al., 2001b), which represents the maximum likely application of a dispersant. Field applications of dispersants are likely to be lower (e.g., 1:20 or 1:50; Davies et al., 1998) but toxicity testing of a high

Table 1
CROSERF toxicity test procedures and recommended refinements

Parameter	CROSERF procedure[a]	Recommendations
Oil weathering	Fresh Weathered (20%)[b]	Fresh Weathered (20%)
Oil loading	Variable (e.g., 0.01–25 g/l)	25 g/l
Dispersant (dispersant:oil)	Corexit 9500 and/or 9527 (1:10)	Test both Corexit 9500 and 9527 (1:10)
Mixing energy/time	18 h moderate energy (Slow stir WAF[c])	Same energy for physically and chemically dispersed oil
Test concentrations	Variable loading	Variable dilutions
Oil exposure regime	4 d static-renewal; open chamber 6 8 h flow-through; airtight chamber	Static: open chamber 8 h and 7 days
Analytical chemistry[d]	TPH + VOCs (GC/FID)	TPH (GC/FID or GC/MS) PAHs (GC/MS SIM)
Species/life stage	Temperate species[e]	Calanoid copepod Subarctic sea urchin larvae Pacific herring larvae
Temperature; salinity	6–8 ffC; 31.5 & (Tanner crab)	10 ffC; 15 & 3 ffC; 32 &
Light regime	Laboratory (fluorescent)	Incorporate limited UV
Toxicity endpoint	Impaired mobility Death	Also delayed impairment/death
Bioaccumulation	Not measured	Tissue PAHs (GC/MS SIM) at 1 and 7 days in copepods

[a] Source: ADEC (2000), Singer et al. (2000); Clark et al. (2001), Rhoton et al. (2001), Singer et al. (2001a).
[b] Distillation to 200 ffC (Singer et al., 2001b).
[c] Approximately 18 h mix time, 20–25% vortex. Use Clark et al. (2001) modification of standard CROSERF mixing energy protocol for physically dispersed oil, followed by 6 h settling time.
[d] TPH: total petroleum hydrocarbons; VOC: volatile organic compounds (<10 carbon alkanes and MAHs); PAHs: polycyclic aromatic hydrocarbons; GC: gas chromatography; FID: flame ionization detection; MS: mass spectrometry; SIM: single ion mode.
[e] ADEC (2000) recommended topsmelt (a non-Alaskan fish) and purple sea urchin for subarctic testing.

dispersant:oil ratio ensures maximum dispersion of oil under the laboratory test conditions. The toxicity of chemically dispersed oil is expected to be similar to physically dispersed oil in cases of poor dispersant efficacy. Consistent with the CROSERF approach, a 1:10 dispersant:oil ratio is recommended for assessing dispersant toxicity under subarctic conditions because it provides the greatest potential to detect differences in the toxicity of chemically dispersed and physically dispersed oil. Only oil dispersed with Corexit 9500 has been tested for toxicity under subarctic conditions using CROSERF protocols (Rhoton et al., 2001), and Barron et al. (2003) tested Corexit 9527 with high mixing energy mixing and other non-CROSERF test conditions. Testing of both Corexit 9500 and 9527 is recommended because both are available for use in Alaska.

CROSERF protocols use moderate mixing energy (e.g., 20–25% vortex) in preparing chemically dispersed WAF, but differ by investigator regarding the mixing energy of the physically dispersed oil. Singer et al. (2001b) recommend low mixing energy (<5% vortex) for physically dispersed oil to limit entrainment of particulate oil greater than 1 lm, but acknowledged that the use of WAF preparations of differing energy results in less comparable results. Blenkinsopp et al. (1996) evaluated the repeatability of mixing procedures and also recommended a minimal vortex to ensure reproducibility in the chemical composition of WAF.

In contrast, the same moderate mixing energy for producing physically and chemically dispersed oil was used by Clark et al. (2001) because it was considered to be more representative of environmental conditions during an oil spill. The Clark et al. (2001) CROSERF procedure of the same moderate mixing energy (20–25% vortex) in preparing WAF of both physically dispersed and chemically dispersed oil is recommended. We also agree with the CROSERF procedure of an 18 h mixing time followed by a 6 h settling time for WAF of both physically dispersed and chemically dispersed oil. Singer et al. (2000) considered a 20–25% vortex to be representative of field conditions that did not produce identified dispersion or emulsification of Prudhoe Bay crude.

3.3. Water accommodated fractions of oil

Two general approaches are available for producing a series of toxicity test solutions of decreasing concentration of either chemically or physically dispersed oil: variable loading and variable dilution (Fig. 1). Variable loading uses a series of decreasing concentrations of applied oil, whereas variable dilution using the same oil to water ratio and a series of increasing water dilutions. In both procedures, a series of decreasing concentrations of WAF is generated and tested to define the toxicity of the dispersed oil. Also, in both procedures the recommended dispersant:oil ratio of 1:10 is maintained in the chemically dispersed WAF.

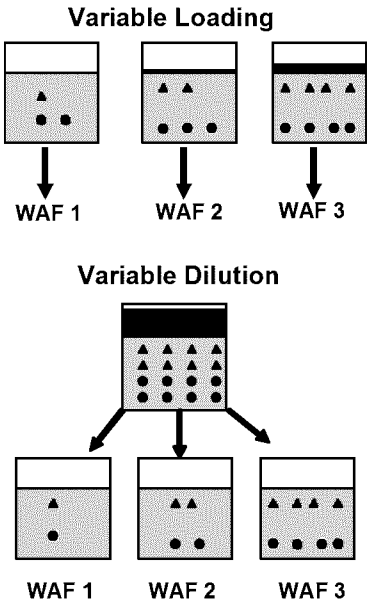

Fig. 1. Illustration of variable loading and variable dilution approaches for preparing water accommodated fractions (WAF) of oil (black fill) and water (grey fill). Triangles and circles represent different concentrations of aqueous phase petroleum compounds.

Singer et al. (2001b) have argued for using variable loading in the CROSERF test protocols because of the more realistic oil:water ratios and the approach allows the loading rate to be determined (e.g., 0.01–10 g oil per l). One concern is that the variable loading approach results in compositional changes in the aqueous phase oil (Fig. 1). This may be problematic in interpreting and comparing the toxicity of different oil products because the estimated measure of toxicity (e.g., median lethal concentration; LC50) is confounded with changes in both concentration and potency, as well as the applied loading rate. For example, Rhoton et al. (2001) and Singer et al. (2000) have shown that the proportion of MAHs relative to total aqueous phase petroleum concentrations increases with increasing oil loading rate. Blenkinsopp et al. (1996) recommended against serial dilution because it underestimated concentrations of some MAHs, but this recommendation was made in the context of extrapolating WAF toxicity to a specified loading rate. Clark et al. (2001) has stated that while loading rate has merit, it was not a relevant measure of exposure for interpreting the toxicity of chemically dispersed oil. Also, loading rate did not correlate well with the toxicity of chemically dispersed oil in the Clark et al. (2001) CROSERF tests.

Although the merits of WAF preparation procedures are a matter of scientific debate, a variable dilution approach is recommended for preparing WAF for dispersed oil testing. The variable dilution will provide a

consistent chemical composition in a test series for each oil–dispersant combination and provides some economies in analytical costs by reducing the need to analyze the composition of every tested concentration. The variable dilution approach will also provide greater comparability of the toxicity of chemically and physically dispersed oil by standardizing the oil:water ratio used in preparing WAF. Various CROSERF tests have used a maximum of 25 g oil per l of water, whereas Blenkinsopp et al. (1996) have recommended a maximum loading rate of 10 g/l. To ensure that test concentrations exceed median lethal concentrations for physically dispersed oil, a maximum loading rate of 25 g/l is recommended for testing under subarctic conditions. This loading is equivalent to a 1:40 oil to water ratio, which is substantially lower than typical non-CROSERF WAF preparations (1:10 ratio) used to make serial dilutions (e.g., Anderson, 1985; Barron et al., 1999). Other CROSERF procedures for preparing WAF are considered appropriate, including preparing WAF in the dark in sealed containers using a fixed head space to water volume of approximately 20% (Singer et al., 2000).

3.4. Analytical chemistry

CROSERF protocols recommend measuring TPH and specific volatile organic compounds, which are comprised of some MAHs and alkanes with 6–9 carbons. PAHs are not specifically recommended, although Singer et al. (2000) identified PAHs as optional analytes in CROSERF testing. PAHs are considered to be determinants of both the acute and chronic toxicity of spilled oil, and are standard analytes used to assess the source, persistence, and toxicity of spilled oil (e.g., Neff and Burns, 1996; French McCay, 2002). We recommend measuring the standard suite of approximately 40 PAHs including the alkyl homologs (e.g., Short and Harris, 1996), as well as TPH because it quantifies total petroleum exposure and is particularly relevant in low PAH oils (Barron et al., 1999).

TPH and PAH concentrations should be measured at test initiation and test end to quantify average exposure concentrations. Additionally, PAHs should be quantified in tissues of selected test species to quantify bioaccumulation potential and hydrocarbon persistence (see below). Measurement of MAHs should be considered optional because, as discussed below, they are not persistent in spilled oil and are no longer routinely measured during oil spill events. Evaporative losses of volatiles during oils spills can be extensive, thus a focus on measurements of specific volatile organic compounds is not merited. For example in experimental slicks in the North Sea, ANS weathered for 5 h had an approximately 30% loss of volume from evaporation, and a lighter oil (Forties blend crude oil) lost approximate 30% volume within 2 h primarily via evaporation (Davies et al., 1998).

3.5. Test species and life stage

A variety of aquatic organisms have been tested using CROSERF protocols, with most reports on the early life stages of temperate species (e.g., Gulf of Mexico and Pacific crustaceans and fish). Only one CROSERF investigation (Rhoton et al., 2001; ADEC, 2000) has evaluated the toxicity of a subarctic species. That study determined that tanner crab larvae were more sensitive to ANS than a standard Gulf of Mexico fish species (Menidia berylina), and had similar sensitivity as a standard mysid shrimp test species. ADEC (2000) noted that most Alaskan marine species were either (1) not commercially available and thus required field collections, (2) of large size and not conducive to CROSERF recommended test chambers, and/or (3) could only be obtained for a relatively limited time each year. Because of these concerns, ADEC (2000) recommended testing the topsmelt (a non-Alaskan fish) and a species of Alaskan echinoderm (purple or green sea urchins), or developing cultures of Alaskan mysids or copepods.

A variety of subarctic marine species have been evaluated for the sensitivity to ANS or Prudhoe Bay crude oil under non-CROSERF test conditions including salmon, flounder, amphipods, crabs, shrimp, shellfish, copepods, herring, and sand lance (Rice et al., 1976; Anderson, 1985; Moles, 1998; Duesterloh et al., 2002; Barron et al., 2003). Based on available information and experience, three Alaskan species are recommended for evaluating dispersed oil toxicity under subarctic conditions: a calanoid copepod, a sea urchin species, and larval Pacific herring. These species are known to be sensitive to petroleum, are important ecologically and/or economically, and can be maintained under the static test conditions recommended below (EPA, 1995; Duesterloh et al., 2002; Barron et al., 2003).

Ecological relevance and known sensitivity should outweigh logistical concerns with collection and rearing, as long as high culture and control survival can be achieved. Collection and testing have been previously demonstrated for both herring and copepods (e.g., Carls et al., 1999; Duesterloh et al., 2002; Barron et al., 2003), and standardized tests with purple sea urchins have been developed (EPA, 1995). Herring were considered an excellent candidate test species in ADEC (2000), with the caveat that they are only available seasonally. Herring larvae are recommended over herring embryos because of similar sensitivity, and more rapid bioaccumulation and toxicity responses. As noted by ADEC (2000), sea urchins occur in Alaska, are sensitive to oil, and an Alaskan species may be more available than alternative subarctic species. A calanoid copepod is also recommended because of the capacity of copepods such

as Calanus marshallae to bioaccumulate petroleum compounds (Duesterloh et al., 2002), making them ideal for assessing and comparing the bioavailability of chemically and physically dispersed oil.

3.6. Oil exposure regime

The oil exposure regime in a dispersed oil toxicity test must be balanced between environmental realism (i.e., persistence of spilled oil in the environment), concerns for chronic toxicity (i.e., potential for sublethal and/or delayed effects), and the practicability and costs of testing. Two CROSERF protocols are available, each with a total duration of four days (e.g., Singer et al., 2001a,b): static-renewal exposure in open test chambers and flow-through exposures in airtight test chambers. The static-renewal exposure consists of the addition of newly prepared WAF to an open glass test chamber every 24 h; the chamber is aerated as necessary to maintain dissolved oxygen levels. The static-renewal regime results in an initial spike then declining concentrations of oil every 24 h, which CROSERF participants conclude will overestimate the potential environmental toxicity of oil. The oil exposure regime considered most representative of oil toxicity by CROSERF participants is a flow-through exposure with clean seawater that results in dilution of the initial WAF to non-detectable levels within 8 h (half-life *ff*2 h; Clark et al., 2001). Various CROSERF reports state that the duration of oil exposure in the flow-through regime is most representative of the persistence of spilled oil in the environment, but quantitative support and literature citations for this assertion are generally not provided in the available reports (e.g., Singer et al., 1999, 2000, 2001a,b; Clark et al., 2001; Rhoton et al., 2001). Singer et al. (1991) noted that dispersant concentrations in field experiments declined to non-detected concentrations "within a matter of hours". ARRT (1999) noted that marine field experiments have indicated disappearance of dispersed oil in 5–12 h. However, Fingas (2002) has recently highlighted the need for caution in interpreting previous field experiments with dispersants because of the concern for inadequate tracking and sampling of the dispersed phase plume, which can have a different trajectory than the oil slick.

The short duration of the CROSERF flow-through exposure protocol requires reassessment for its relevancy to subarctic conditions where evaporative losses are expected to be substantially slower than in temperate environments (e.g., Payne et al., 1991). For example, aqueous phase oil was present in some intertidal areas during the Exxon Valdez oil spill for weeks rather than hours. Total PAH concentrations in the water column of Snug Harbor, Herring Bay, and Northwest Bay (Knight Island and Eleanor Island, Alaska) ranged between 1 and 10 lg/l for 2 to over 5 weeks after the spill (Neff and Burns, 1996; Short and Harris, 1996). Large spills in temperate environments have also persisted for days rather than hours (French, 1998), although this is considered to be less likely. For example, a toxic plume of oil persisted in the North Cape, Rhode Island fuel oil spill for multiple days (>10 lg/l aqueous phase PAHs for over 6 days; French, 1998). Additionally, aqueous phase petroleum is expected to be generally more persistent when spilled oil is chemically dispersed than when it is only physically dispersed (French McCay and Payne, 2001). In conclusion, the CROSERF flow-through protocol may be representative of most spills in temperate environments, but they do not appear to adequately represent the spills of greatest concern in subarctic environments.

An additional concern is the CROSERF protocol of using airtight chambers in the flow-through exposure regime. These test chambers were designed to minimize test variability, but artificially elevate concentrations of MAHs (e.g., Rhoton et al., 2001). Wave tank experiments and Exxon Valdez oil spill measurements indicate the majority of MAHs are lost within the first 24 to 48 h of a spill of ANS (Payne et al., 1991; Wolfe et al., 1994). As previously noted, North Sea field trials have shown rapid and extensive evaporative losses of volatile compounds in both ANS and a lighter crude oil (Davies et al., 1998). Use of airtight chambers without the prescribed CROSERF flow-through dilution would likely result in persistent MAH exposure and thus are not recommended.

An alternative oil exposure regime consisting of a static exposure in open chambers is recommended over the two CROSERF test protocols. A static exposure regime provides an intermediate duration of oil exposure because test concentrations are not renewed, but they are not diluted. Persistence of oil in the static test chambers will be determined at least in part by the properties of the oil (solubility, Henryffs law constant) and test conditions (e.g., salinity and temperature representative of subarctic environments), rather than manipulated dilution or renewal regimes. Testing of each species under two test durations is recommended to encompass a range of environmental exposures to dispersed oil: 8 h and 7 days. Although longer oil exposures are plausible, 7 days is considered sufficient to detect delayed effects, hydrocarbon concentrations will have declined to non-detectable levels within 7 days, and longer exposures may lead to artifacts caused by declining organism health in the test system unrelated to oil exposure. Anderson (1985) has previously noted the importance of a latency period in determining the toxicity of chemically dispersed oil to Pacific herring larvae. Both calanoid copepods and Pacific herring larvae have been shown to have high control survival under static conditions and water quality was not impaired (Duesterloh et al., 2002; Barron et al., 2003). Mortality should

also be monitored daily to allow reporting of LC50s at standard reporting times of 2 and 4 days. If possible, delayed mortality should also be assessed in the 8 h exposure, by transferring test organisms to clean water.

3.7. Salinity and temperature

Dispersant effectiveness generally increases with increasing salinity and increasing temperature (Fingas, 2002; Moles et al., 2002). Temperature and salinity effects on the toxicity of chemically dispersed oil are largely unknown because toxicity tests are usually performed at the preferred temperature and salinity of each test species. There have been only a few toxicity tests of chemically dispersed oil under conditions of subarctic salinity and temperature. Rhoton et al. (2001) assessed the toxicity of Prudhoe Bay crude oil dispersed with Corexit 9500 using tanner crab larvae and CROSERF protocols. Using non-CROSERF methods and subarctic conditions, Anderson (1985) reported the toxicity of Prudhoe Bay crude oil dispersed with either fdispersant Effor Corexit 9527, and Barron et al. (2003) evaluated the toxicity of ANS dispersed with Corexit 9527. Dispersant use under subarctic conditions may occur over a range of salinities and temperatures, thus dispersant testing should encompass conditions that reflect potential environmental exposures. We recommend testing at two different temperature–salinity regimes reflective of a range of subarctic conditions: 10 ffC at 15 & and 3 ffC at 32 & . Cold temperatures at high salinity are reflective of subarctic winter conditions and higher temperatures and lower salinity are reflective of spring and summer conditions with increased dilution from snow and ice melt.

3.8. Light regime

Recent research indicates that aqueous phase petroleum is phototoxic, with toxicity increasing 2 to greater than 100-fold for fresh and weathered crude oils and fuel oils in the presence of the UV that occurs in aquatic habitats (e.g., Pelletier et al., 1997; Calfee et al., 1999; Duesterloh et al., 2002). The vast majority of oil toxicity tests have been performed under fluorescent lighting (minimal UV), and only one study has investigated the photoenhanced toxicity of chemically dispersed oil. Barron et al. (2003) observed a 2 to 450-fold increase in the toxicity of weathered ANS chemically dispersed with Corexit 9527 in 5 h sunlight exposures compared to chemically dispersed ANS tested under fluorescent lighting. The growing body of evidence for the photoenhanced toxicity of oil, and its likely ecological relevance in aquatic environments (Barron et al., 2000; Barron and Kafaihue, 2001) indicates that a UV light regime should be incorporated into a chemical dispersant toxicity testing program.

A limited UV exposure is a recommended addition to the CROSERF protocols, and has been successfully evaluated with both calanoid copepods and Pacific herring larvae using non-CROSERF methods (Duesterloh et al., 2002; Barron et al., 2003). A brief (e.g., less than 8 h) exposure to either sunlight or simulated sunlight should be included that is equivalent to 5–10% of the clear sky UV at a latitude of 60ffN on the summer solstice. This equates to a UV dose of 2–5 mW h cm^2 in the A region of UV (320–400 nm) and 0.02–0.05 mW h cm^2 in the B region of UV (280–320 nm) (Barron and Kafaihue, 2001). The UV light regime should be applied after 24 h of initiation of WAF exposure to allow initial bioaccumulation of phototoxic petroleum hydrocarbons and heterocycles.

3.9. Test endpoints

CROSERF test endpoints are generally focused on mortality, although morbidity (e.g., impaired mobility) has been assessed in some tests. An assessment of petroleum bioaccumulation has not been previously recommended in tests using CROSERF protocols, although Wolfe et al. (1999) evaluated the bioavailability and trophic transfer of phenanthrene in from algae to rotifers exposed to physically and chemically dispersed Prudhoe Bay crude oil. Recommended changes to the CROSERF protocols include addition of an assessment of delayed mortality and morbidity by extending the test duration to 7 days, determination of toxicity endpoints based on petrogenic PAHs in WAF, and assessment of PAH bioaccumulation in a calanoid copepod. As discussed by Duesterloh et al. (2002), calanoid copepods have rapid and extensive uptake of PAHs with a bioconcentration factor (tissue:water) of 8000 for total PAHs in C. marshallae. The bioaccumulation assessment should be performed at 1 and 7 days following oil exposure to provide an estimate of both uptake and elimination of PAHs. More comprehensive bioaccumulation sampling would yield more definitive estimates of uptake and persistence (e.g., Barron et al., 1990), but would be an expensive addition to the recommended testing regimes.

4. Conclusions and recommendations

The CROSERF protocols have substantial merit in meeting the objectives of standardizing toxicity testing procedures and reducing test variability. Blenkinsopp et al. (1996) reported on variability in WAF chemistry, and interlaboratory testing has been performed, although the results are not yet available. A number of refinements are recommended to adapt the protocols for testing with subarctic species under conditions of expected longer oil persistence. WAF preparation should

include testing both a fresh and moderately weathered oil under conditions of moderate mixing energy (20–25% vortex), and preparing toxicity test solutions using variable dilutions rather than variable loading. We recommend that tests be performed with a species of sea urchin, calanoid copepod, and Pacific herring using static exposures in open chambers under a range of subarctic conditions of temperature and salinity. Test durations of 8 h and 7 days are recommended to encompass a range of environmental exposure scenarios and allow assessment of delayed mortality. The potential for photoenhanced toxicity should be assessed by incorporating a limited exposure to sunlight or simulated natural sunlight. Petrogenic PAHs should be measured in WAF and selected test solutions, and in copepod tissue to allow an assessment of hydrocarbon bioaccumulation and persistence. These recommendations are intended to increase the utility of the test results for interpreting the toxicity of chemically dispersed oil and for making risk management decisions regarding dispersant use under subarctic conditions. Future investigations should include assessment of the toxicity and photoenhanced toxicity of the dispersed oil plume in field trials as well as mesocosm studies.

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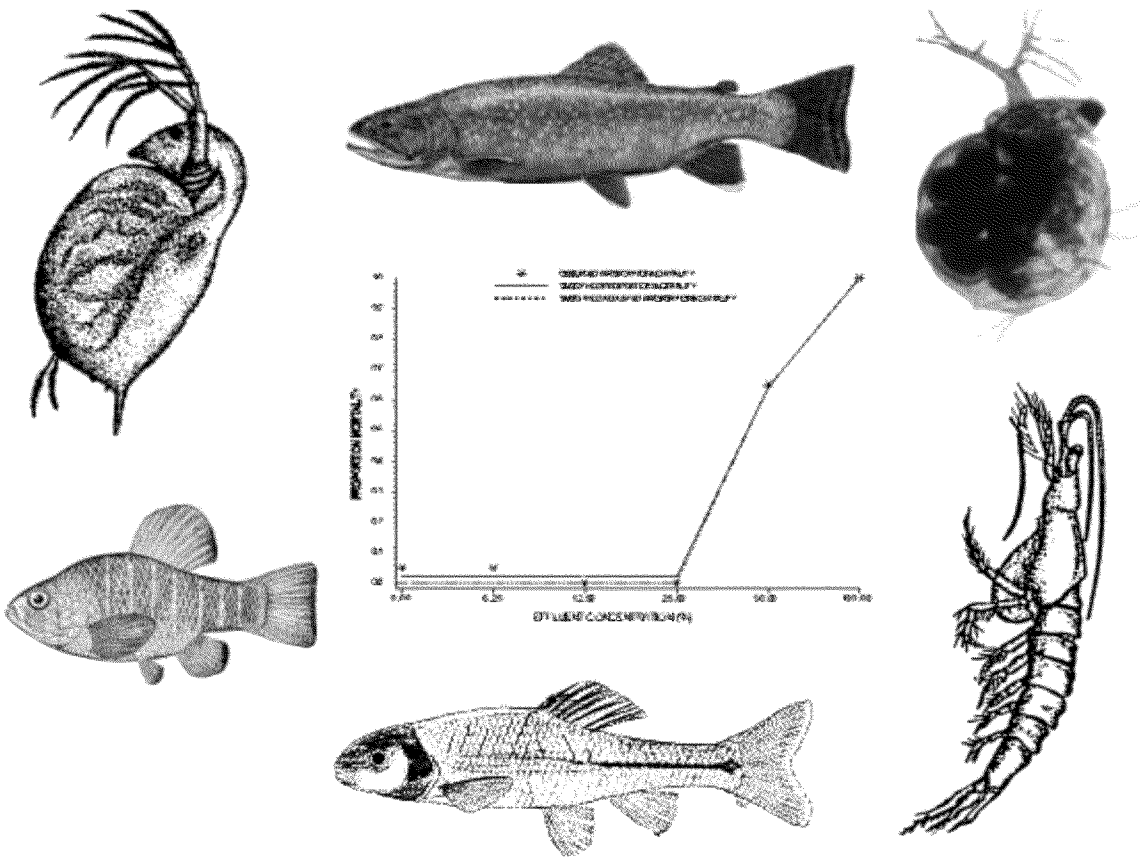

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SECTION 1

INTRODUCTION

1.1 This manual describes acute toxicity tests for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in acutely toxic concentrations. With the exception of the *Holmesimysis costata* Acute Test (Table 19), the methods included in this manual are referenced in Table IA, 40 CFR Part 136 regulations and, therefore, constitute approved methods for acute toxicity tests. They are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a central laboratory or on-site, by the regulatory agency or the permittee. The *Holmesimysis costata* Acute Test (Table 19) is specific to Pacific Coast waters and is not listed at 40 CFR Part 136 for nationwide use. This method has been proposed but not yet approved at 40 CFR Part 136.

1.2 The data are used for NPDES permits development and to determine compliance with permit toxicity limits. Data can also be used to predict potential acute and chronic toxicity in the receiving water, based on the LC50 and appropriate dilution, application, and persistence factors. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations. Data from acute toxicity tests performed as part of permit requirements are evaluated during compliance evaluation inspections and performance audit inspections.

1.3 Modifications of these tests are also used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, and to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA, 1988a; USEPA, 1988b; USEPA, 1989a; USEPA, 1989b; USEPA, 1991a).

1.4 This methods manual serves as a companion to the short-term chronic toxicity test methods manuals for freshwater and marine organisms (USEPA, 2002a; USEPA, 2002b), the NPDES compliance inspection manual (USEPA, 1988c), and the manual for evaluation of laboratories performing aquatic toxicity tests (USEPA, 1991b). In 2002, EPA revised previous editions of each of the three methods manuals (USEPA, 1993a; USEPA, 1994a; USEPA, 1994b).

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991c).

1.6 The use of any test species or test conditions other than those described in Tables 12-18 in this manual and referenced in Table 1A, 40 CFR 136.3, shall be considered a major modification to the method and subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.

1.7 These methods are restricted to use by, or under the supervision of, analysts experience in the use or conduct of, and interpretation of data from, aquatic toxicity tests. Each analyst must demonstrate the ability to generate acceptable test results with the methods using the procedures described in this methods manual.

1.8 This manual was prepared in the established EMSL-Cincinnati format (USEPA, 1983a).

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SECTION 2

TYPES OF TESTS

2.1 The selection of the test type will depend on the NPDES permit requirements, the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity.

2.2 Effluent acute toxicity is generally measured using a multi-concentration, or definitive test, consisting of a control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that is lethal to 50% of the test organisms (LC50) within the prescribed period of time (24-96 h), or the highest effluent concentration in which survival is not statistically significantly different from the control.

2.3 Use of pass/fail tests consisting of a single effluent concentration (e.g., the receiving water concentration or RWC) and a control is not recommended. If the NPDES permit has a whole effluent toxicity limit for acute toxicity at the RWC, it is prudent to use that permit limit as the midpoint of a series of five effluent concentrations. This will ensure that there is sufficient information on the dose-response relationship. For example, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) (RWC + 100)/2, (3) RWC, (4) RWC/2, and (5) RWC/4. More specifically, if the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

2.4 Receiving (ambient) water toxicity tests commonly employ two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

2.5 A negative result from an acute toxicity test does not preclude the presence of chronic toxicity. Also, because of the potential temporal variability in the toxicity of effluents, a negative test result with a particular sample does not preclude the possibility that samples collected at some other time might exhibit acute (or chronic) toxicity.

2.6 The frequency with which acute toxicity tests are conducted under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

2.7 Tests may be static (static non-renewal or static renewal), or flow-through.

2.7.1 STATIC TESTS

2.7.1.1 Static non-renewal tests - The test organisms are exposed to the same test solution for the duration of the test.

2.7.1.2 Static-renewal tests - The test organisms are exposed to a fresh solution of the same concentration of sample every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

2.7.2 FLOW-THROUGH TESTS

2.7.2.1 Two types of flow-through tests are in common use: (1) sample is pumped continuously from the sampling point directly to the dilutor system; and (2) grab or composite samples are collected periodically, placed in a tank adjacent to the test laboratory, and pumped continuously from the tank to the dilutor system. The flow-through method employing continuous sampling is the preferred method for on-site tests. Because of the large volume (often 400 L/day) of effluent normally required for flow-through tests, it is generally considered too costly and impractical to conduct these tests off-site at a central laboratory.

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2.8 Advantages and disadvantages of the types of tests are as follows:

2.8.1 STATIC NON-RENEWAL TESTS

2.8.1.1 Advantages:

1. Simple and inexpensive.
2. Very cost effective in determining compliance with permit conditions.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform many more tests in the same amount of time.
4. Smaller volume of effluent required than for static renewal or flow-through tests.

2.8.1.2 Disadvantages:

1. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
2. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Generally less sensitive than static renewal or flow-through tests, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.8.2 STATIC-RENEWAL, ACUTE TOXICITY TESTS

2.8.2.1 Advantages:

1. Reduced possibility of dissolved oxygen (DO) depletion from high chemical oxygen demand (COD) and/or biological oxygen demand (BOD), or ill effects from metabolic wastes from organisms in the test solutions.
2. Reduced possibility of loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Test organisms that rapidly deplete energy reserves are fed when the test solutions are renewed, and are maintained in a healthier state.

2.8.2.2 Disadvantages:

1. Require greater volume of effluent that non-renewal tests.
2. Generally less sensitive than flow-through tests, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.8.3 FLOW-THROUGH TESTS

2.8.3.1 Advantages:

1. Provide a more representative evaluation of the acute toxicity of the source, especially if sample is pumped continuously directly from the source and its toxicity varies with time.
2. DO concentrations are more easily maintained in the test chambers.
3. A higher loading factor (biomass) may be used.
4. The possibility of loss of toxicant due to volatilization, adsorption, degradation, and uptake is reduced.

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2.8.3.2 Disadvantages:

1. Large volumes of sample and dilution water are required.
2. Test equipment is more complex and expensive, and requires more maintenance and attention.
3. More space is required to conduct tests.
4. Because of the resources required, it would be very difficult to perform multiple or overlapping sequential tests.

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SECTION 3

HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

3.1.1 Development and maintenance of an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management, and includes (1) the appointment of a laboratory health and safety officer with the responsibility and authority to develop and maintain a safety program, (2) the preparation of a formal, written, health and safety plan, which is provided to each laboratory staff member, (3) an ongoing training program on laboratory safety, and (4) regularly scheduled, documented, safety inspections.

3.1.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

3.1.3 Prior to sample collection and laboratory work, personnel must determine that all required safety equipment and materials have been obtained and are in good condition.

3.1.4 Guidelines for the handling and disposal of hazardous materials must be strictly followed.

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

3.2.1.1 Personnel must use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes.

3.2.2 LABORATORY SAFETY EQUIPMENT

3.2.2.1 Each laboratory (including mobile laboratories) must be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, and eye fountains.

3.2.2.2 Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1 Guidance in Material Safety Data Sheets should be followed for reagents and other chemicals purchased from supply houses. Incompatible materials should not be stored together.

3.3.2 Work with effluents must be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see Safety Manuals, Subsection 3.5). Personnel collecting samples and performing toxicity tests should not work alone.

3.3.3 Because the chemical composition of effluents is usually only poorly known, they must be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the test areas must be used whenever necessary.

3.3.4 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

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3.3.5 All containers must be adequately labeled to indicate their contents.

3.3.6 Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or under an exhaust canopy over the work area.

3.3.7 Good housekeeping contributes to safety and reliable results.

3.3.8 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.9 Mobile laboratories must be properly grounded to protect against electrical shock.

3.4 DISEASE PREVENTION

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against hepatitis B, tetanus, typhoid fever, and polio.

3.5 SAFETY MANUALS

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general industrial safety manuals, including USEPA (1986) and Walters and Jameson (1984).

3.6 WASTE DISPOSAL

3.6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state, and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in, performing testing activities. Local fire officials should be notified of any potentially hazardous conditions.

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SECTION 4

QUALITY ASSURANCE

4.1 INTRODUCTION

4.1.1 Development and maintenance of a toxicity test laboratory quality assurance (QA) program requires an ongoing commitment by laboratory management, and includes the following: (1) appointment of a laboratory quality assurance officer with the responsibility and authority to develop and maintain a QA program; (2) preparation of a quality assurance plan with data quality objectives; (3) preparation of written descriptions of laboratory standard operating procedures (SOP's) for test organism culturing, toxicity testing, instrument calibration, sample chain-of-custody, laboratory sample tracking system, etc.; and (4) provision of adequate, qualified technical staff and suitable space and equipment to assure reliable data.

4.1.2 QA practices within an aquatic toxicology laboratory must address all activities that affect the quality of the final effluent toxicity data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition and operation of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

4.1.3 Quality control practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance, and general guidance on good laboratory practices related to toxicity testing, see: FDA, 1978; USEPA, 1975; USEPA, 1979a; USEPA, 1980a; USEPA, 1980b; USEPA, 1991b; DeWoskin, 1984; and Taylor, 1987.

4.1.4 Guidance for the evaluation of laboratories performing toxicity tests and laboratory evaluation criteria may be found in USEPA 1991b.

4.2 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.2.1 Separate test organism culturing and toxicity testing areas should be provided to avoid possible loss of cultures due to cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into organism culturing or toxicity testing areas, and from toxicity test laboratories and sample preparation areas into culture rooms.

4.2.2 Laboratory and toxicity test temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities and Equipment).

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are listed in Section 6, Test Organisms. The organisms should appear healthy, behave normally, feed well, and have low mortality in cultures, during holding, and in test controls. Test organisms should be positively identified to species.

4.4 LABORATORY WATER USED FOR CULTURING AND TEST DILUTION WATER

4.4.1 The quality of water used for test organism culturing and for dilution water used in toxicity tests is extremely important. Water for these two uses should come from the same source. The dilution water used in effluent toxicity tests will depend in part on the objectives of the study and logistical constraints, as discussed in detail in Section 7, Dilution Water. The dilution water used for internal quality assurance tests with organisms, food, and reference toxicants should be the water routinely used with success in the laboratory. Types of water are discussed in Section

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5, Facilities and Equipment. Water used for culturing and test dilution should be analyzed for toxic metals and organics at least annually or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. The concentration of the metals, Al, As, Cr, Co, Cu, Fe, Pb, Ni, and Zn, expressed as total metal, should not exceed 1 µg/L each, and Cd, Hg, and Ag, expressed as total metal, should not exceed 100 ng/L each. Total organochlorine pesticides plus PCBs should be less than 50 ng/L (APHA, 1992). Pesticide concentrations should not exceed USEPA's Ambient Water Quality chronic criteria values where available.

4.5 EFFLUENT SAMPLING AND SAMPLE HANDLING

4.5.1 Sample holding times and temperatures must conform to conditions described in Section 8, Effluent and Receiving Water Sampling and Sample Handling.

4.6 TEST CONDITIONS

4.6.1 The temperature of test solutions must be measured by placing the thermometer or probe directly into the test solutions, or by placing the thermometer in equivalent volumes of water in surrogate vessels positioned at appropriate locations among the test vessels. Temperature should be recorded continuously in at least one vessel during the duration of each test. Test solution temperatures should be maintained within the limits specified for each test. DO concentration and pH in test chambers should be checked daily throughout the test period, as prescribed in Section 9, Acute Toxicity Test Procedures.

4.7 QUALITY OF TEST ORGANISMS

4.7.1 The health of test organisms is primarily assessed by the performance (survival, growth, and/or reproduction) of organisms in control treatments of individual tests. The health and sensitivity of test organisms is also assessed by reference toxicant testing. In addition to documenting the sensitivity and health of test organisms, reference toxicant testing is used to initially demonstrate acceptable laboratory performance (Subsection 4.14) and to document ongoing laboratory performance (Subsection 4.15).

4.7.2 Regardless of the source of test organisms (in-house cultures or purchased from external suppliers), the testing laboratory must perform at least one acceptable reference toxicant test per month for each toxicity test method conducted in that month (Subsection 4.15). If a test method is conducted only monthly, or less frequently, a reference toxicant test must be performed concurrently with each effluent toxicity test.

4.7.3 When acute or short-term chronic toxicity tests are performed with effluents or receiving waters using test organisms obtained from outside the test laboratory, concurrent toxicity tests of the same type must be preformed with a reference toxicant, unless the test organism supplier provides control chart data from at least the last five monthly acute toxicity tests using the same reference toxicant and test conditions.

4.7.4 The supplier should also certify the species identification of the test organisms, and provide the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted.

4.7.5 If a routine reference toxicant test fails to meet test acceptability criteria, then the reference toxicant test must be immediately repeated.

4.8 FOOD QUALITY

4.8.1 The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. This is especially true for the unsaturated fatty acid content of brine shrimp nauplii, *Artemia*. Suitable trout chow, *Artemia*, and other foods must be obtained as described in this manual.

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4.8.2 Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. If a batch of food is suspected to be defective, the performance of organisms fed with the new food can be compared with the performance of organisms fed with a food of known quality in side-by-side tests. If the food is used for culturing, its suitability should be determined using a short-term chronic test which will determine the effect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source. Where applicable, foods used only in acute toxicity tests can be compared with a food of known quality in side-by-side, multi-concentration acute tests, using the reference toxicant regularly employed in the laboratory QA program.

4.8.3 New batches of food used in culturing and testing should be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum test acceptability criteria for control survival and reproduction or growth. If the concentration of total organochlorine pesticides exceeds 0.15 µg/g wet weight, or the concentration of the total organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight, or toxic metals (Al, As, Cr, Co, Cu, Pb, Ni, Zn, expressed as total metal) exceed 20 µg/g wet weight, the food should not be used (for analytical methods see AOAC, 1990 and USDA, 1989). For foods (e.g., such as YCT) which are used to culture and test organisms, the quality of food should meet the requirements for the laboratory water used for culturing and test dilution water as described in Section 4.4 above.

4.9 ACCEPTABILITY OF ACUTE TOXICITY TEST RESULTS

4.9.1 For the test results to be acceptable, control survival must equal or exceed 90%.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of the test will depend on the experience and professional judgment of the laboratory analyst and the reviewing staff of the regulatory authority. Any deviation from test specifications must be noted when reporting data from a test.

4.10 ANALYTICAL METHODS

4.10.1 Routine chemical and physical analyses for culture and dilution water, food, and test solutions, must include established quality assurance practices outlined in Agency methods manuals (USEPA, 1979a; USEPA, 1993b).

4.10.2 Reagent containers should be dated when received from the supplier, and the shelf life should not be exceeded. Also, working solutions should be dated when prepared, and the recommended shelf life should be observed.

4.11 CALIBRATION AND STANDARDIZATION

4.11.1 Instruments used for routine measurements of chemical and physical parameters such as pH, DO, temperature, conductivity, salinity, alkalinity, and hardness must be calibrated and standardized prior to use each day according to the instrument manufacturer's procedures as indicated in the general section on quality assurance (see EPA Methods 150.1, 360.1, 170.1, and 120.1; USEPA, 1979b). Calibration data are recorded in a permanent log.

4.11.2 Wet chemical methods used to measure hardness, alkalinity, and total residual chlorine must be standardized prior to use each day according to the procedures for those specific EPA methods (see EPA Methods 130.2 and 310.1; USEPA 1979b).

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4.12 REPLICATION AND TEST SENSITIVITY

4.12.1 The sensitivity of toxicity tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data.

4.13 VARIABILITY IN TOXICITY TEST RESULTS

4.13.1 Factors which can affect test success and precision include: the experience and skill of the laboratory analyst; test organism age, condition, and sensitivity; dilution water quality; temperature control; and the quality and quantity of food provided. The results will depend upon the species used and the strain or source of the test organisms, and test conditions such as temperature, DO, food, and water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. Jensen (1972) discussed the relationship between sample size (numbers of fish) and the standard error of the test, and considered 20 fish per concentration as optimum for Probit Analysis.

4.13.2 Test precision can be estimated by using the same strain of organisms under the same test conditions, and employing a known toxicant, such as a reference toxicant. The single-laboratory (intra-laboratory) and multi-laboratory (inter-laboratory) precision of acute toxicity tests with several common test species and reference toxicants are listed in Tables 1-4. Intra- and inter-laboratory precision are described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated endpoints from the replicated tests.

4.13.3 Intra-laboratory precision data from 268 acute toxicity tests with four species and five reference toxicants are listed in Tables 1 and 2. The precision, expressed as CV%, ranged from 3% to 86%. More recent CV values reported by Jop et al. (1986), Dorn and Rogers (1989), Hall et al. (1989), and Cowgill et al. (1990), fell in a somewhat lower range (8% to 41%).

4.13.4 Inter-laboratory precision of acute toxicity tests from 253 reference toxicant tests with seven species, listed in Tables 2, 3, 4, and 5 (expressed as CV% for LC50s), ranged from 11% to 167%. Table 6 shows interlaboratory precision data from a study of acute toxicity test methods using reference toxicant, effluent, and receiving water sample types (USEPA, 2001a; USEPA, 2001b). Averaged across sample types, total interlaboratory precision (expressed as CV% for LC50s) ranged from 13% to 38.5% for the acute methods.

4.13.5 No clear pattern of differences were noted in the intra- or inter-laboratory test precision with the species listed, although the test results with some toxicants, such as cadmium, appear to more variable than those with other reference toxicants.

4.13.6 Additional information on toxicity test precision is provided in the Technical Support Document for Water Quality-Based Toxics Control (see pp. 2-4, and 11-15; USEPA, 1991c).

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TABLE 1. INTRA-LABORATORY PRECISION OF LC50S FROM STATIC ACUTE TOXICITY TESTS WITH AQUATIC ORGANISMS USING REFERENCE TOXICANTS[1]

		REFERENCE TOXICANT [2]								
		SDS			NAPCP			CD		
TEST ORGANISM		N	LC50	CV (%)	N	LC50	CV (%)	N	LC50	CV (%)
Pimephales promelas	(96 h, 21EC)[3]	9	8.6	20	12	0.14	40	9	0.15	120
Daphnia magna	(24 h, 20EC)[4]	8	20.9	28	10	0.69	14	11	0.121	49
Daphnia magna	(24 h, 26EC)[4]	10	12.9	48	9	0.67	25	9	0.026	77
Daphnia magna	(48 h, 20EC)[4]	10	13.5	29	10	0.42	21	9	0.038	58
Daphnia magna	(48 h, 26EC)[4]	9	10.8	33	9	0.48	23	8	0.009	35
Daphnia pulex	(24 h, 20EC)[4]	9	18.4	23	9	0.64	15	5	0.147	30
Daphnia pulex	(24 h, 26EC)[4]	10	13.9	25	9	0.62	25	10	0.063	45
Daphnia pulex	(48 h, 20EC)[4]	10	12.6	32	9	0.48	16	10	0.042	45
Daphnia pulex	(48 h, 26EC)[4]	9	10.2	36	8	0.47	32	6	0.006	14
Mysidopsis bahia	(96 h, 25EC)[5]							13	0.346	9

[1] Precision expressed as percent coefficient of variation, where CV% = (standard deviation X 100)/mean.

[2] SDS = Sodium dodecyl (lauryl) sulfate; NAPCP = Sodium pentachlorophenate; CD = Cadmium; N = Number of tests; toxicant concentration in mg/L.

[3] *Pimephales promelas* tests were performed in soft, synthetic freshwater; total hardness, 40-48 mg/L as $CaCO_3$, by J. Dryer, Aquatic Biology Section, EMSL-Cincinnati.

[4] *Daphnia* data from Lewis and Horning, 1991. Tests with *D. magna* used hard reconstituted water (total hardness, 180-200 mg/L as $CaCO_3$); tests with *D. pulex* used moderately-hard reconstituted water (total hardness, 80-100 mg/L as $CaCO_3$).

[5] Mysid tests were performed in 25 ppt salinity, natural seawater. Data were provided by Steve Ward, Environmental Services Division, U.S. Environmental Protection Agency, Edison, New Jersey. Personal communication, November 14, 1990.

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TABLE 2. INTRA- AND INTER-LABORATORY PRECISION OF ACUTE TOXICITY TESTS WITH *DAPHNIA MAGNA*, USING A STANDARD EFFLUENT[1,2]

LABORATORY	INTER-LABORATORY PRECISION: LC50s FROM REPLICATE TESTS		INTRA-LABORATORY PRECISION[3]
	24 H	48 H	
INDUSTRY			
1	14.4	4.2	
	11.4	4.9	---
2	13.9	6.8	
	16.6	6.1	
	13.7	6.1	6.4
3	11.7	3.5	
	17.4	7.1	---
GOVERNMENT			
1	14.0	4.4	
	10.0	4.4	
	10.8	4.1	4.0
2	13.2	4.5	
	14.1	4.5	---
3	11.6	4.2	---
COMMERCIAL			
1	20.1	4.9	
	20.1	4.7	---
2	8.9	3.7	
	12.3	5.6	---
3	14.8	9.0	
	25.4	9.1	
	26.4	8.6	3.0
N	20	20	3
MEAN	15.0	5.52	4.47
SD	4.75	1.75	1.75
CV%	31.6	31.6	39.1

[1] From Table 2, p. 191, Grothe and Kimerle, 1985. Tests performed at 20EC ±2EC; dilution water hardness, 100mg/L as $CaCO_3$; dilution water alkalinity, 76 mg/L as $CaCO_3$; effluent hardness, approx. 1000 mg/L as $CaCO_3$; effluent alkalinity, 310 mg/L as $CaCO_3$; effluent dilutions - 56%, 32%, 18%, 10%, 5.6%, 3.1%, 1.7%.

[2] LC50 expressed in percent effluent.

[3] Intra-laboratory precision expressed as the weighted mean CV(%).

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TABLE 3. INTER-LABORATORY PRECISION OF ACUTE TOXICITY TESTS WITH AQUATIC ORGANISMS, USING REFERENCE TOXICANTS[1]

	REFERENCE TOXICANT					
	SILVER			ENDOSULFAN		
TEST ORGANISM	N	LC50	CV (%)	N	LC50	CV (%)
1. *Pimephales promelas* (96 h, 22EC)						
96-h static test (Meas)	10	14.0	53	12	2.03	38
96-h flow-through test (Meas)	9	7.49	40	12	0.96	46
2. *Oncorhyncus mykiss* (96 h, 12EC)						
96-h static test (Meas)	10	34.5	88	12	1.15	50
96-h flow-through test (Meas)	9	11.5	33	12	0.40	42
3. *Daphnia magna* (48 h, 20EC)						
48-h static (Meas)	12	10.6	166	11	328	51
4. *Mysidopsis bahia* (96 h, 22EC)						
96-h static test (Nom)	6	210	27	5	0.84	62
96-h flow-through test (Nom)	6	251	22	6	1.02	58
96-h flow-through test (Meas)	6	192	58	5	0.94	167
5. *Cyprinodon variegatus* (96 h, 22EC)						
96-h static test (Nom)	4	1122	35	6	2.41	37
96-h flow-through test (Nom)	5	1573	50	6	1.69	46
96-h flow-through test (Meas)	5	1216	50	6	0.81	46

[1] Data for *Pimephales promelas* (fathead minnow), *Oncorhyncus mykiss* (rainbow trout), and *Daphnia magna* were taken from USEPA, 1983b.

Data for, *Mysidopsis bahia*, and *Cyprinodon variegatus* (sheepshead minnow) were taken from USEPA, 1981. Six laboratories participated in each study. Test salinity was 28‰.

LC50s expressed in µg/L.

In the studies with the freshwater organisms, the water hardness for five of the six laboratories ranged between 36 and 75 mg/L. However, the water hardness for the sixth laboratory was 255 mg/L, resulting in LC50 values for silver more than an order of magnitude larger than for the other five. These values were rejected in calculating the CV%. The mean weights of test fish were from 0.05-0.26 g for fathead minnows, and 0.22-1.32 g for rainbow trout. *Daphnia* were #24-h old.

In studies with the marine organisms, only one LC50 (presumably the combined LC50 from duplicate tests) was reported for each toxicity test. LC50s for flow-through tests with *Mysidopsis bahia* and Cyprinodon *variegatus* were calculated two different ways -- (1) on the basis of the nominal toxicant concentrations (Nom), and (2) on the basis of measured (Meas) toxicant concentrations. Test organism age was #2 days for *Mysidopsis bahia*, and 28 days for *Cyprinodon variegatus*. The salinity of test solutions was 28‰.

N, the total number of LC50 values used in calculating the CV(%) varied with organism and toxicant because some data were rejected due to water hardness, lack of concentration measurements, and/or because some of the LC50s were not calculable.

[2] CV% = Percent coefficient of variation = (standard deviation x 100)/mean.

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TABLE 4. INTER-LABORATORY STUDY OF ACUTE TOXICITY TEST PRECISION, 1990: SUMMARY OF RESPONSES USING KCL AS THE REFERENCE TOXICANT[1]

TEST TYPE	NO. LABS SUBMITTING VALID DATA	TEST PRECISION (CV%)[2] GRAPH[3] METHOD			STAT[4] METHOD			TOTAL[5]		
		N	LC50	CV%	N	LC50	CV%	N	LC50	CV%
Pimephales promelas (96 h, 22EC)[6]	17	6	944	28.8	13	832	27.8	17	864	29.6
Pimephales promelas (24 h, 25EC)[7]	6	6	832	11.5	6	832	11.5	–	–	–
Ceriodaphnia dubia (48 h, 25EC)[7]	11	11	256	53.1	11	264	48.5	–	–	–
Mysidopsis bahia (96 h, 22EC)[8]	14	7	292	32.9	11	250	36.0	14	268	37.3

[1] Interlaboratory study of toxicity test precision conducted in 1990 by the Environmental Monitoring Systems Laboratory - Cincinnati, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268, in cooperation with the states of New Jersey and North Carolina, and the Office of Water Enforcement and Permits, U.S. Environmental Protection Agency, Washington, DC.

[2] Percent coefficient of variation = (standard deviation X 100)/mean. Calculated for LC50 from acute tests. LC50s expressed as mg/L KCl added to the dilution water.

[3] LC50 estimated by the Graphical Method.

[4] LC50 estimated by Probit, Litchfield-Wilcoxon, or Trimmed Spearman-Karber method.

[5] LC50 usually reported for only one method of analysis for each test. Where more than one LC50 was reported for a test, the lowest value was used to calculate the statistics for "Total."

[6] Data from the New Jersey Department of Environmental Protection: static daily-renewal tests, using moderately-hard synthetic freshwater.

[7] Data from North Carolina certified laboratories: static non-renewal tests, using moderately-hard reconstituted freshwater.

[8] Data from the New Jersey Department of Environmental Protection: static daily-renewal tests, using 25 ppt salinity, FORTY FATHOMS® synthetic seawater.

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TABLE 5. NATIONAL INTERLABORATORY STUDY OF ACUTE TOXICITY TEST PRECISION, 1991: SUMMARY OF RESPONSES USING REFERENCE TOXICANTS[1]

Test Type	No. Labs Submitting Data	LC50	CV%[2]
Pimephales promelas (48 h, 25EC)[3]	203	896[4]	28.6
Ceriodaphnia dubia (48 h, 25EC)[3]	171	432[4]	39.8
Mysidopsis bahia (48 h, 25EC)[5]	61	532[4]	30.1
Menidia beryllina (48 h, 25EC)[5]	39	164[6]	42.2

[1] From a national study of interlaboratory precision of toxicity test data performed in 1991 by the Environmental Monitoring Systems Laboratory - Cincinnati, U.S. Environmental Protection Agency, Cincinnati, OH 45268. Participants included Federal, state, and private laboratories engaged in NPDES permit compliance monitoring. LC50s were estimated by the graphical or Spearman-Karber method.

[2] Percent coefficient of variation = (standard deviation X 100)/mean.

[3] Static non-renewal tests, using moderately-hard synthetic freshwater (total hardness = 80-100 mg/L as $CaCO_3$).

[4] Expressed as mg KCl added per liter of dilution water.

[5] Static non-renewal tests, using 30 ppt modified GP2 artificial seawater.

[6] Expressed as Fg Cu^{++} added per liter of dilution water.

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TABLE 6. NATIONAL INTERLABORATORY STUDY OF ACUTE TOXICITY TEST PRECISION, 2000: PRECISION OF LC50 POINT ESTIMATES FOR REFERENCE TOXICANT, EFFLUENT, AND RECEIVING WATER SAMPLE TYPES[1].

Method	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
Pimephales promelas	KCl	7.62	19.7	21.1
	Municipal effluent	10.3	19.2	21.8
	Receiving water	-	-	17.2
	Average	8.96	19.4	20.0
Ceriodaphnia dubia	KCl	14.6	15.2	21.1
	Municipal effluent	9.68	32.8	34.2
	Receiving water	-	-	31.8
	Average	12.1	24.0	29.0
Cyprinodon variegatus	KCl	-	-	26.0
	Municipal effluent	-	-	19.4
	Receiving water	-	-	32.5
	Average	-	-	26.0
Menidia beryllina	$CuSO_4$[6]	-	-	-
	Industrial effluent	9.91	49.7	50.7
	Receiving water	-	-	26.3
	Average	9.91	49.7	38.5
Holmesimysis costata[7]	Zn (48 h test)	19	-	
	Zn (96 h test)	23	-	
	Zn (interlaboratory trial 1)	-	-	24
	Zn (interlaboratory trial 2)	-	-	1
	Average	21		13

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type. The study design also did not provide within-laboratory replication for the *Cyprinodon variegatus* Acute Method.

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[3] The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

[4] The between-laboratory component of variability for duplicate samples tested at different laboratories.

[5] The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

[6] Precision estimates were not calculated for the reference toxicant sample type since the majority of results for this sample type were outside of the test concentration range (ie., >100).

[7] *Holmesimysis costata* Acute Test data were from Martin *et al.* (1989). Zn was tested in two intralaboratory trials and in two interlaboratory trials. Data from this study was only reported to two significant figures.

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4.14 DEMONSTRATING ACCEPTABLE LABORATORY PERFORMANCE

4.14.1 It is a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes. To meet this requirement, the intra-laboratory precision, expressed as percent coefficient of variation (CV%), of each type of test to be used in a laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (i.e., the same test duration, type of dilution water, age of test organisms, feeding, etc.), and same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations.

4.15 DOCUMENTING ONGOING LABORATORY PERFORMANCE

4.15.1 Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method conducted in the laboratory during that month. For a given test method, successive tests must be performed with the same reference toxicant, at the same concentrations, in the same dilution water, using the same data analysis methods. Precision may vary with the test species, reference toxicant, and type of test. Each laboratory's reference toxicity data will reflect conditions unique to that facility, including dilution water, culturing, and other variables; however, each laboratory's reference toxicity results should reflect good repeatability.

4.15.2 A control chart should be prepared for each combination of reference toxicant, test species, test condition, and endpoint. Toxicity endpoints from five or six tests are adequate for establishing the control charts. In this technique, a running plot is maintained for the toxicity values (X_i) from successive tests with a given reference toxicant (Figure 1), and endpoints (LC50s) are examined to determine if they are within prescribed limits. The types of control charts illustrated (see USEPA, 1979a) are used to evaluate the cumulative trend of results from a series of samples, thus reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. The mean ($\bar{x}$) and upper and lower control limits (±2S) are re-calculated with each successive test result. After two years of data collection, or a minimum of 20 data points, the control chart should be maintained using only the 20 most recent data points.

4.15.3 Laboratories should compare the calculated CV (i.e., standard deviation / mean) of the LC50 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-3 in USEPA, 2000b). If the calculated CV exceeds the 75th percentile of CVs reported nationally, the laboratory should use the 75th and 90th percentiles to calculate warning and control limits, respectively, and the laboratory should investigate options for reducing variability.

4.15.4 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified. At the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone. If more than one out of 20 reference toxicant tests fall outside the control limits, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. In those instances when the laboratory can document the cause for the outlier (e.g., operator error, culture health or test system failure), the outlier should be excluded from the future calculations of the control limits. If two or more consecutive tests do not fall within the control limits, the results must be explained and the reference toxicant test must be immediately repeated. Actions taken to correct the problem must be reported.

4.15.5 If the toxicity value from a given test with the reference toxicant falls <u>well</u> outside the expected range for the test organisms when using the standard dilution water, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Performance should improve with experience, and the control limits for point estimates should gradually narrow. However, control limits of ±2S, by definition, will be exceeded 5% of the time, regardless of how well a laboratory performs. Highly proficient laboratories which develop a very narrow control limit may be unfairly penalized if a test which falls just outside the control limits is rejected *de facto*. For this reason, the width

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of the control limits should be considered in determining whether or not a reference toxicant test result falls "well" outside the expected range. The width of the control limits may be evaluated by comparing the calculated CV (i.e., standard deviation / mean) of the LC50 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-3 in USEPA, 2000b). In determining whether or not a reference toxicant test result falls "well" outside the expected range, the result also may be compared with upper and lower bounds for ±3S, as any result outside these control limits would be expected to occur by chance only 1 out of 100 tests (Environment Canada, 1990). When a result from a reference toxicant test is outside the 99% confidence intervals, the laboratory must conduct an immediate investigation to assess the possible causes for the outlier.

4.15.6 Reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. Reference toxicant testing is used for evaluating the health and sensitivity of organisms over time and for documenting initial and ongoing laboratory performance. While reference toxicant test results should not be used as a *de facto* criterion for test rejection, effluent and receiving water test results should be reviewed and interpreted in the light of reference toxicant test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increased test organism sensitivity or toward decreased test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test.

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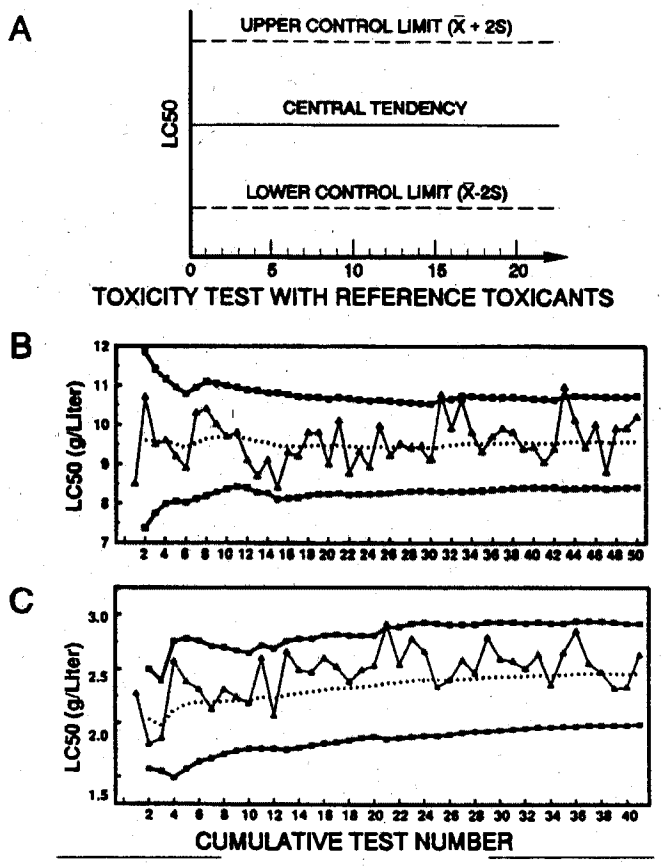

Figure 1. Control (cusum) charts: A, General case; B and C, 48-h acute tests with sodium chloride. (B) Fathead minnow (*Pimephales promelas*), and (C) *Ceriodaphnia dubia*, with the individual LC50s (Triangles), cumulative LC50 means (dotted line), and upper and lower control limits of two standard deviations (squares). (Provided by the Environmental Services Division, U.S. Environmental Protection Agency, Kansas City, KS).

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4.16 REFERENCE TOXICANTS

4.16.1 Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), are suitable for use in the NPDES and other Agency programs requiring aquatic toxicity tests. EMSL-Cincinnati hopes to release EPA-certified solutions of cadmium and copper, with accompanying toxicity data for the recommended test species, for use as reference toxicants through cooperative research and development agreements with commercial suppliers, and will continue to develop additional reference toxicants for future release. Standard reference materials can be obtained from commercial supply houses, or can be prepared inhouse using reagent grade chemicals. The regulatory agency should be consulted before reference toxicant(s) are selected and used.

4.17 RECORD KEEPING

4.17.1 Proper record keeping is important. A complete file should be maintained for each individual toxicity test or group of tests on closely related samples. This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions employed; and results of reference toxicant tests. Laboratory data should be recorded on a real-time basis to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed and dated by the laboratory personnel performing the tests.

4.17.2 The regulatory authority should retain records pertaining to discharge permits. Permittees are required to retain records pertaining to permit applications and compliance for a minimum of 3 years [40 CFR 122.41(j)(2)].

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SECTION 5

FACILITIES AND EQUIPMENT

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities should include equipment for rearing and/or holding organisms.

5.1.2 The facilities must be well ventilated and free of toxic fumes. Sample preparation, culturing, and toxicity testing areas should be separated to avoid cross contamination of cultures or toxicity test solutions with toxic fumes. Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories and/or sample handling areas is not circulated to test organism culture rooms or toxicity test rooms, or that air from toxicity test rooms does not contaminate culture areas. Air pressure differentials between such rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely-fitting doors.

5.1.3 Control of test solution temperature can best be achieved using circulating water baths, heat exchangers, or environmental chambers. Photoperiod can be controlled using automatic timers in the laboratory or environmental chambers.

5.1.4 Water used for rearing, holding, and testing organisms may be reconstituted synthetic water, ground water, surface water, or dechlorinated tap water. Dechlorination can be accomplished by carbon filtration, laboratory water conditioning units, or the use of sodium thiosulfate. After dechlorination, total residual chlorine should be non-detectable. Sodium thiosulfate may be toxic to the test organisms, and if used for dechlorination, paired controls with and without sodium thiosulfate should be incorporated in effluent toxicity tests. Use of 3.6 mg (anhydrous) sodium thiosulfate/L will reduce l.0 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable.

5.1.4.1 A good quality, laboratory grade deionized water, providing a resistance of 18 megaohm-cm, must be available in the laboratory and in sufficient quantity for laboratory needs. Deionized water may be obtained from MILLIPORE®, Milli-Q®, MILLIPORE QPAK™$_2$ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deionizer with preconditioned water from a CULLIGAN®, CONTINENTAL®, or equivalent, mixed-bed water treatment system.

5.1.5 Air used for aeration must be free of oil and fumes. Oil-free air pumps should be used where possible. Particulates can be removed from the air using BALSTON® Grade BX or equivalent filters (Balston, Inc., Lexington, MA), and oil and other organic vapors can be removed using activated carbon filters (BALSTON®, C-1 filter, or equivalent).

5.1.6 During rearing, holding, and testing, test organisms should be shielded from external disturbances such as rapidly changing light conditions (especially salmonids) and pedestrian traffic.

5.1.7 Materials used for exposure chambers, tubing, etc., that come in contact with the effluent and dilution water should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON®) should be used whenever possible to minimize sorption and leaching of toxic substances, and may be reused after cleaning. Containers made of plastics, such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used to ship, store, and transfer effluents and receiving waters, but they should not be reused unless absolutely necessary, because they could carry over adsorbed toxicants from one test to another. However, these containers may be repeatedly reused for storing uncontaminated waters such as deionized or laboratory-prepared dilution waters and receiving waters. Glass or disposable polystyrene containers can be used as test chambers. The use of large ($20 L) glass carboys is discouraged for safety reasons.

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5.1.8 New plastic products should be tested for toxicity before general use by exposing organisms to them under ordinary test conditions.

5.1.9 Equipment which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below. Fiberglass, in addition to the previously mentioned materials, can be used for holding and dilution water storage tanks, and in the water delivery system. All material should be flushed or rinsed thoroughly with dilution water before using in the test.

5.1.10 Copper, galvanized material, rubber, brass, and lead must not come in contact with holding or dilution water, or with effluent samples and test solutions. Some materials, such as neoprene rubber (commonly used for stoppers), may be toxic and should be tested before use.

5.1.11 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

5.2 CLEANING TEST CHAMBERS AND LABORATORY APPARATUS

5.2.1 New plasticware used for effluent or dilution water collection or organism test chambers does not require thorough cleaning before use. It is sufficient to rinse new sample containers once with sample dilution water before use. New glassware must be soaked overnight in 10% acid (see below) and rinsed well in deionized water and dilution water.

5.2.2 All non-disposable sample containers, test vessels, tanks, and other equipment that has come in contact with effluent must be washed after use in the manner described below to remove surface contaminants as described below:

1. Soak 15 min in tap water, and scrub with detergent, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh, dilute (10%, V:V) hydrochloric or nitric acid to remove scale, metals, and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

5.2.3 All test chambers and equipment should be thoroughly rinsed with the dilution water immediately prior to use in each test.

5.3 APPARATUS AND EQUIPMENT FOR CULTURING AND TOXICITY TESTS

5.3.1 Culture units -- see Appendix. It is preferable to obtain test organisms from in-house culture units. If it is not feasible to maintain cultures in-house, test organisms can be obtained from commercial sources, and should be shipped to the laboratory in well oxygenated water in insulated containers to minimize excursions in water temperature during shipment. The temperature of the water in the shipping containers should be measured on arrival, to determine if the organisms were subjected to obvious undue thermal stress.

5.3.2 Samplers -- automatic samplers, preferably with sample cooling capability, that can collect a 24-h composite sample of 2 L or more.

5.3.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

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5.3.4 Environmental chamber or equivalent facility with temperature control (20EC or 25EC)

5.3.5 Water purification system -- MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$, or equivalent. Depending on the quantity of high grade water needed, a first-stage pre-conditioner deionizer, such as a Culligan® or Continental® System, or equivalent, may be needed to provide feed water to the high-purity system.

5.3.6 Balance -- analytical, capable of accurately weighing to 0.0001 g.

5.3.7 Reference weights, Class S -- for documenting the performance of the analytical balance(s). The balance(s) should be checked with reference weights which are at the upper and lower ends of the range of the weighings made when the balance is used. A balance should be checked at the beginning of each series of weighings, periodically (such as every tenth weight) during a long series of weighings, and after the last weight of a series is taken.

5.3.8 Test chambers -- borosilicate glass or non-toxic disposable plastic test chambers are suitable. Test chamber volumes are indicated in the method summaries. To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic, 6 mm (¼ in) thick.

5.3.9 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

5.3.10 Volumetric pipets -- Class A, 1-100 mL.

5.3.11 Serological pipets -- 1-10 mL, graduated.

5.3.12 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

5.3.13 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring test organisms.

5.3.14 Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

5.3.15 Glass or electronic thermometers -- for measuring water temperature.

5.3.16 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

5.3.17 National Bureau of Standards Certified thermometer (see USEPA Method 170.1; USEPA 1979b).

5.3.18 pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

5.3.19 Refractometer -- for measuring effluent, receiving, and test solution salinity.

5.3.20 Amperometric titrator -- for measuring total residual chlorine.

5.4 REAGENTS AND CONSUMABLE MATERIALS

5.4.1 Reagent water -- defined as MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$ or equivalent water (see Subsection 5.3.5 above).

5.4.2 Effluent, dilution water, and receiving water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling and Sample Handling.

5.4.3 Reagents for hardness and alkalinity tests (see USEPA Methods 130.2 and 310.1; USEPA l979b).

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5.4.4 Standard pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for instrument calibration (see USEPA Method 150.1; USEPA 1979b).

5.4.5 Specific conductivity and salinity standards (see USEPA Method 120.1; USEPA 1979b).

5.4.6 Laboratory quality control check samples and standards for the above chemistry methods.

5.4.7 Reference toxicant solutions (see Section 4, Quality Assurance).

5.4.8 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1; USEPA 1979b), or reagents for modified Winkler analysis.

5.4.9 Sources of Food for Cultures and Toxicity Tests.

5.4.9.1 All food should be tested for nutritional suitability, and chemically analyzed for organic chlorine, PCBs, and toxic metals (see Section 4, Quality Assurance).

5.4.9.2 Brine Shrimp (*Artemia*) -- see Appendix A.

1. Brine Shrimp (*Artemia*) Cysts.

   There are many commercial sources of brine shrimp cysts. The quality of the cysts may vary from one batch to another, and the cysts in each new batch (can or lot) should be evaluated for nutritional suitability and chemical contamination. The nutritional suitability (see Leger et al., 1985, 1986) of each new batch is checked against known suitable reference cysts by performing a side-by-side growth and/or reproduction tests using the "new" and "reference" cysts. If the results of tests for nutritional suitability or chemical contamination do not meet standards, the *Artemia* should not be used.

2. Frozen Adult Brine Shrimp

   Frozen adult brine shrimp are available from pet stores and other commercial sources.

5.4.9.3 Trout Chow

Starter or No. 1 pellets, prepared according to current U.S. Fish and Wildlife Service specifications, are available from commercial sources. (The flake food, TETRAMIN® or BIORIL®, can be used regularly as a substitute for trout chow in preparing food for daphnids, and can be used as a short-term substitute for trout chow in feeding fathead minnows.)

5.4.9.4 Dried, Powdered Leaves (CEROPHYLL®)

Dried, powdered, cereal leaves (e.g., CEROPHYLL® or equivalent) are available from commercial suppliers. Dried, powdered, alfalfa leaves obtained from health food stores have been found to be a satisfactory substitute for cereal leaves.

5.4.9.5 Yeast

Packaged dry yeast, such as Fleischmann's, or equivalent, can be purchased at the local grocery store.

5.4.9.6 Flake Fish Food

The flake foods, TETRAMIN® and BIORIL®, are available at most pet supply shops.

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5.5 TEST ORGANISMS

5.5.1 Test organisms are obtained from inhouse cultures or commercial suppliers (see Section 6, Test Organisms).

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SECTION 6

TEST ORGANISMS

6.1 TEST SPECIES

6.1.1 The species used in characterizing the acute toxicity of effluents and/or receiving waters will depend on the requirements of the regulatory authority and the objectives of the test. It is essential that good quality test organisms be readily available throughout the year from inhouse or commercial sources to meet NPDES monitoring requirements. The organisms used in toxicity tests must be identified to species. If there is any doubt as to the identity of the test organisms, representative specimens should be sent to a taxonomic expert to confirm the identification.

6.1.2 Toxicity test conditions and culture methods are provided in this manual for the following principal test organisms:

Freshwater Organisms:

1. *Ceriodaphnia dubia* (daphnid) (Table 12).
2. *Daphnia pulex* and *D. magna* (daphnids) (Table 13).
3. *Pimephales promelas* (fathead minnow) (Table 14).
4. *Oncorhynchus mykiss* (rainbow trout) and *Salvelinus fontinalis* (brook trout) (Table 15).

Estuarine and Marine Organisms:

1. *Mysidopsis bahia* (mysid) (Table 16).[1]
2. *Cyprinodon variegatus* (sheepshead minnow) (Table 17).
3. *Menidia beryllina* (inland silverside), *M. menidia* (Atlantic silverside), and *M. peninsulae* (tidewater silverside) (Table 18).

6.1.3 The test species (AFS, 1991) listed in Subsection 6.1.2 are the recommended acute toxicity test organisms. They are easily cultured in the laboratory, are sensitive to a variety of pollutants, and are generally available throughout the year from commercial sources. Summaries of test conditions for these species are provided in Tables 12-18. Guidelines for culturing and/or holding the organisms are provided in Appendix A.

6.1.4 Additional species may be suitable for toxicity tests in the NPDES Program. A list of alternative acute toxicity test species and minimal testing requirements (i.e., temperature, salinity, and life stage) for these species are provided in Appendix B. Table 19 provides a summary of test conditions for *Holmesimysis costata*, which should also be considered an alternative acute toxicity test species. The *Holmesimysis costata* Acute Test (Table 19) is specific to Pacific Coast waters and is not listed at 40 CFR Part 136 for nationwide use. It is important to note that these species may not be as easily cultured or tested as the species on the list in 6.1.2, and may not be available from commercial sources.

6.1.5 Some states have developed culturing and testing methods for indigenous species that may be as sensitive or more sensitive than the species recommended in 6.1.2. However, EPA allows the use of indigenous species only where state regulations require their use or prohibit importation of the species in 6.1.2. Where state regulations prohibit importation or use of the recommended test species, permission must be requested from the appropriate state agency prior to their use.

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[1] The genus name of this organism was formally changed to *Americamysis* (Price *et al.*, 1994); however, the method manual will continue to refer to *Mysidopsis bahia* to maintain consistency with previous versions of the method.

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6.1.6 Where states have developed culturing and testing methods for indigenous species other than those recommended in this manual, data comparing the sensitivity of the substitute species and one or more of the recommended species must be obtained in side-by-side toxicity tests with reference toxicants and/or effluents, to ensure that the species selected are at least as sensitive as the recommended species. These data must be submitted to the permitting authority (State or Region) if required. EPA acknowledges that reference toxicants prepared from pure chemicals may not always be representative of effluents. However, because of the observed and/or potential variability in the quality and toxicity of effluents, it is not possible to specify a representative effluent.

6.1.7 Guidance for the selection of test organisms where the salinity of the effluent and/or receiving water requires special consideration is provided in the Technical Support Document for Water Quality-Based Toxics Control (USEPA, 1991c).

1. Where the salinity of the receiving water is <1‰, freshwater organisms are used regardless of the salinity of the effluent.
2. Where the salinity of the receiving water is $1‰, the choice of organisms depends on state water quality standards and/or permit requirements.

6.2 SOURCES OF TEST ORGANISMS

6.2.1 INHOUSE CULTURES

6.2.1.1 Inhouse cultures should be established wherever it is cost effective. If inhouse cultures cannot be maintained, test organisms should be purchased from experienced commercial suppliers (see Appendix for sources).

6.2.2 COMMERCIAL SUPPLIERS

6.2.2.1 All of the principal test organisms listed in Subsection 6.1.2 are available from commercial suppliers.

6.2.3 FERAL (NATURAL OCCURRING, WILD CAUGHT) ORGANISMS

6.2.3.1 The use of test organisms taken from the receiving water has strong appeal, and would seem to be the logical approach. However, it is impractical for the following reasons:

1. Sensitive organisms may not be present in the receiving water because of previous exposure to the effluent or other pollutants.
2. It is often difficult to collect organisms of the required age and quality from the receiving water;
3. Most states require collection permits, which may be difficult to obtain. Therefore, it is usually more cost effective to culture the organisms in the laboratory or obtain them from private, state, or Federal sources. Fish such as fathead minnows, sheepshead minnows, and silversides, and invertebrates such as daphnids and mysids, are easily reared in the laboratory or purchased.
4. The required QA/QC records, such as the single laboratory precision data, would not be available.
5. Since it is mandatory that the identity of test organisms is known to the species level, it would necessary to examine each organism caught in the wild to confirm its identity, which would usually be impractical or, at the least, very stressful to the organisms.
6. Test organisms obtained from the wild must be observed in the laboratory for a minimum of one week prior to use, to assure that they are free of signs of parasitic or bacterial infections and other adverse effects. Fish captured by electroshocking must not be used in toxicity testing.

6.2.3.2 Guidelines for collection of feral organisms are provided in USEPA, 1973; USEPA 1990a.

6.2.4 Regardless of their source, test organisms should be carefully observed to ensure that they are free of signs of stress and disease, and in good physical condition. Some species of test organisms, such as trout, can be obtained from stocks certified as "disease-free."

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6.3 LIFE STAGE

6.3.1 Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as first instars of daphnids and juvenile mysids and fish, is required for all tests. In a given test, all organisms should be approximately the same age and should be taken from the same source. Since age may affect the results of the tests, it would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility.

6.4 LABORATORY CULTURING

6.4.1 Instructions for culturing and/or holding the recommended test organisms are included in Appendix A.

6.5 HOLDING AND HANDLING TEST ORGANISMS

6.5.1 Test organisms should not be subjected to changes of more than 3EC in water temperature or 3‰ in salinity in any 12 h period.

6.5.2 Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch dry surfaces or are injured during handling must be discarded. Dipnets are best for handling larger organisms. These nets are commercially available or can be made from small-mesh nylon netting, silk bolting cloth, plankton netting, or similar material. Wide-bore, smooth glass tubes (4 to 8 mm inside diameter) with rubber bulbs or pipettors (such as a PROPIPETTE® or other pipettor) should be used for transferring smaller organisms such as daphnids, mysids, and larval fish.

6.5.3 Holding tanks for fish are supplied with a good quality water (see Section 5, Facilities and Equipment) with a flow-through rate of at least two tank-volumes per day. Otherwise, use a recirculation system where the water flows through an activated carbon or undergravel filter to remove dissolved metabolites. Culture water can also be piped through high intensity ultraviolet light sources for disinfection, and to photodegrade dissolved organics.

6.5.4 Crowding should be avoided. The DO must be maintained at a minimum of 4.0 mg/L for marine and warm water, freshwater species, and 6.0 mg/L for cold-water, freshwater species. The solubility of oxygen depends on temperature, salinity, and altitude. Aerate if necessary.

6.5.5 Fish should be fed as much as they will eat at least once a day with live or frozen brine shrimp or dry food (frozen food should be completely thawed before use). Brine shrimp can be supplemented with commercially prepared food such as Tetramin® or BioRil® flake food, or equivalent. Excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning.

6.5.6 Fish should be observed carefully each day for signs of disease, stress, physical damage, and mortality. Dead and abnormal specimens should be removed as soon as observed. It is not uncommon to have some fish (5-10%) mortality during the first 48 h in a holding tank because of individuals that refuse to feed on artificial food and die of starvation.

6.5.7 A daily record of feeding, behavioral observations, and mortality should be maintained.

6.6 TRANSPORTATION TO THE TEST SITE

6.6.1 Organisms are transported from the base or supply laboratory to a remote test site in culture water or standard dilution water in plastic bags or large-mouth screw-cap (500 mL) plastic bottles in styrofoam coolers. Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags. Another method commonly used to maintain sufficient DO during shipment is to aerate with an airstone which is supplied from a portable pump. The DO concentration must not fall below 4.0 mg/L for marine and warm-water, freshwater species, and 6.0 mg/L for cold-water, freshwater species.

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6.6.2 Upon arrival at the test site, organisms are transferred to receiving water if receiving water is to be used as the test dilution water. All but a small volume of the holding water (approximately 5%) is removed by siphoning, and replaced slowly over a 10 to 15 min period with dilution water. If receiving water is used as dilution water, caution must be exercised in exposing the test organisms to it, because of the possibility that it might be toxic. For this reason, it is recommended that only approximately 10% of the test organisms be exposed initially to the dilution water. If this group does not show excessive mortality or obvious signs of stress in a few hours, the remainder of the test organisms are transferred to the dilution water.

6.6.3 A group of organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed, or if mortality appears to exceed 10% preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. The mortality may be due to the presence of toxicity, if receiving water is used as dilution water, rather than a diseased condition of the test organisms. If the acclimation process is repeated with a new group of test organisms and excessive mortality occurs, it is recommended that an alternative source of dilution water be used.

6.6.4 In static tests, marine organisms can be used at all concentrations of effluent by adjusting the salinity of the effluent to a standard salinity (such as 25‰) or to the salinity approximating that of the receiving water, by adding sufficient dry ocean salts, such as Forty Fathoms®, or equivalent, GP2 or hypersaline brine.

6.6.5 Saline dilution water can be prepared with deionized water or a freshwater such as well water or a suitable surface water. If dry ocean salts are used, care must be taken to ensure that the added salts are completely dissolved and the solution is aerated 24 h before the test organisms are placed in the solutions. The test organisms should be acclimated in synthetic saline water prepared with the dry salts. Caution: addition of dry ocean salts to dilution water may result in an increase in pH. (The pH of estuarine and coastal saline waters is normally 7.5-8.3.)

6.6.6 All effluent concentrations and the control(s) used in a test should have the same salinity. However, if this is impractical because of the large volumes of water required, such as in flow-through tests, the highest effluent concentration (lowest salinity) that could be tested would depend upon the salinity of the receiving water and the tolerance of the test organisms. The required salinities for toxicity tests with estuarine and marine species are listed in Tables 16-19. However, the tolerances of other candidate test species would have to be determined by the investigator in advance of the test.

6.6.7 Because of the circumstances described above, when performing flow-through tests of effluents discharged to saline waters, it is advisable to acclimate groups of test organisms to each of three different salinities, such as 10, 20, and 30‰, prior to transporting them to the test site. It may also be advisable to maintain cultures of these test organisms at a series of salinity levels, including at least 10, 20, and 30‰, so that the change in salinity upon acclimation at the desired test dilutions does not exceed 6‰.

6.7 TEST ORGANISM DISPOSAL

6.7.1 When the toxicity test is concluded, all test organisms (including controls) should be humanely destroyed and disposed of in an appropriate manner.

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SECTION 7

DILUTION WATER

7.1 TYPES OF DILUTION WATER

7.1.1 The type of dilution water used in effluent toxicity tests will depend largely on the objectives of the study:

7.1.1.1 If the objective of the test is to estimate the absolute acute toxicity of the effluent, a synthetic (standard) dilution water is used. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.2 If the objective of the test is to estimate the acute toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of receiving water (if non-toxic), collected either upstream and outside the influence of the outfall, or with other uncontaminated natural water (ground or surface water) or standard dilution water having approximately the same characteristics (hardness and/or salinity) as the receiving water. Seasonal variations in the quality of surface waters may affect effluent toxicity. Therefore, the hardness of fresh receiving water, and the salinity of saline receiving water samples should be determined before each use. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.3 If the objective of the test is to determine the additive or mitigating effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected immediately upstream or outside the influence of the outfall. A second set of controls, using culture water, should be included in the test.

7.1.2 An acceptable dilution water is one which is appropriate for the objectives of the test; supports adequate performance of the test organisms with respect to survival, growth, reproduction, or other responses that may be measured in the test (i.e., consistently meets test acceptability criteria for control responses); is consistent in quality; and does not contain contaminants that could produce toxicity. Receiving waters, synthetic waters, or synthetic waters adjusted to approximate receiving water characteristics may be used for dilution provided that the water meets the above listed qualifications for an acceptable dilution water. USEPA (2000a) provides additional guidance on selecting appropriate dilution waters.

7.1.3 When dual controls (one control using culture water and one control using dilution water) are used (see Subsections 7.1.1.1 - 7.1.1.3 above), the dilution water control should be used to determine test acceptability. It is also the dilution water control that should be compared to effluent treatments in the calculation and reporting of test results. The culture water control should be used to evaluate the appropriateness of the dilution water source. Significant differences between organism responses in culture water and dilution water controls could indicate toxicity in the dilution water and may suggest an alternative dilution water source. USEPA (2000a) provides additional guidance on dual controls.

7.2 STANDARD, SYNTHETIC DILUTION WATER

7.2.1 Standard, synthetic, dilution water is prepared with deionized water and reagent grade chemicals or mineral water (Tables 7 and 8) and commercial sea salts (FORTY FATHOMS®, HW MARINEMIX®) (Table 9). The source water for the deionizer can be groundwater, or tap water.

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7.2.2 DEIONIZED WATER USED TO PREPARE STANDARD, SYNTHETIC, DILUTION WATER

7.2.2.1 Deionized water is obtained from a MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$, or equivalent system. It is advisable to provide a preconditioned (deionized) feed water by using a Culligan®, Continental®, or equivalent, system in front of the MILLIPORE® System to extend the life of the MILLIPORE® cartridges.

7.2.2.2 The recommended order of the cartridges in a four-cartridge deionizer (i.e., MILLI-Q® System or equivalent) is: (1) ion exchange, (2) ion exchange, (3) carbon, and (4) organic cleanup (such as ORGANEX-Q®, or equivalent), followed by a final bacteria filter. The QPAK™$_2$ water system is a sealed system which does not allow for the rearranging of the cartridges. However, the final cartridge is an ORGANEX-Q® filter, followed by a final bacteria filter. Commercial laboratories using this system have not experienced any difficulty in using the water for culturing or testing. Reference to the MILLI-Q® systems throughout the remainder of the manual includes all MILLIPORE® or equivalent systems.

7.2.3 STANDARD, SYNTHETIC FRESHWATER

7.2.3.1 To prepare 20 L of standard, synthetic, moderately hard, reconstituted water, use the reagent grade chemicals in Table 7 as follows:

1. Place 19 L of MILLI-Q®, or equivalent, deionized water in a properly cleaned plastic carboy.
2. Add 1.20 g of $MgSO_4$, 1.92 g $NaHCO_3$, and 0.080g KCl to the carboy.
3. Aerate overnight.
4. Add 1.20 g of $CaSO_4 \cdot 2\ H_2O$ to 1 L of MILLI-Q® or equivalent deionized water in a separate flask. Stir on magnetic stirrer until calcium sulfate is dissolved, add to the 19 L above, and mix well.
5. For *Ceriodaphnia* culture and testing, add sufficient sodium selenate (Na_2SeO_4) to provide 2 µg selenium per liter of final dilution water.
6. Aerate the combined solution vigorously for an additional 24 h to dissolve the added chemicals and stabilize the medium.
7. The measured pH, hardness, etc., should be as listed in Table 7.

7.2.3.2 To prepare 20 L of standard, synthetic, moderately hard, reconstituted water, using 20% mineral water such as PERRIER® Water, or equivalent (Table 8), follow the instructions below.

1. Place 16 L of MILLI-Q® or equivalent deionized water in a properly cleaned plastic carboy.
2. Add 4 L of PERRIER® Water, or equivalent.
3. Aerate vigorously for 24 h to stabilize the medium.
4. The measured pH, hardness, and alkalinity of the aerated water will be as indicated in Table 8.
5. This synthetic water is referred to as diluted mineral water (DMW) in the toxicity test methods.

7.2.4 STANDARD, SYNTHETIC SEAWATER

7.2.4.1 To prepare 20 L of a standard, synthetic, reconstituted seawater (modified GP2), with a salinity of 31‰ (Table 9), follow the instructions below. Other salinities can be prepared by making the appropriate dilutions.

1. Place 20 L of MILLI-Q® or equivalent deionized water in a properly cleaned plastic carboy.
2. Weigh reagent grade salts listed in Table 9 and add, one at a time, to the deionized water. Stir well after adding each salt.
3. Aerate the final solution at a rate of 1 L/h for 24 h.
4. Check the pH and salinity.

Larger or smaller volumes of modified GP2 can be prepared by using proportionately larger or smaller amounts of salts and dilution water.

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7.2.4.2 Synthetic seawater can also be prepared by adding commercial sea salts, such as FORTY FATHOMS®, HW MARINEMIX® or equivalent, to deionized water. For example, thirty-one parts per thousand (31‰) FORTY FATHOMS® can be prepared by dissolving 31 g of product per liter of deionized water. The salinity of the resulting solutions should be checked with a refractometer.

7.3 USE OF RECEIVING WATER AS DILUTION WATER

7.3.1 If the objectives of the test require the use of uncontaminated surface water as dilution water, and the receiving water is uncontaminated, it may be possible to collect a sample of the receiving water close to the outfall, but upstream from or beyond the influence of the effluent. However, if the receiving water is contaminated, it may be necessary to collect the sample in an area "remote" from the discharge site, matching as closely as possible the physical and chemical characteristics of the receiving water near the outfall.

7.3.2 The sample should be collected immediately prior to the test, but never more than 96 h before the test begins. Except where it is used within 24 h, or in the case where large volumes are required for flow-through tests, the sample should be chilled to 4°C during or immediately following collection, and maintained at that temperature prior to use in the test.

7.3.3 In the case of freshwaters, the regulatory authority may require that the hardness of the dilution water be comparable to the receiving water at the discharge site. This requirement can be satisfied by collecting an uncontaminated surface water with a suitable hardness, or adjusting the hardness of an otherwise suitable surface water by addition of reagents as indicated in Table 7.

7.3.4 In an estuarine environment, the investigator should collect uncontaminated water having a salinity as near as possible to the salinity of the receiving water at the discharge site. Water should be collected at slack high tide, or within one hour after high tide. If there is reason to suspect contamination of the water in the estuary, it is advisable to collect uncontaminated water from an adjacent estuary. At times it may be necessary to collect water at a location closer to the open sea, where the salinity is relatively high. In such cases, deionized water or uncontaminated freshwater is added to the saline water to dilute it to the required test salinity. Where necessary, the salinity of a surface water can be increased by the addition of artificial sea salts, such as FORTY FATHOMS® or equivalent, a natural seawater of higher salinity, or hypersaline brine. Instructions for the preparation of hypersaline brine by concentrating natural seawater are provided below.

7.3.5 Receiving water containing debris or indigenous organisms, that may be confused with or attack the test organisms, should be filtered through a sieve having 60 µm mesh openings prior to use.

TABLE 7. PREPARATION OF SYNTHETIC FRESHWATER USING REAGENT GRADE CHEMICALS[1]

	Reagent Added (mg/L)[2]				Approximate Final Water Quality		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	pH[3]	Hardness[4]	Alkalinity[4]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	57-64
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

[1]Taken in part from Marking and Dawson (1973).
[2]Add reagent grade chemicals to deionized water.
[3]Approximate equilibrium pH after 24 h of aeration.
[4]Expressed as mg $CaCO_3$/L.

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TABLE 8. PREPARATION OF SYNTHETIC FRESHWATER USING MINERAL WATER[1]

Water Type	Volume of Mineral Water Added (mL/L)[2]	Proportion of Mineral Water (%)	Approximate Final Water Quality		
			pH[3]	Hardness[4]	Alkalinity[4]
Very Soft	50	2.5	7.2-8.1	10-13	10-13
Soft	100	10.0	7.9-8.3	40-48	30-35
Moderately Hard	200	20.0	7.9-8.3	80-100	57-64
Hard	400	40.0	7.9-8.3	160-180	110-120
Very Hard[5]	---	---	---	---	---

[1]From Mount et al., 1987; data provided by Philip Lewis, EMSL-Cincinnati.
[2]Add mineral water to MILLI-Q® water or equivalent to prepare DMW (Diluted Mineral Water).
[3]Approximate equilibrium pH after 24 h of aeration.
[4]Expressed as mg $CaCO_3$/L.
[5]Dilutions of PERRIER® Water form a precipitate when concentrations equivalent to "very hard water" are aerated.

TABLE 9. PREPARATION OF SYNTHETIC SEAWATER USING REAGENT GRADE CHEMICALS[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
Kcl	0.61	12.2
Kbr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1]Modified GP2.
[2]The constituent salts and concentrations were taken from USEPA, 1990b. The salinity is 30.89 G/L.
[3]GP2 can be diluted with deionized (DI) water to the desired test salinity.

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7.3.6 When receiving water is used as dilution water in flow-through tests, it is preferable to pump the dilution water continuously to the acclimation chamber and/or dilutor. However, where it is not feasible to pump the dilution water continuously, grab samples of the dilution water are transported to the test site in tanks, and continuously pumped from the tanks to the acclimation chamber and/or dilutor.

7.3.7 HYPERSALINE BRINE

7.3.7.1 Hypersaline brine (HSB) has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to deionized water to prepare dilution water, or to effluents or surface waters to increase their salinity.

7.3.7.2 The ideal container for making HSB from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is used, use only oil-free air compressors to prevent contamination.

7.3.7.3 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several thorough deionized water rinses. High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

7.3.7.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

7.3.7.5 After the required salinity is attained, the HSB should be filtered a second time through a 1-Fm filter and poured directly into portable containers (20 L CUBITAINERS® or polycarbonate water cooler jugs are suitable). The containers should be capped and labeled with the date the brine was generated and its salinity. Containers of HSB should be stored in the dark and maintained under room temperature until used.

7.3.7.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and brine before mixing in the effluent.

7.3.7.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 25‰, 100‰ divided by 25‰ = 4.0. The proportion of brine is 1 part in 4 (one part brine to three parts deionized water).

7.3.7.8 To make 1 L of seawater at 25‰ salinity from a hypersaline brine of 100‰, 250 mL of brine and 750 mL of deionized water are required.

7.4 USE OF TAP WATER AS DILUTION WATER

7.4.1 The use of tap water as dilution water is discouraged unless it is dechlorinated and fully treated. Tap water can be dechlorinated by deionization, carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg/L (anhydrous) sodium thiosulfate will reduce 1.0 mg chlorine/L (APHA, 1992, p. 4-36). Following dechlorination, total residual chlorine should not exceed 0.01 mg/L. Because of the possible toxicity of thiosulfate to test organisms, a control lacking thiosulfate should be included in toxicity tests utilizing thiosulfate-dechlorinated water.

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7.4.2 To be adequate for general laboratory use following dechlorination, the tap water is passed through a deionizer and carbon filter to remove toxic metals and organics, and to control hardness and alkalinity.

7.5 DILUTION WATER HOLDING

7.5.1 A given batch of dilution water should not be used for more than 14 days following preparation because of the possible build-up of bacterial, fungal, or algal slime growth and the problems associated with it. The container should be kept covered and the contents should be protected from light.

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SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING AND SAMPLE HANDLING

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point is ordinarily the same as that specified in the NPDES discharge permit (USEPA, 1979c). Conditions for exception would be: (1) better access to a sampling point between the final treatment and the discharge outfall; (2) if the effluent is chlorinated prior to discharge to the receiving waters, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to publicly owned treatment works or separate process waters in industrial facilities prior to their being combined with other process waters or non-contact cooling water, additional sampling points may be chosen.

8.1.2 The decision on whether to collect grab or composite samples is based on the requirements of the NPDES permit, the objectives of the test, and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times within the treatment facility are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitive number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and average the quality of the effluent over the sampling period. Sampling recommendations are provided below.

8.1.3 Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

8.1.4 Details of date, time, location, duration, and procedures used for effluent sample and dilution water collection should be recorded.

8.2 EFFLUENT SAMPLE TYPES

8.2.1 The advantages and disadvantages of effluent grab and composite samples are listed below:

8.2.1.1 Grab Samples

Advantages:

1. Easy to collect; require a minimum of equipment and on-site time.
2. Provide a measure of instantaneous toxicity. Toxicity spikes are not masked by dilution.

Disadvantages:

1. Samples are collected over a very short period of time and on a relatively infrequent basis. The chances of detecting a spike in toxicity would depend on the frequency of sampling, and the probability of missing spikes is high.

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8.2.1.2 Composite Samples:

Advantages:

1. A single effluent sample is collected over a 24-h period.
2. The sample is collected over a much longer period of time than grab samples and contains all toxicity spikes.

Disadvantages:

1. Sampling equipment is more sophisticated and expensive, and must be placed on-site for at least 24 h.
2. Toxicity spikes may not be detected because they are masked by dilution with less toxic wastes.

8.3 EFFLUENT SAMPLING RECOMMENDATIONS

8.3.1 When tests are conducted on-site, test solutions can be renewed daily with freshly collected samples.

8.3.2 When tests are conducted off-site, samples are collected once, or daily, and used for test initiation and renewal.

8.3.3 Sufficient sample must be collected to perform the required toxicity and chemical tests. A 4-L (1-gal) CUBITAINER® will provide sufficient sample volume for most tests (see Tables 12-19).

8.3.4 The following effluent sampling methods are recommended:

8.3.4.1 Continuous Discharges

1. If the facility discharge is continuous, but the calculated retention time of the continuously discharged effluent is less than 14 days and the variability of the effluent toxicity is unknown, at a minimum, four grab samples or four composite samples are collected over a 24-h period. For example, a grab sample is taken every 6 h (total of four samples) and each sample is used for a separate toxicity test, or four successive 6-h composite samples are taken and each is used in a separate test.

2. If the calculated retention time of a continuously discharged effluent is greater than 14 days, or if it can be demonstrated that the wastewater does not vary more than 10% in toxicity over a 24-h period, regardless of retention time, a single grab sample is collected for a single toxicity test.

3. The retention time of the effluent in the wastewater treatment facility may be estimated from calculations based on the volume of the retention basin and rate of wastewater inflow. However, the calculated retention time may be much greater than the actual time because of short-circuiting in the holding basin. Where short-circuiting is suspected, or sedimentation may have reduced holding basin capacity, a more accurate estimate of the retention time can be obtained by carrying out a dye study.

8.3.4.2 Intermittent Discharges

8.3.4.2.1 If the facility discharge is intermittent, a grab sample is collected midway during each discharge period. Examples of intermittent discharges are:

1. When the effluent is continuously discharged during a single 8-h work shift (one sample is collected), or two successive 8-h work shifts (two samples are collected).

2. When the facility retains the wastewater during an 8-h work shift, and then treats and releases the wastewater as a batch discharge (one sample is collected).

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3. When the facility discharges wastewater to an estuary only during an outgoing tide, usually during the 4 h following slack high tide (one sample is collected).

8.3.4.3 At the end of a shift, clean up activities may result in the discharge of a slug of toxic waste, which may require sampling and testing.

8.4 RECEIVING WATER SAMPLING

8.4.1 Logistical problems and difficulty in securing sampling equipment generally preclude the collection of composite receiving water samples for toxicity tests. Therefore, it is common practice to collect a single grab sample and use it throughout the test.

8.4.2 The sampling point is determined by the objectives of the test. In rivers, grab samples should be collected at mid-stream and mid-depth, if accessible. At estuarine and marine sites, samples should be collected at mid-depth.

8.4.3 To determine the extent of the zone of toxicity in the receiving water downstream from the outfall, receiving water samples are collected at several distances downstream from the discharge. The time required for the effluent-receiving-water mixture to travel to sampling points downstream from the outfall, and the rate and degree of mixing, may be difficult to ascertain. Therefore, it may not be possible to correlate downstream toxicity with effluent toxicity at the discharge point unless a dye study is performed. The toxicity of receiving water samples from five stations downstream from the discharge point can be evaluated using the same number of test vessels and test organisms as used in one effluent toxicity test with five effluent dilutions.

8.5 EFFLUENT AND RECEIVING WATER SAMPLE HANDLING, PRESERVATION, AND SHIPPING

8.5.1 Unless the samples are used in an on-site toxicity test the day of collection (or hand delivered to the testing laboratory for use on the day of collection), it is recommended that they be held at 0-6°C until used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances.

8.5.2 Composite samples should be chilled as they are collected. Grab samples should be chilled immediately following collection.

8.5.3 If the effluent has been chlorinated, total residual chlorine must be measured immediately following sample collection.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of each grab or composite sample must not exceed 36 h. EPA believes that 36 h is adequate time to deliver the samples to the laboratories performing the tests in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the USEPA Regional Administrator under 40 CFR 136.3(e) should include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However, in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, each grab or composite sample may also be used to prepare test solutions for renewal at 24 h, 48 h, and/or 72 h after first use, if stored at 0-6°C, with minimum head space, as described in Subsection 8.5. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

8.5.5 To minimize the loss of toxicity due to volatilization of toxic constituents, all sample containers should be "completely" filled, leaving no air space between the contents and the lid.

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8.5.6 SAMPLES USED IN ON-SITE TESTS

8.5.6.1 Samples collected for on-site tests should be used within 24 h.

8.5.7 SAMPLES SHIPPED TO OFF-SITE FACILITIES

8.5.7.1 Samples collected for off-site toxicity testing are to be chilled to 0-6°C during or immediately after collection, and shipped iced to the performing laboratory. Sufficient ice should be placed with the sample in the shipping container to ensure that ice will still be present when the sample arrives at the laboratory and is unpacked. Insulating material should not be placed between the ice and the sample in the shipping container unless required to prevent breakage of glass sample containers.

8.5.7.2 Samples may be shipped in one or more 4-L (1 gal) CUBITAINERS® or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. After use with receiving water or effluents, CUBITAINERS® and plastic jugs are punctured to prevent reuse.

8.5.7.3 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. Saturday and Sunday shipping and receiving schedules of private carriers vary with the carrier.

8.6 SAMPLE RECEIVING

8.6.1 Upon arrival at the laboratory, samples are logged in and the temperature is measured and recorded. If the samples are not immediately prepared for testing, they are stored at 0-6°C until used.

8.6.2 Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory, and the sample holding time should not exceed 36 h before first use unless a variance has been granted by the NPDES permitting authority.

8.7 PERSISTENCE OF EFFLUENT TOXICITY DURING SAMPLE SHIPMENT AND HOLDING

8.7.1 The persistence of the toxicity of an effluent prior to its use in a toxicity test is of interest in assessing the validity of toxicity test data, and in determining the possible effects of allowing an extension of the holding time. Where a variance in holding time (>36 h, but −72 h) is requested by a permittee (see Subsection 8.5.4 above), information on the effects of the extension in holding time on the toxicity of the samples must be obtained by comparing the results of multi-concentration acute toxicity tests performed on effluent samples held 36 h with toxicity test results using the same samples after they were held for the requested, longer period. The portion of the sample set aside for the second test must be held under the same conditions as during shipment and holding.

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SECTION 9

ACUTE TOXICITY TEST PROCEDURES

9.1 PREPARATION OF EFFLUENT AND RECEIVING WATER SAMPLES FOR TOXICITY TESTS

9.1.1 When aliquots are removed from the sample container, the head space above the remaining sample should be held to a minimum. Air which enters a container upon removal of sample should be expelled by compressing the container before reclosing, if possible (i.e., where a CUBITAINER® used), or by using an appropriate discharge valve (spigot).

9.1.2 It may be necessary to first coarse-filter samples through a sieve having 2-4 mm mesh openings to remove debris and/or break up large floating or suspended solids. If samples contain indigenous organisms that may attack or be confused with the test organisms, the samples must be filtered through a sieve with 60 µm mesh openings. Caution: filtration may remove some toxicity.

9.1.3 At a minimum, pH, conductivity or salinity, and total residual chlorine are measured in the undiluted effluent or receiving water, and pH and conductivity are measured in the dilution water.

9.1.4 It is recommended that total alkalinity and total hardness also be measured in the undiluted test water (effluent or receiving water) and the dilution water.

9.1.5 Total ammonia is measured in effluent and receiving water samples where toxicity may be contributed by unionized ammonia (i.e., where total ammonia $5 mg/L). The concentration (mg/L) of unionized (free) ammonia in a sample is a function of temperature and pH, and is calculated using the percentage value obtained from Table 10, under the appropriate pH and temperature, and multiplying it by the concentration (mg/L) of total ammonia in the sample.

9.1.6 Effluents and receiving waters can be dechlorinated using 6.7 mg/L anhydrous sodium thiosulfate to reduce 1 mg/L chlorine (Standard Methods, 18th Edition, APHA, 1992, p. 9-32; note that the amount of thiosulfate required to dechlorinate effluents is greater than the amount needed to dechlorinate tap water). Since thiosulfate may contribute to sample toxicity, a thiosulfate control should be used in the test in addition to the normal dilution water control.

9.1.7 The DO concentration in the samples should be near saturation prior to use. Aeration may be used to bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH. However, aeration during collection, transfer, and preparation of samples should be minimized to reduce the loss of volatile chemicals.

9.1.8 If the samples must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved oxygen and nitrogen may become a problem. To avoid this problem, samples may be warmed slowly in open test containers. If DO is still above 100% saturation after warming to test temperature, samples should be aerated moderately (approximately 500 mL/minimum) for a few minutes using an airstone. If DO is below 4.0 mg/L after warming to test temperature, the solutions must be aerated moderately (approximately 500 mL/min) for a few minutes, using an airstone, until the DO is within the prescribed range ($4.0 mg/L when using warm water species, or $6.0 mg/L when using cold water species). Caution: avoid excessive aeration.

9.1.9 Mortality due to pH alone may occur if the pH of the sample falls outside the range of 6.0-9.0. Thus, the presence of other forms of toxicity (metals and organics) in the sample may be masked by the toxic effects of low or high pH. The question about the presence of other toxicants can be answered only by performing two parallel tests, one with an adjusted pH, and one without an adjusted pH. Freshwater samples are adjusted to pH 7.0, and marine samples are adjusted to pH 8.0, by adding 1N NaOH or 1N HCl dropwise, as required, being careful to avoid overadjustment.

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TABLE 10. PERCENT UNIONIZED NH_3 IN AQUEOUS AMMONIA SOLUTIONS: TEMPERATURES 15-26EC AND pH's 6.0-8.9[1]

pH	Temperature (°C)											
	15	16	17	18	19	20	21	22	23	24	25	26
6.0	0.0274	0.0295	0.0318	0.0343	0.0369	0.0397	0.0427	0.0459	0.0493	0.0530	0.0568	0.0610
6.1	0.0345	0.0372	0.0400	0.0431	0.0464	0.0500	0.0537	0.0578	0.0621	0.0667	0.0716	0.0768
6.2	0.0434	0.0468	0.0504	0.0543	0.0584	0.0629	0.0676	0.0727	0.0781	0.0901	0.0901	0.0966
6.3	0.0546	0.0589	0.0634	0.0683	0.0736	0.0792	0.0851	0.0915	0.0983	0.1134	0.1134	0.1216
6.4	0.0687	0.0741	0.0799	0.0860	0.0926	0.0996	0.107	0.115	0.124	0.133	0.143	0.153
6.5	0.0865	0.0933	0.1005	0.1083	0.1166	0.1254	0.135	0.145	0.156	0.167	0.180	0.193
6.6	0.109	0.117	0.127	0.136	0.147	0.158	0.170	0.182	0.196	0.210	0.226	0.242
6.7	0.137	0.148	0.159	0.171	0.185	0.199	0.214	0.230	0.247	0.265	0.284	0.305
6.8	0.172	0.186	0.200	0.216	0.232	0.250	0.269	0.289	0.310	0.333	0.358	0.384
6.9	0.217	0.234	0.252	0.271	0.292	0.314	0.338	0.363	0.390	0.419	0.450	0.482
7.0	0.273	0.294	0.317	0.342	0.368	0.396	0.425	0.457	0.491	0.527	0.566	0.607
7.1	0.343	0.370	0.399	0.430	0.462	0.497	0.535	0.575	0.617	0.663	0.711	0.762
7.2	0.432	0.466	0.502	0.540	0.581	0.625	0.672	0.722	0.776	0.833	0.893	0.958
7.3	0.543	0.586	0.631	0.679	0.731	0.786	0.845	0.908	0.975	1.05	1.12	1.20
7.4	0.683	0.736	0.793	0.854	0.918	0.988	1.061	1.140	1.224	1.31	1.41	1.51
7.5	0.858	0.925	0.996	1.07	1.15	1.24	1.33	1.43	1.54	1.65	1.77	1.89
7.6	1.08	1.16	1.25	1.35	1.45	1.56	1.67	1.80	1.93	2.07	2.21	2.37
7.7	1.35	1.46	1.57	1.69	1.82	1.95	2.10	2.25	2.41	2.59	2.77	2.97
7.8	1.70	1.83	1.97	2.12	2.28	2.44	2.62	2.82	3.02	3.24	3.46	3.71
7.9	2.13	2.29	2.46	2.65	2.85	3.06	3.28	3.52	3.77	4.04	4.32	4.62
8.0	2.66	2.87	3.08	3.31	3.56	3.82	4.10	4.39	4.70	5.03	5.38	5.75
8.1	3.33	3.58	3.85	4.14	4.44	4.76	5.10	5.46	5.85	6.25	6.68	7.14
8.2	4.16	4.47	4.80	5.15	5.52	5.92	6.34	6.78	7.25	7.75	8.27	8.82
8.3	5.18	5.56	5.97	6.40	6.86	7.34	7.85	8.39	8.96	9.56	10.2	10.9
8.4	6.43	6.90	7.40	7.93	8.48	9.07	9.69	10.3	11.0	11.7	12.5	13.3
8.5	7.97	8.54	9.14	9.78	10.45	11.16	11.90	12.7	13.5	14.4	15.2	16.2
8.6	9.83	10.5	11.2	12.0	12.8	13.6	14.5	15.5	16.4	17.4	18.5	19.5
8.7	12.07	12.9	13.8	14.7	15.6	16.6	17.6	18.7	19.8	21.0	22.2	23.4
8.8	14.7	15.7	16.7	17.8	18.9	20.0	21.2	22.5	23.7	25.1	26.4	27.8
8.9	17.9	19.0	20.2	21.4	22.7	24.0	25.3	26.7	28.2	29.6	31.1	32.6

[1]Table provided by Teresa Norberg-King, Environmental Research Laboratory, Duluth, Minnesota. Also see Emerson, et. al., 1975, Thurston, et. al, 1974, and USEPA, 1985.

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9.2 PRELIMINARY TOXICITY RANGE-FINDING TESTS

9.2.1 USEPA Regional and State personnel generally have observed that it is not necessary to conduct a toxicity range-finding test prior to initiating a static, acute, definitive toxicity test. However, when preparing to perform a static test with an sample of completely unknown quality, or before initiating a flow-through test, it is advisable to conduct a preliminary toxicity range-finding test.

9.2.2 A toxicity range-finding test ordinarily consists of a down-scaled, abbreviated static acute test in which groups of five organisms are exposed to several widely-spaced sample dilutions in a logarithmic series, such as 100%, 10.0%, 1.00%, and 0.100%, and a control, for 8-24 h. **Caution**: if the sample must also be used for the full-scale definitive test, the 36-h limit on holding time (Section 8, , Effluent and Receiving Water Sampling and Sample Handling, Subsection 8.5.4) must not be exceeded before the definitive test is initiated.

9.2.3 It should be noted that the toxicity (LC50) of a sample observed in a range-finding test may be significantly different from the toxicity observed in the follow-up definitive test because: (1) the definitive test is usually longer; and (2) the test may be performed with a sample collected at a different time, and possibly differing significantly in the level of toxicity.

9.3 MULTI-CONCENTRATION (DEFINITIVE) EFFLUENT TOXICITY TESTS

9.3.1 The tests recommended for use in determining discharge permit compliance in the NPDES program are multi-concentration, or definitive, tests which provide (1) a point estimate of effluent toxicity in terms of a LC50, or (2) a no-observed-adverse-effect concentration (NOAEC) defined in terms of mortality, and obtained by hypothesis testing. The tests may be static non-renewal, static renewal, or flow-through.

9.3.2 The tests consist of a control and a minimum of five effluent concentrations. USEPA recommends the use of a $0.5 dilution factor for selecting effluent test concentrations. Effluent test concentrations of 6.25%, 12.5%, 25%, 50%, and 100% are commonly used, however, test concentrations should be selected independently for each test based on the objective of the study, the expected range of toxicity, the receiving water concentration, and any available historical testing information on the effluent. USEPA (2000a) provides additional guidance on choosing appropriate test concentrations.

9.3.3 When these tests are used in determining compliance with permit limits, effluent test concentrations should be selected to bracket the receiving water concentration (RWC). This may be achieved by selecting effluent test concentrations in the following manner: (1) 100% effluent, (2) [RWC + 100]/2, (3) RWC, (4) RWC/2, and (5) RWC/4. For example, where the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

9.3.4 If acute/chronic ratios are to be determined by simultaneous acute and short-term chronic tests with a single species, using the same sample, both types of tests must use the same test conditions, i.e., temperature, water hardness, salinity, etc.

9.4 RECEIVING WATER TESTS

9.4.1 Receiving water toxicity tests generally consist of 100% receiving water and a control. The total hardness or salinity of the control should be comparable to the receiving water.

9.4.2 The data from the two treatments are analyzed by hypothesis testing to determine if test organism survival in the receiving water differs significantly from the control. A minimum of four replicates and 10 organisms per replicate are required for each treatment (see Tables 12-19).

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9.4.3 In cases where the objective of the test is to estimate the degree of toxicity of the receiving water, a definitive, multi-concentration test is performed by preparing dilutions of the receiving water, using a $0.5 dilution series, with a suitable control water.

9.5 STATIC TESTS

9.5.1 Static tests may be non-renewal or renewal.

9.5.2 An excess volume of each dilution is prepared to provide sufficient material for toxicity testing and routine chemical analyses. The solutions are well mixed with a glass rod, TEFLON® stir bar, or other means. Aliquots of each sample concentration are delivered to the test chambers, and the chambers are arranged in random order. The test solutions are brought to the required temperature, and the test organisms are added. The remaining volumes of each sample concentration are used, as necessary, for the chemical analyses.

9.5.3 Saline dilution water can be prepared by adding dry salts (FORTY FATHOMS® or equivalent, or modified GP2) or hypersaline brine to de-ionized water, or a suitable surface freshwater, to adjust the salinity of the entire dilution series. If saline receiving water is used as the diluent, a salinity control must be prepared using deionized water and dried sea salts to determine if the addition of sea salts alone has an adverse effect on the test organisms. It may be desirable to conduct static toxicity tests at several salinities.

9.5.4 If the effluent has low salinity, but the test is to be conducted with a salt water organism, the test solutions may be prepared by adding dry ocean salts or hypersaline brine to a sufficient quantity of 100% effluent to raise the salinity to the required level, which will depend on the objectives of the test and the policy of the regulatory agency. After the addition of the dried salts, stir gently for 30 to 60 min, preferably with a magnetic stirrer, to ensure that the salts are in solution. It is important to check the final salinity with a refractometer.

9.5.5 Addition of dry salts to effluents and dilution water may change the pH and affect the toxicity of the waste. If the objective of the test is to determine the toxicity of the effluent at the original pH, the pH of the salinity-adjusted solutions can be brought to the required level by dropwise addition of 1N HCl or 1N NaOH. It is recommended that a concurrent test be conducted with salinity-adjusted effluent in which the pH has not been altered after adding the salt.

9.5.6 The volume of the effluent used must be sufficient to prepare all percent concentrations of the effluent needed for the toxicity test and for routine chemical analysis. For example, to conduct tests with *Menidia*, the use of 200 mL of test solution in each of duplicate exposure vessels and five concentrations of effluent (10 exposure vessels), would require a total of 1 L of 100% effluent. However, to provide sufficient volumes of test solutions for routine chemical analysis and for toxicity testing, additional effluent would be required (1.5-2.0 L).

9.5.7 A standard control lacking thiosulfate should be included in tests where the dilution water was prepared by dechlorinating tap water with thiosulfate.

9.5.8 If, within 1 h of the start of the test, 100% mortality has occurred in the higher effluent concentrations (such as 100% and 50%), additional concentrations of effluents, such as 3.1%, 1.6%, and 0.8%, are added to the test at the lower end of the concentration series.

9.5.9 pH drift during acute, static-renewal, or non-renewal toxicity tests may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. This problem can be minimized by conducting a test in a static-renewal mode rather than a non-renewal mode, or the problem can be avoided by conducting the test in a flow-through mode, rather than a static-renewal or non-renewal mode.

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9.6 FLOW-THROUGH TESTS

9.6.1 Flow-through tests are usually performed with the same effluent concentrations that are used for static tests, except that where the receiving water is saline and the effluent is not, 100% effluent cannot be tested with a marine organism. Examples of flow-through test systems are provided in the Appendix. Small organisms, such as mysids and daphnids, are confined in screened enclosures placed in the flow-through chambers. More than one species may be used in the same test chamber in a given test, if segregated.

9.6.2 The dilutor system should be operated long enough prior to adding the test organisms to calibrate the dilutor and make the necessary adjustments in the temperature, flow rate through the test chambers, and aeration. The flow rate through the proportional dilutor must provide for a minimum of five 90% replacements of water volume in each test chamber every 24 h (see Figure 2). This replacement rate should provide sufficient flow to maintain an adequate concentration of dissolved oxygen. The dilutor should also be capable of maintaining the test concentration at each dilution within 5% of the starting concentration for the duration of the test. The calibration of the dilutor should be checked carefully before the test begins to determine the volume of effluent and dilution water used in each portion of the effluent delivery system and the flow rate through each test chamber. The general operation of the dilutor should be checked at least at the beginning and end of each day during the test.

9.6.3 The control consists of the same dilution water, test conditions, procedures, and organisms used in testing the effluent. In the event a test is to be conducted with salt water organisms, where each effluent dilution has a different salinity, a static control is prepared for the lowest (or highest, in the case of high salinity, e.g. brine wastes) salinity level used in the flow-through test to determine if salinity alone has any adverse effects on the test organisms.

9.7 NUMBER OF TEST ORGANISMS

9.7.1 A minimum of 20 organisms of a given species are exposed to each effluent concentration (Jensen, 1972). Small fish and invertebrates are captured with 4- to 8-mm inside diameter pipettes. Organisms larger than 10 mm can be captured by dip net. In a typical toxicity test involving five effluent concentrations and a control (six concentrations x 20 organisms per concentration), fish and other large test organisms are captured from a common pool and distributed sequentially to the test chambers until the required number of organisms are placed in each. The test chambers are then positioned randomly. To avoid carryover of excess culture water in transferring small organisms to the test chambers, it may be advantageous to distribute small organisms, such as daphnids, mysids, and larval fish, first to small holding vessels, such as weighing boats, petri dishes, or small beakers. The water in the intermediary holding vessels is then drawn down to a small volume and the entire lot is transferred to a test chamber. In the case of daphnids, both excessive handling and carryover of culture water and can be avoided by placing the tip of the transfer pipettes below the surface of the water in the test chambers and allowing the organisms to swim out of the pipettes without discharging the contents.

9.8 REPLICATE TEST CHAMBERS

9.8.1 Two or more test chambers are provided for each effluent concentration and the control. Although the data from duplicate chambers are usually combined to determine the LC50 and confidence interval, the practice of dividing the test population for each effluent concentration between two or more replicate chambers has several advantages and is considered good laboratory practice because it: (1) permits easier viewing and counting of test organisms; (2) more easily avoids possible violations of loading limits, which might occur if all of the test organisms are placed in a single test vessel; and (3) ensures against the invalidation of the test which might result from accidental loss of a test vessel, where all of the test organisms for a given treatment are in a single chamber.

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9.9 LOADING OF TEST ORGANISMS

9.9.1 A limit is placed on the loading (weight) of organisms per liter of test solution to minimize the depletion of dissolved oxygen, the accumulation of injurious concentrations of metabolic waste products, and/or stress induced by crowding, any of which could significantly affect the test results. However, the probability of exceeding loading limits is greatly reduced with the use of very young test organisms.

9.9.2 For both renewal and non-renewal static tests, loading in the test solutions must not exceed the following live weights: 1.1 g/L at 15°C, 0.65 g/L at 20°C, or 0.40 g/L at 25°C.

9.9.3 For flow-through tests, the live weight of test organisms in the test chambers must not exceed 7.0 g/L of test solution at 15°C, or 2.5 g/L at 25°C.

9.10 ILLUMINATION

9.10.1 Light of the quality and intensity normally obtained in the laboratory during working hours is adequate (10-20 $\mu E/m^2/s$ or 50-100 ft-c). A uniform photoperiod of 16 h light and 8 h darkness can be achieved in the laboratory or environmental chamber, using automatic timers.

9.11 FEEDING

9.11.1 Where indicated in the test summary tables (Tables 12-19), food is made available to test organisms while holding before they are placed in the test chambers. The organisms are fed at test renewal, 48 h after the test is initiated, if Regional or State policy requires a 96-h test duration.

9.11.2 Where *Artemia* nauplii are fed, the nauplii are first concentrated on a NITEX® screen and then are resuspended in fresh or salt water, depending on the salinity of the test solutions, using just enough water to form a slurry that can be transferred by pipette. It should be noted that *Artemia* nauplii placed in freshwater usually die in 4 h, generally are not eaten after death, and decay rapidly, whereas those placed in saline water remain viable and can serve as food for the duration of the test.

9.11.3 Problems caused by feeding, such as the possible alteration of the toxicant concentration, the build-up of food and metabolic wastes and resulting oxygen demand, are common in static test systems. Where feeding is necessary, excess food should be removed daily by aspirating with a pipette.

9.11.4 Feeding does not cause the above problems in flow-through systems. However, it is advisable to remove excess food, fecal material, and any particulate matter that settles from the effluent, from the bottom of the test vessels daily by aspirating with a pipette.

9.12 TEST TEMPERATURE

9.12.1 Test temperature will depend on the test species and objectives of the test (see Tables 12-19). Where acute and short-term chronic toxicity tests are performed simultaneously with the same species to determine acute:chronic ratios, both tests must be performed at the chronic test temperature. The average daily temperature of the test solutions should be maintained within ±1°C of the selected test temperature, for the duration of the test. This can be accomplished for static tests by use of a water bath or environmental chamber, and in flow-through tests by passing the effluent and/or dilution water through separate coils immersed in a heating or cooling water bath prior to entering the dilutor system. Coils should be made from materials recommended in Section 5, Facilities and Equipment.

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9.13 **STRESS**

9.13.1 Minimize stress on test organisms by avoiding unnecessary disturbances.

9.14 **DISSOLVED OXYGEN CONCENTRATION**

9.14.1 Aeration during the test may alter the results and should be used only as a last resort to maintain the required DO. Aeration can reduce the apparent toxicity of the test solutions by stripping them of highly volatile toxic substances, or increase its toxicity by altering the pH. However, the DO in the test solution should not be permitted to fall below 4.0 mg/L for warm water species and 6.0 mg/L for cold water species. Oxygen saturation values in fresh and saline waters can be determined from Figure 3 and Table 11, respectively.

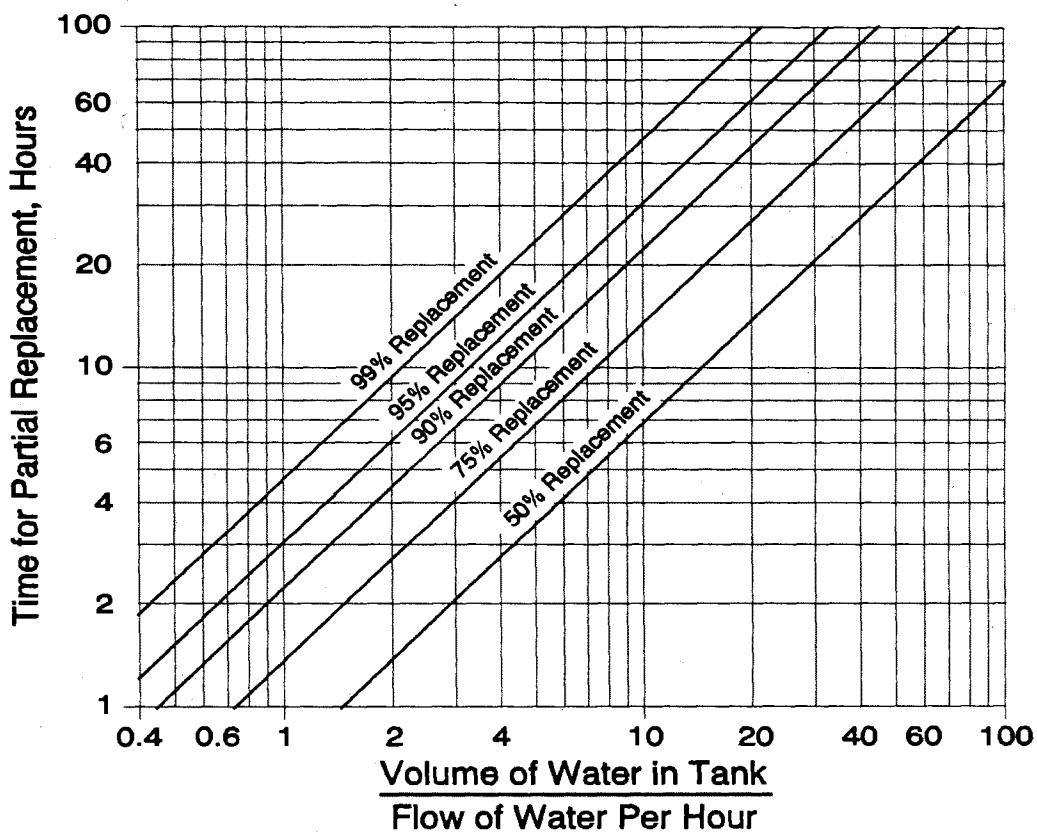

Figure 2. Approximate times required to replace water in test chambers in flow-through tests. For example: for a chamber containing 4 L, with a flow of 2 L/h, the above graph indicates that 90% of the water would be replaced every 4.8 h. The same time period (such as hours) must be used on both axes, and the same unit of volume (such as liters) must be used for both volume and flow (From: Sprague, 1969).

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TABLE 11. OXYGEN SOLUBILITY (MG/L) IN WATER AT EQUILIBRIUM WITH AIR AT 760 MM HG (AFTER RICHARDS AND CORWIN, 1956)

Temp	Salinity (‰)									
°C	0	5	10	15	20	25	30	35	40	45
0	14.2	13.8	13.4	12.9	12.5	12.1	11.7	11.2	10.8	10.6
1	13.8	13.4	13.0	12.6	12.2	11.8	11.4	11.0	10.6	10.3
2	13.4	13.0	12.6	12.2	11.9	11.5	11.1	10.7	10.3	10.0
3	13.1	12.7	12.3	11.9	11.6	11.2	10.8	10.4	10.0	9.8
4	12.7	12.3	12.0	11.6	11.3	10.9	10.5	10.1	9.8	9.5
5	12.4	12.0	11.7	11.3	11.0	10.6	10.2	9.8	9.5	9.3
6	12.1	11.7	11.4	11.0	10.7	10.3	10.0	9.6	9.3	9.1
8	11.5	11.2	10.8	10.5	10.2	9.8	9.5	9.2	8.9	8.7
10	10.9	10.7	10.3	10.0	9.7	9.4	9.1	8.8	8.5	8.3
12	10.5	10.2	9.9	9.6	9.3	9.0	8.7	8.4	8.1	7.9
14	10.0	9.7	9.5	9.2	8.9	8.6	8.3	8.1	7.8	7.6
16	9.6	9.3	9.1	8.8	8.5	8.3	8.0	7.7	7.5	7.3
18	9.2	9.0	8.7	8.5	8.2	8.0	7.7	7.5	7.2	7.1
20	8.9	8.6	8.4	8.1	7.9	7.7	7.4	7.2	6.9	6.8
22	8.6	8.4	8.1	7.9	7.6	7.4	7.2	6.9	6.7	6.6
24	8.3	8.1	7.8	7.6	7.4	7.2	6.9	6.7	6.5	6.4
26	8.1	7.8	7.6	7.4	7.2	7.0	6.7	6.5	6.3	6.1
28	7.8	7.6	7.4	7.2	7.0	6.8	6.5	6.3	6.1	6.0
30	7.6	7.4	7.1	6.9	6.7	6.5	6.3	6.1	5.9	5.8
32	7.3	7.1	6.9	6.7	6.5	6.3	6.1	5.9	5.7	5.6

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CORRECTION FACTORS FOR OXYGEN SATURATION AT VARIOUS ALTITUDES

ALTITUDE		PRESSURE	
FT	M	MM	FACTOR
0	0	760	1.00
330	100	750	1.01
665	200	741	1.03
980	300	732	1.04
1310	400	723	1.05
1640	500	714	1.06
1970	600	705	1.08
2300	700	696	1.09
2630	800	687	1.11
2950	900	679	1.12
3280	1000	671	1.13
3610	1100	663	1.15
3940	1200	655	1.16
4270	1300	647	1.17
4600	1400	639	1.19
4930	1500	631	1.20
5250	1600	623	1.22
5580	1700	615	1.24
5910	1800	608	1.25
6240	1900	601	1.26
6560	2000	594	1.28
6900	2100	587	1.30
7220	2200	580	1.31
7550	2300	573	1.33
7880	2400	566	1.34
8200	2500	560	1.36

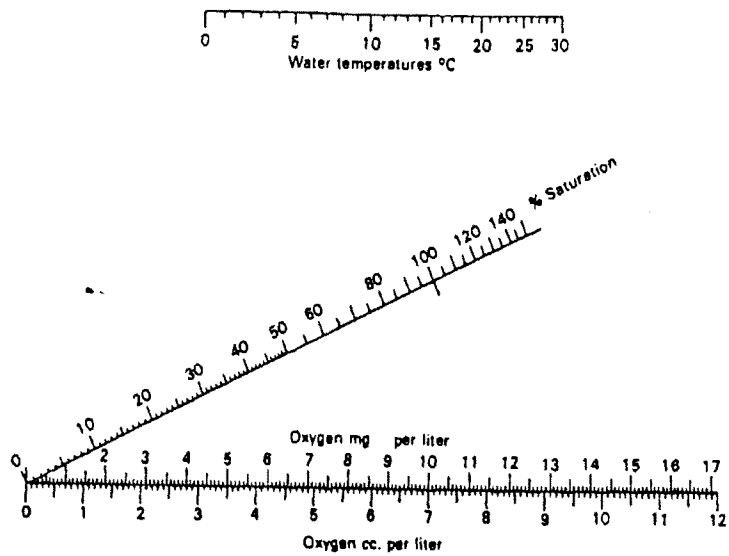

Figure 3. Rawson's nomograph for obtaining oxygen saturation values in freshwater at different temperatures at sea level. When a straightedge is used to connect the water temperature on the upper scale and the concentration on the lower scale, the percent saturation can be read from the point of intersection on the diagonal scale. To determine the percent saturation at locations above sea level, factors are provided to convert oxygen concentrations measured at various altitudes to sea level values in the table at the upper left. For example, an oxygen concentration of 6.4 mg/L measured in a body of water at an altitude of 1000 m and a temperature of 15°C would be equivalent to a concentration of 6.4 x 1.13, or 7.2 mg/L, at sea level. To determine the percent saturation, a straightedge is used to connect the point at 15°C on the temperature scale with the point, 7.2 mg/L on the concentration scale, and the percent saturation is read at the point of intersection (68%) on the diagonal scale. (From Welch, 1948).

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9.14.2 In static tests, low DOs commonly occur in the higher concentrations of wastewater. Aeration is accomplished by bubbling air through a pipet at the rate of 100 bubbles/min. If aeration is necessary, all test solutions must be aerated. It is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test is started. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.

9.14.3 In most flow-through tests, DO depletion is not a problem in the test chambers because aeration occurs as the liquids pass through the dilutor system. If the DO decreases to a level that would be a source of additional stress, the turnover rate of the solutions in the test chambers must be increased sufficiently to maintain acceptable DO levels. If the increased turnover rate does not maintain adequate DO levels, aerate the dilution water prior to the addition of the effluent, and aerate all test solutions. To reduce the potential for driving off volatile compounds in the wastewater, aeration may be accomplished by bubbling air through a 1 mL pipet at a rate of no more than 100 bubbles/min, using an air valve to control the flow.

9.14.4 Caution must be exercised to avoid excessive aeration. Turbulence caused by aeration should not result in a physical stress to the test organisms. When aeration is used, the methodology must be detailed in the report. For safety reasons, pure oxygen should not be used to aerate test solutions.

9.15 TEST DURATION

9.15.1 Test duration may vary from 24 to 96 h depending on the objectives of the test and the requirements of the regulatory authority. For specific information on test duration, see the tables summarizing the test conditions below.

9.16 ACCEPTABILITY OF TEST RESULTS

9.16.1 For the test results to be acceptable, survival in controls must be at least 90%. Tests in which the control survival is less than 90% are invalid, and must be repeated. In tests with specific chemicals, the concentration of the test material must not vary more than 20% at any treatment level during the exposure period.

9.16.2 Upon subsequent completion of a valid test, the results of all tests, valid and invalid, are reported to the regulatory authority with an explanation of the tests performed and results.

9.17 SUMMARY OF TEST CONDITIONS FOR THE PRINCIPAL TEST ORGANISMS

9.17.1 Summaries of the test conditions for the daphnids, *Ceriodaphnia dubia*, *Daphnia pulex*, and *D. magna*, fathead minnows, *Pimephales promelas*, rainbow trout, *Oncorhynchus mykiss*, brook trout, *Salvelinus fontinalis*, the mysids, *Mysidopsis bahia* and *Holmesimysis costata*, sheepshead minnows, *Cyprinodon variegatus*, and silversides, *Menidia beryllina, M. menidia*, and *M. peninsulae*, are provided in Tables 12-19.

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TABLE 12. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *CERIODAPHNIA DUBIA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2002.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:[2]	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (recommended) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	30 mL (recommended minimum)
8. Test solution volume:	15 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	Less than 24-h old (required)
11. No. organisms per test chamber:	5 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	4 for effluent and receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent and receiving water tests (required minimum)
14. Feeding regime:	Feed YCT and *Selenastrum* while holding prior to the test; newly-released young should have food available a minimum of 2 h prior to use in a test; add 0.1 mL each of YCT and *Selenastrum* 2 h prior to test solution renewal at 48 h (recommended)
15. Test chamber cleaning:	Cleaning not required
16. Test chamber aeration:	None (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and dilution water.

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TABLE 12. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *CERIODAPHNIA DUBIA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2002.0) (CONTINUED)

17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness (available options)
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: $0.5 dilution series (recommended) Receiving Waters: None, or $0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	1 L (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

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TABLE 13. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *DAPHNIA PULEX* AND *D. MAGNA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2021.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:[2]	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	30 mL (recommended minimum)
8. Test solution volume:	25 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	Less than 24-h old (required)
11. No. organisms per test chamber:	5 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	4 for effluent and receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent and receiving water tests (required minimum)
14. Feeding regime:	Feed YCT and *Selenastrum* while holding prior to the test; newly-released young should have food available a minimum of 2 h prior to use in a test; add 0.1 mL each of YCT and *Selenastrum* 2 h prior to test solution renewal at 48 h (recommended)
15. Test chamber cleaning:	Cleaning not required
16. Test chamber aeration:	None (recommended)
17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness (available options)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and dilution water.

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TABLE 13. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *DAPHNIA PULEX* AND *D. MAGNA* ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATER (TEST METHOD 2021.0) (CONTINUED)

18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: $0.5 dilution series (recommended) Receiving Waters: None, or $0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	1 L (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

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TABLE 14. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2000.0)[1,2]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:[3]	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	250 mL (recommended minimum)
8. Test solution volume:	200 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	1-14 days; less than or equal to 24-h range in age (required)
11. No. organisms per test chamber:	10 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent tests (required minimum) 40 for receiving water tests (required minimum)
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h (recommended)
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min (recommended)

[1] *Cyprinella leedsi* (Bannerfish shiner, formerly *Notropis leedsi*; AFS, 1991) can be used with the test conditions in this table, where it is the required test organism in NPDES permits.

[2] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[3] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and dilution water.

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TABLE 14. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS[1] (TEST METHOD 2000.0) (CONTINUED)

17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness. (available options)
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: $0.5 dilution series (recommended) Receiving Waters: None, or $ 0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	2 L for effluents and receiving waters (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

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TABLE 15. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, AND BROOK TROUT, *SALVELINUS FONTINALIS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2019.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:	12°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness. Light intensity should be raised gradually over a 15 min period at the beginning of the photoperiod, and lowered gradually at the end of the photoperiod, using a dimmer switch or other suitable device. (recommended)
7. Test chamber size:	5 L (recommended minimum) (test chambers should be covered to prevent fish from jumping out)
8. Test solution volume:	4 L (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	Rainbow Trout: 15-30 days (after yolk sac absorption to 30 days) (required) Brook Trout: 30-60 days (required)
11. No. organisms per test chamber:	10 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent tests (required minimum) 40 for receiving water tests (required minimum)
14. Feeding regime:	Feeding not required
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 6.0 mg/L; rate should not exceed 100 bubbles/min (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

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TABLE 15. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, AND BROOK TROUT, *SALVELINUS FONTINALIS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2019.0) (CONTINUED)

17. Dilution water:	Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW (see Section 7, Dilution Water), receiving water, ground water, or synthetic water, modified to reflect receiving water hardness (available options)
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: $ 0.5 dilution series (recommended) Receiving Waters: None, or $ 0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	20 L for effluents (recommended) 40 L for receiving waters (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

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TABLE 16. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR MYSID, *MYSIDOPSIS BAHIA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2007.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature[2]:	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	250 mL (recommended minimum)
8. Test solution volume:	200 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	1-5 days; less than or equal to 24-h range in age (required)
11. No. organisms per test chamber:	10 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent tests (required minimum) 40 for receiving water tests (required minimum)
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; feed 0.2 mL of concentrated suspension of *Artemia* nauplii # 24-h old, daily (approximately 100 nauplii per mysid) (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature, salinity, and dilution water.

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TABLE 16. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR MYSID, *MYSIDOPSIS BAHIA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS[1] (TEST METHOD 2007.0) (CONTINUED)

15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min (recommended)
17. Dilution water:	5-30‰ ±10%; Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q® or equivalent deionized water (see Section 7, Dilution Water); or receiving water (available options)
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: $0.5 dilution series (recommended) Receiving Waters: None, or $0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	1 L for effluents (recommended) 2 L for receiving waters (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

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TABLE 17. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2004.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:[2]	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	250 mL (recommended minimum)
8. Test solution volume:	200 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	1-14 days; less than or equal to 24-h range in age (required)
11. No. organisms per test chamber:	10 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent tests (required minimum) 40 for receiving water tests (required minimum)
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h (recommended)
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature, salinity, and dilution water.

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TABLE 17. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2004.0) (CONTINUED)

17. Dilution water:	5-32‰ ±10%; Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q® or equivalent deionized water (see Section 7, Dilution Water); or receiving water (available options)
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: $0.5 dilution series (recommended) Receiving Waters: None, or $ 0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	1 L for effluents (recommended) 2 L for receiving waters (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

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TABLE 18. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SILVERSIDE, *MENIDIA BERYLLINA, M. MENIDIA*, AND *M. PENINSULAE*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2006.0)[1]

1. Test type:	Static non-renewal, static-renewal, or flow-through (available options)
2. Test duration:	24, 48, or 96 h (available options)
3. Temperature:[2]	20°C ±1°C; or 25°C ±1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4. Light quality:	Ambient laboratory illumination (recommended)
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels) (recommended)
6. Photoperiod:	16 h light, 8 h darkness (recommended)
7. Test chamber size:	250 mL (recommended minimum)
8. Test solution volume:	200 mL (recommended minimum)
9. Renewal of test solutions:	After 48 h (required minimum)
10. Age of test organisms:	9-14 days; less than or equal to 24-h range in age (required)
11. No. organisms per test chamber:	10 for effluent and receiving water tests (required minimum)
12. No. replicate chambers per concentration:	2 for effluent tests (required minimum) 4 for receiving water tests (required minimum)
13. No. organisms per concentration:	20 for effluent tests (required minimum) 40 for receiving water tests (required minimum)
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; add 0.2 mL *Artemia* nauplii concentrate 2 h prior to test solution renewal at 48 h (recommended)
15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 12.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature, salinity, and dilution water.

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TABLE 18. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SILVERSIDE, *MENIDIA BERYLLINA, M. MENIDIA*, AND *M. PENINSULAE*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 2006.0) (CONTINUED)

17. Dilution water:	5-32‰ ±10%; Uncontaminated source of seawater, deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, modified GP2, or equivalent) prepared with MILLI-Q® or equivalent deionized water (see Section 7, Dilution Water); or receiving water (available options) 1-32‰ ±10% for *M. beryllina*; 15-32‰ ±10% for *M. menidia*; and *M. peninsulae*
18. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Waters: 100% receiving water and a control (recommended)
19. Dilution series:	Effluents: $0.5 dilution series (recommended) Receiving Waters: None, or $0.5 dilution series (recommended)
20. Endpoint:	Effluents: Mortality (required) Receiving Waters: Mortality (required)
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period (required) Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period (recommended)
22. Sample volume required:	1 L for effluents (recommended) 2 L for receiving waters (recommended)
23. Test acceptability criterion:	90% or greater survival in controls (required)

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TABLE 19. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR WEST COAST MYSID, *HOLMESIMYSIS COSTATA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS[1]

NOTE: This method is specific to Pacific Coast waters and is not listed at 40 CFR Part 136 for nationwide use. This method has been proposed but not yet approved at 40 CFR Part 136.

1. Test type:	Static non-renewal or static-renewal
2. Test duration:	24, 48, or 96 h
3. Temperature:	15°C ± 1°C for organisms collected South of Pt Conception, CA 13°C ± 1°C for organisms collected North of Pt Conception, CA
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	1000 mL (minimum)
8. Test solution volume:	200 mL (minimum)
9. Renewal of test solutions:	Minimum, at 48 h
10. Age of test organisms:	3 to 4 days post-hatch juveniles
11. No. organisms per test chamber:	Minimum, 5 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 5 for effluent tests and receiving water tests
13. No. organisms per concentration:	Minimum, 25 for effluent tests and receiving water tests
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; feed 0.2 mL of concentrated suspension of *Artemia* nauplii #24-h old, daily approximately 40 nauplii per mysid)

[1] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and salinity.

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TABLE 19. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR WEST COAST MYSID, *HOLMESIMYSIS COSTATA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	34 ± 2‰ salinity; Uncontaminated seawater (1 µm filtered) or hypersaline brine or equivalent (see Section 7, Dilution Water)
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: $0.5 dilution series Receiving Waters: None, or $0.5 dilution series
20. Endpoint:	Effluents: Mortality Receiving Waters: Mortality
21. Sampling and sample holding requirements:	Effluents: Grab or composite sample first used within 36 h of completion of the sampling period Receiving Waters: Grab or composite sample first used within 36 h of completion of the sampling period
22. Sample volume required:	1 L for effluents 2 L for receiving waters
23. Test acceptability criterion:	90% or greater survival in controls

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SECTION 10

TEST DATA

10.1 BIOLOGICAL DATA

10.1.1 Death is the "effect" used for determining toxicity to aquatic organisms in acute toxicity tests.

10.1.2 Death is not as easily determined for some organisms. The criteria usually employed in establishing death are: (1) no movement of gills or appendages; and (2) no reaction to gentle prodding.

10.1.3 The death of some organisms, such as mysids and larval fish, is easily detected because of a change in appearance from transparent or translucent to opaque. General observations of appearance and behavior, such as erratic swimming, loss of reflex, discoloration, excessive mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism, should also be noted in the daily record.

10.1.4 The test chambers should be checked for early mortality during the first few hours of the test. The number of surviving organisms in each test chamber is recorded at the end of each 24-h period (Figure 4). When recognizable, dead organisms should be removed during each observation period.

10.1.5 The species, source, and age of the test organisms should be recorded.

10.2 CHEMICAL AND PHYSICAL DATA

10.2.1 In static tests, at a minimum, pH, salinity or conductivity, and total residual chlorine are measured in the highest concentration of test solution and in the dilution water at the beginning of the test, at test solution renewal, and at test termination. If total residual chlorine is not detected in effluent or dilution water at test initiation, it is unnecessary to measure total residual chlorine at test solution renewal or at test termination. It is also unnecessary to measure total residual chlorine in laboratory prepared synthetic dilution waters. DO, pH, and temperature are measured in the control and all test concentrations at the beginning of the test, daily thereafter, and at test termination.

10.2.1.1 It is recommended that total alkalinity and total hardness also be measured in the control and highest effluent concentration at the beginning of the test and at test solution renewal.

10.2.1.2 Total ammonia is measured in samples where toxicity may be contributed by unionized ammonia (where total ammonia might be $5 mg/L).

10.2.1.3 The DO should be monitored closely (every 2 h) for the first 4 to 8 h, to guard against rapid DO depletion, and is measured daily thereafter in all effluent concentrations in which there are surviving organisms, and at test termination. It is recommended that test solution DO be recorded continuously in the test chamber at the highest test solution concentration or in a surrogate vessel at a comparable test solution concentration and containing the standard complement of test organisms.

10.2.1.4 At a minimum, test solution temperature is measured at the beginning of the test, and daily thereafter. Temperature measurements are made by placing thermometers or other temperature sensing devices directly in test solutions or in a comparable volumes of water in chambers positioned in several locations among the test vessels to determine test solution temperatures. It is recommended that test solution temperature be recorded continuously in at least one test chamber or in a comparable volume of water in a surrogate vessel which is comparable to the test chambers.

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10.2.2 In flow-through tests, at a minimum, pH, salinity or conductivity, total alkalinity, total hardness, and total residual chlorine are measured daily in the highest effluent concentration. DO and temperature are measured at the beginning of the test, daily thereafter in the control and all test concentrations, and at test termination.

10.2.3 The measurement of specific conductance is recommended because it is a very useful parameter in detecting transient fluctuations in the chemical characteristics of effluents, and will indicate errors in test dilutions.

10.2.4 Where acute toxicity test methods are utilized to determine permit limits for toxic chemicals, at a minimum, the concentration of the test material must be measured in each test concentration at test initiation, daily thereafter, and at test termination.

10.2.5 Methods used for chemical analysis should be those specified for Section 304(h) of the CWA (USEPA, 1993b). For salinity measurements, a refractometer may be used if calibrated with a sample of known salinity.

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INDUSTRY/TOXICANT: _____
ADDRESS: _____
CONTACT: _____
EFFLUENT SERIAL NO: _____
NPDES PERMIT NO.: _____
SAMPLE COLLECTOR: _____
GRAB SAMPLE: COLLECTED (1) _____ AM/PM; __/__/__(DATE)
(2) _____ AM/PM; __/__/__(DATE)
(3) _____ AM/PM; __/__/__(DATE)
(4) _____ AM/PM; __/__/__(DATE)

COMPOSITE SAMPLE: COLLECTED (1) FROM: _____ AM/PM; __/__/__(DATE) TO: _____ AM/PM; __/__/__(DATE)
(2) FROM: _____ AM/PM; __/__/__(DATE) TO: _____ AM/PM; __/__/__(DATE)
(3) FROM: _____ AM/PM; __/__/__(DATE) TO: _____ AM/PM; __/__/__(DATE)
(4) FROM: _____ AM/PM; __/__/__(DATE) TO: _____ AM/PM; __/__/__(DATE)

PERSON CONDUCTING TEST: _____
TEST PERIOD:
BEGINNING: DATE _____ TIME _____
ENDING: DATE _____ TIME _____
TEST ORGANISM:
SPECIES: _____
AGE: _____
SOURCE: _____
DILUTION WATER USED: _____
DISSOLVED OXYGEN (mg/L) TOTAL RESIDUAL AMMONIA: _____
0 HRS _____ FREE AMMONIA: _____
24 " _____
48 " _____
72 " _____
96 " _____

Conc. or %	Test Container Number	Number of Live Organisms					Dissolved Oxygen (mg/l)					pH					Total Alkalinity (mg/l as $CaCO_3$)					Total Hardness (mg/l as $CaCO_3$)									
		0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96

Figure 4. Example of data sheet for effluent toxicity tests.

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1. EXPOSURE CHAMBER

Total capacity: _____ mL

Test solution volume: _____ mL

Test solution surface area: _____ cm^2

Water depth (constant): _____ cm
(cyclic): _____ to _____ cm

2. FEEDING SCHEDULE

Not Fed: _____

Fed daily: _____

Fed irregularly:
(describe): _____

Food used: _____

3. AERATION

None: _____

Slow: _____ (Bubbles or mL/min)

Moderate: _____ " "

Vigorous: _____ " "

From: _____ AM/PM; ___/___/___ (DATE)
To: _____ AM/PM; ___/___/___ (DATE)

4. SCREENED ANIMAL ENCLOSURES
Not used: _____

Used: _____
(cm) Diameter

5. Condition/appearance of surviving organisms at end of test: (i.e., alive but immobile; loss of orientation; erratic movement; etc.) _____

6. Comments: _____

NPDES NO: _____ Inspection Date: _____ Outfall number: _____
Facility Name: _____ Test Date: _____ Macro Test: _____
City Name: _____ Inspection Code: _____ Type Macro: _____
County Name: _____ Type Inspection: _____ Expo Time: _____
Receiving Water: _____ Date Info to WSD: _____ Results: _____
Permit Issued: _____ Date Info to State: _____ Fish Test: _____
Permit Expires: _____ Date of WMD Action: _____ Type Fish: _____
SIC Code: _____ Data of Static Action: _____ Expo Time: _____
Present Treatment: _____ Type of Action: _____ Results: _____
Remarks: _____ Annual Status Update: _____ Remarks: _____

Figure 5. Check list on back of effluent toxicity data sheet.

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SECTION 11

ACUTE TOXICITY DATA ANALYSIS

11.1 INTRODUCTION

11.1.1 The objective of acute toxicity tests with effluents and receiving waters is to identify discharges of toxic effluents in acutely toxic amounts. Data are derived from tests designed to determine the adverse effects of effluents and receiving waters on the survival of the test organisms. The recommended effluent toxicity test consists of a control and five or more concentrations of effluent (i.e., multi-effluent-concentration, or definitive tests), in which the endpoint is (1) an estimate of the effluent concentration which is lethal to 50% of the test organisms in the time period prescribed by the test, expressed as the LC50, or (2) the highest effluent concentration at which survival is not significantly different from the control (No-Observed-Adverse-Effect Concentration, or NOAEC). Receiving water tests may be single concentration or multi-concentration tests. The LC50 is determined by the Graphical, Spearman-Karber, Trimmed Spearman-Karber, or Probit Method. The NOAEC is determined by hypothesis testing.

11.1.2 Some states require tests consisting of a control and a single concentration of effluent with a pass/fail endpoint. Control survival must be 90% or greater for an acceptable test. The test "passes" if survival in the control and effluent concentration equals or exceeds 90%. The test "fails" if survival in the effluent is less than 90%, and is significantly different from control survival (which must be 90% or greater), as determined by hypothesis testing.

11.1.3 The toxicity of receiving (surface) water can be determined with (1) a paired test consisting of four replicates each of a suitable control and 100% surface water, or (2) a multi-concentration test. The results of the first type of test (100% receiving water and a control) are analyzed by hypothesis testing. The results of the second type of test may be analyzed by hypothesis testing or used to determine an LC50.

11.1.4 The data analysis methods recommended in this manual have been chosen primarily because they are (1) well-tested and well-documented, (2) applicable to most types of test data sets for which they are recommended, but still powerful, and (3) most easily understood by non-statisticians. Many other methods were considered in the selection process, and it is recognized that the methods selected are not the only possible methods of analysis of acute toxicity data.

11.1.5 ROLE OF THE STATISTICIAN

11.1.5.1 The use of the statistical methods described in this manual for routine data analysis does not require the assistance of a statistician. However, if the data appear unusual in any way, or fail to meet the necessary assumptions, a statistician should be consulted. The choice of a statistical method to analyze toxicity test data and the interpretation of the results of the analysis of the data can become problematic if there are anomalies in the data. Analysts who are not proficient in statistics are strongly advised to seek the assistance of a statistician before selecting alternative methods of analysis and using the results.

11.1.6 INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

11.1.6.1 A critical assumption in the statistical analysis of toxicity data is statistical independence among observations. Statistical independence means that given knowledge of the true mean for a given concentration or control, knowledge of the error in any one actual observation would provide no information about the error in any other observation. One of the best ways to insure independence is to properly follow randomization procedures. The purpose of randomization is to avoid situations where test organisms are placed serially, by level of concentration, into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.

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11.1.6.2 Another area for potential bias of results is the presence of outliers. An outlier is an inconsistent or questionable data point that appears unrepresentative of the general trend exhibited by the majority of the data. Outliers may be detected by tabulation of the data, plotting, and by an analysis of the residuals. An explanation should be sought for any questionable data points. Without an explanation, data points should be discarded only with extreme caution. If there is no explanation, the statistical analysis should be performed both with and without the outlier, and the results of both analyses should be reported. For a discussion of techniques for evaluating outliers, see Draper and John (1981).

11.2 DETERMINATION OF THE LC50 FROM DEFINITIVE, MULTI-EFFLUENT-CONCENTRATION ACUTE TOXICITY TESTS

11.2.1 The method used to estimate the LC50 from multi-concentration acute toxicity tests depends on the shape of the tolerance distribution, and how well the effluent concentrations chosen characterize the cumulative distribution function for the tolerance distribution (i.e., the number of partial mortalities). A review of effluent acute toxicity data from the last 248 tests performed by the Ecological Support Branch, Environmental Services Division, EPA Region 4, indicated the following pattern in the number of partial mortalities: (1) no partial mortalities (all or nothing response) - 28%; (2) one partial mortality - 54%; (3) two or more partial mortalities - 16%; (4) LC50 occurring a one of the test concentrations - 2%.

11.2.1.1 Four methods for estimating the LC50 are presented below: the Graphical Method, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, and the Probit Method. The analysis scheme is shown in Figure 6. Included in the presentation of each method is a description of the method, the requirements for the method, a description of the calculations involved in the method or a description of the computer program, and an example of the calculations.

11.2.1.2 The Probit Method, the Spearman-Karber Method, and the Trimmed Spearman-Karber Method are designed to produce LC50 values and associated 95% confidence intervals. It should be noted that software used to calculate point estimates occasionally may not provide associated 95% confidence intervals. This situation may arise when test data do not meet specific assumptions required by the statistical methods, when point estimates are outside of the test concentration range, and when specific limitations imposed by the software are encountered. USEPA (2000a) provides guidance on confidence intervals under these circumstances.

11.2.2 THE GRAPHICAL METHOD

11.2.2.1 Description

1. The Graphical Method is a mathematical procedure for calculating the LC50.

2. The procedure estimates the LC50 by linearly interpolating between points of a plot of observed percent mortality versus the base 10 logarithm ($\log_{10}$) of percent effluent concentration.

3. It does not provide a confidence interval for the LC50 estimate.

4. Use of the Graphical Method is only recommended when there are no partial mortalities.

11.2.2.2 Requirements

1. The only requirement for the Graphical Method is that the observed percent mortalities bracket the 50%.

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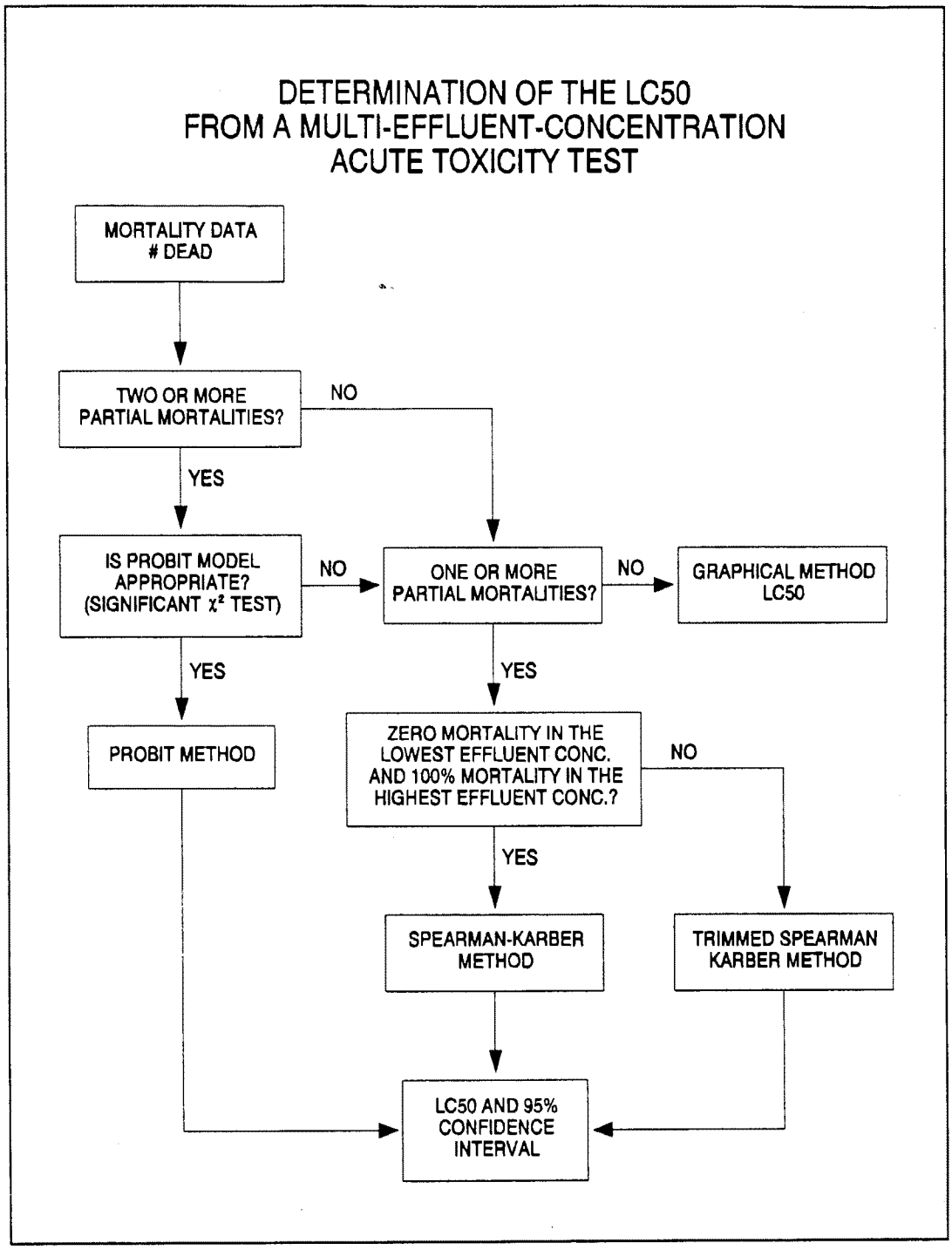

Figure 6. Flowchart for determination of the LC50 for multi-effluent-concentration acute toxicity tests.

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11.2.2.3 General Procedure

1. Let $p_o, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_o \# ... \# p_k$. The smoothing replaces any adjacent p_i's that do not conform to $p_o \# ... \# p_k$, with their average. For example, if p_i is less than p_{i-1}, then:

$$p_{i\&1}^{s} \; ' \; p_i^{s} \; ' \; (p_i \% p_{i\&1})/2$$

where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

2. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^{a} \; ' \; (p_i^{s} \& p_0^{s}) \; / \; (1 \& p_0^{s})$$

where: p_o^s = the smoothed observed proportion mortality for the control.

3. Plot the smoothed, adjusted data on 2-cycle semi-log graph paper with the logarithmic axis (the *y* axis) used for percent effluent concentration and the linear axis (the *x* axis) used for observed percent mortality.

4. Locate the two points on the graph which bracket 50% mortality and connect them with a straight line.

5. On the scale for percent effluent concentration, read the value for the point where the plotted line and the 50% mortality line intersect. This value is the estimated LC50 expressed as a percent effluent concentration.

11.2.2.4 Example Calculation

1. All-or-nothing data (Graphical Method) in Table 20 are used in the calculations. Note that in this case, the data must be smoothed and adjusted for mortality in the controls.

2. To smooth the data, the observed proportion mortality for the control and the lower three effluent concentrations must be averaged. The smoothed observed proportion mortalities are as follows: 0.0125, 0.0125, 0.0125, 0.0125, 1.0, and 1.0.

3. The smoothed responses are adjusted for control mortality (see 11.2.2.3), where the smoothed response for the control (p_o^s) = 0.0125. The smoothed, adjusted response proportions for the effluent concentrations are as follows: 0.0, 0.0, 0.0, 1.0, and 1.0.

4. A plot of the smoothed, adjusted data is shown in Figure 7.

5. The two points on the graph which bracket the 50% mortality line (0% mortality at 25% effluent, and 100% mortality at 50% effluent) are connected with a straight line.

6. The point at which the plotted line intersects the 50% mortality line is the estimated LC50. The estimated LC50 = 35% effluent.

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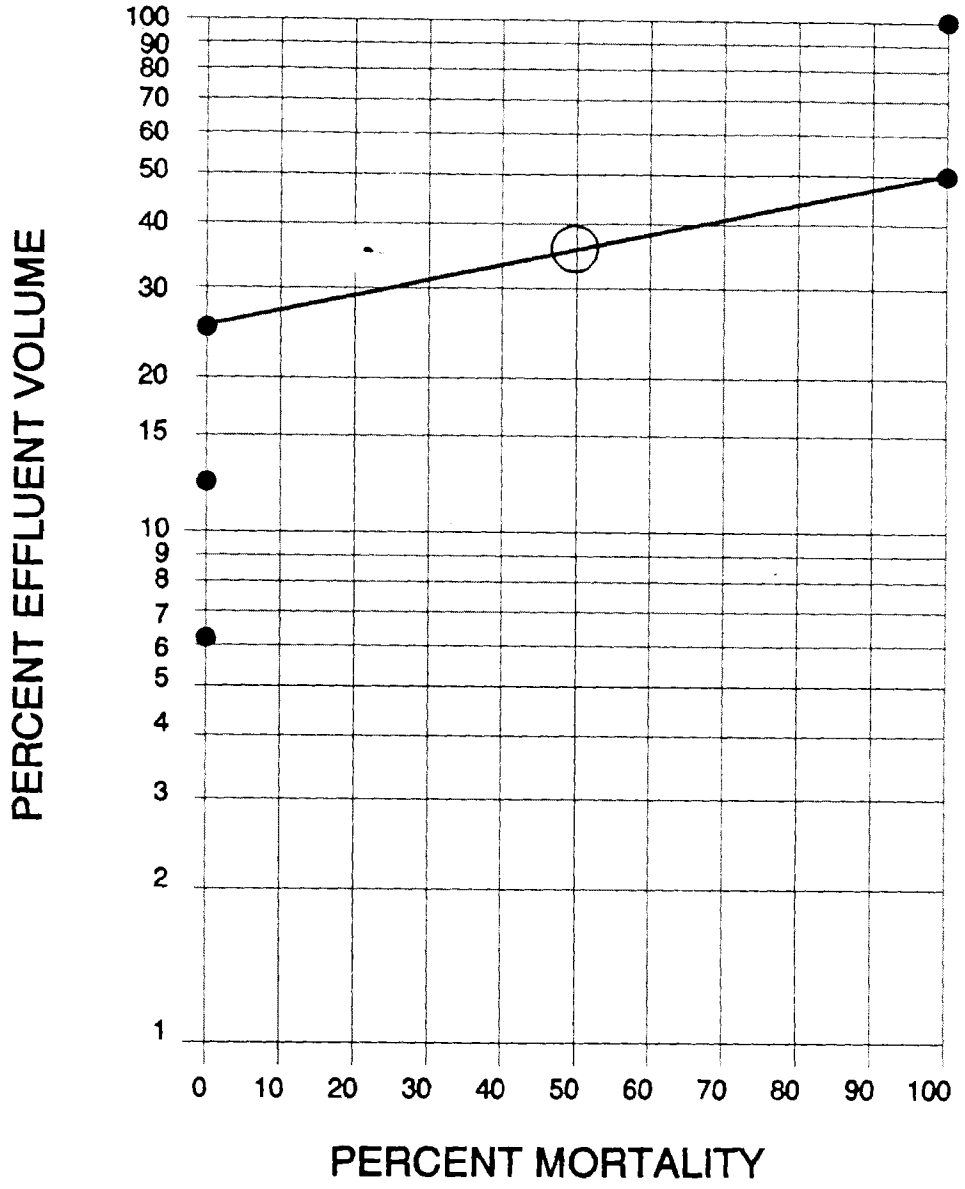

Figure 7. Plotted data and fitted line for graphical method, using all-or-nothing data.

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TABLE 20. MORTALITY DATA (NUMBER OF DEAD ORGANISMS) FROM ACUTE TOXICITY TESTS USED IN EXAMPLES OF LC50 DETERMINATIONS (20 ORGANISMS IN THE CONTROL AND ALL TEST CONCENTRATIONS)

Effluent Conc. (%)	Method of Analysis			
	Graphical	Spearman-Karber	Trimmed Spearman-Karber	Probit
CONTROL	1	1	1	0
6.25%	0	1	0	0
12.5%	0	0	2	3
25.0%	0	0	0	9
50.0%	20	13	0	20
100.0%	20	20	16	20

11.2.3 THE SPEARMAN-KARBER METHOD

11.2.3.1 Description

1. The Spearman-Karber Method is a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978).

2. This procedure estimates the mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the mean is equivalent to an estimate of the median of the log tolerance distribution.

3. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data are smoothed.

4. Abbott's procedure is used to "adjust" the test results for mortality occurring in the control.

5. Use of the Spearman-Karber Method is recommended when partial mortalities occur in the test solutions, but the data do not fit the Probit model.

11.2.3.2 Requirements

1. To calculate the LC50 estimate, the following must be true:
   a. The smoothed adjusted proportion mortality for the lowest effluent concentration (not including the control) must be zero.
   b. The smoothed adjusted proportion mortality for the highest effluent concentration must be one.

2. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed adjusted proportion mortalities must be between zero and one.

11.2.3.3 General Procedure

1. The first step in the estimation of the LC50 by the Spearman-Karber Method is to smooth the observed response proportions, p_i, if they do not satisfy p_o #... #p_k (see 11.2.2.3, Step 1).

2. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (see 11.2.2.3, Step 2).

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3. Plot the smoothed adjusted data on 2-cycle semi-log graph paper with the logarithmic axis (the *y* axis) used for percent effluent concentration and the linear axis (the *x* axis) used for observed percent mortality.

4. Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k-1} \frac{(p_{i+1}^{a} - p_i^{a})\ (X_i + X_{i+1})}{2}$$

where: p_i^a = the smoothed adjusted proportion mortality at concentration i
X_i = the $\log_{10}$ of concentration i
k = the number of effluent concentrations tested, not including the control.

5. Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^{a}(1-p_i^{a})\ (X_{i+1} - X_{i-1})^2}{4(n_i - 1)}$$

where: X_i = the $\log_{10}$ of concentration i
n_i = the number of organisms tested at effluent concentration i
p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i
k = the number of effluent concentrations tested, not including the control.

6. Calculate the 95% confidence interval for m: $m \pm 2.0\sqrt{V(m)}$

7. The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking base_{10} antilogs of the above values.

8. With the exclusion of the plot in item 3, the above calculations can be carried out using the Trimmed Spearman-Karber computer program mentioned in 11.2.4.3 and 11.2.4.4.

11.2.3.4 Example Calculation

1. Mortality data from a definitive, multi-concentration, acute toxicity test are given in Table 20. Note that the data must be smoothed and adjusted for mortality in the controls.

2. To smooth the data, the observed proportion mortality for the control, and the observed proportion mortality for the 6.25%, 12.5%, and 25% effluent concentrations must be averaged. The smoothed observed proportion mortalities are as follows: 0.025, 0.025, 0.025, 0.025, 0.65, and 1.00.

3. To adjust the smoothed, observed proportion mortality in each effluent concentration for mortality in the control group, Abbott's formula must be used. After smoothing and adjusting, the proportion mortalities for the effluent concentrations are as follows: 0.000, 0.000, 0.000; 0.641, and 1.000.

4. The data will not be plotted for this example. For an example of the plotting procedures, see 11.2.2.4.

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5. The $\log_{10}$ of the estimated LC50, m, is calculated as follows:

$$
\begin{aligned}
m = \; & [(0.0000 - 0.0000)(0.7959 + 1.0969)]/2 + \\
& [(0.0000 - 0.0000)(1.0969 + 1.3979)]/2 + \\
& [(0.6410 - 0.0000)(1.3979 + 1.6990)]/2 + \\
& [(1.0000 - 0.6410)(1.6990 + 2.0000)]/2 \\
= \; & 1.656527
\end{aligned}
$$

6. The estimated variance of m, V(m), is calculated as follows:

$$
\begin{aligned}
V(m) = \; & (0.0000)(1.0000)(1.3979 - 0.7959)^2/4(19) + \\
& (0.0000)(1.0000)(1.6990 - 1.0969)^2/4(19) + \\
& (0.6410)(0.3590)(2.0000 - 1.3979)^2/4(19) \\
= \; & 0.0010977
\end{aligned}
$$

7. The 95% confidence interval for m is calculated as follows:

$$1.656527 \pm 2\sqrt{0.0010977} \;\; ' \;\; (1.5902639,\; 1.7227901)$$

8. The estimated LC50 is as follows: antilog(1.656527) = 45.3%.

9. The upper limit of the 95% confidence interval for the estimated LC50 is as follows:

antilog(1.7227901) = 52.8%

10. The lower limit of the 95% confidence interval for the estimated LC50 is as follows:

antilog(1.5902639) = 38.9%

11.2.4 THE TRIMMED SPEARMAN-KARBER METHOD

11.2.4.1 Description

1. The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton, et al, 1977).

2. This procedure estimates the trimmed mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution.

3. Use of the Trimmed Spearman-Karber Method is only appropriate when the requirements for the Probit Method and the Spearman-Karber Method are not met.

11.2.4.2 Requirements

1. To calculate the LC50 estimate with the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5.

2. To calculate a confidence interval for the LC50 estimate, one or more of the smoothed, adjusted, observed proportion mortalities must be between zero and one.

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11.2.4.3 General Procedure

1. Smooth the observed proportion mortalities as described in 11.2.2.3, Step 1.

2. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (see 11.2.2.3, Step 2).

3. Plot the smoothed, adjusted data as described in 11.2.2.3, Step 3.

4. Calculate the amount of trim to use in the estimation of the LC50 as follows:

$$\text{Trim} \;'\; \max(p_1^a,\ 1\ \&\ p_k^a)$$

where: p_1^a = the smoothed, adjusted proportion mortality for the lowest effluent concentration, exclusive of the control.
p_k^a = the smoothed, adjusted proportion mortality for the highest effluent concentration.
k = the number of effluent concentrations, exclusive of the control.

5. Due to the intensive nature of the calculation for the estimated LC50 and the calculation for the associated 95% confidence interval using the Trimmed Spearman-Karber Method, it is recommended that the data be analyzed by computer.

6. A computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed-Karber Method, can be obtained through the Environmental Monitoring and Support Laboratory (EMSL), 26 W. Martin Luther King Drive, Cincinnati, OH 45268. The program can be obtained from EMSL-Cincinnati by sending a diskette with a written request to the above address.

7. The modified program automatically performs the following functions:
   a. Smoothing.
   b. Adjustment for mortality in the control.
   c. Calculation of the trim.
   d. Calculation of the LC50.
   e. Calculation of the associated 95% confidence interval.

11.2.4.4 Example Calculation Using the Computer Program

1. Data from Table 20 are used to illustrate the analysis using the Trimmed Spearman-Karber program.

2. The program requests the following input (see Figure 8):
   a. Output destination (D = disk file or P = printer).
   b. Title for output.
   c. Control data.
   d. Data for each toxicant concentration.

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```
TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5

ENTER DATE OF TEST:
08/19/93
ENTER TEST NUMBER:
1
WHAT IS TO BE ESTIMATED?
 (ENTER "L" FOR LC50 AND "E" FOR EC50)
L

ENTER TEST SPECIES NAME:
Fathead minnow

ENTER TOXICANT NAME:
Effluent

ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT:
%

ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
20

ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
1

ENTER THE NUMBER OF CONCENTRATIONS
 (NOT INCLUDING THE CONTROL;  MAX = 10):
5

ENTER THE 5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
6.25 12.5 25 50 100

ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
Y

ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
20

ENTER UNITS FOR DURATION OF EXPERIMENT
 (ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
H

ENTER DURATION OF TEST:
96

ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:
0 2 0 0 16

WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
Y
```

Figure 8. Example of input for computer program for Trimmed Spearman-Karber Method.

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3. The program output includes the following (see Figure 9):
   a. A table of the concentrations tested, number of organisms exposed, and mortalities.
   b. The amount of trim used in the calculation.
   c. The estimated LC50 and the associated 95% confidence interval.

4. The analysis results for this example are as follows:
   a. The observed proportion mortalities smoothed and adjusted for mortality in the control.
   b. The amount of trim used to calculate the estimate:

$$\text{trim} = \max\{0.00, 0.205\} = 0.205.$$

   c. The estimate of the LC50 is 77.1% with a 95% confidence interval of (69.7%, 85.3%).

11.2.5 THE PROBIT METHOD

11.2.5.1 Description

1. The Probit Method is a parametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978).

2. The analysis consists of transforming the observed proportion mortalities with a probit transformation, and transforming the effluent concentrations to $\log_{10}$.

3. Given the assumption of normality for the $\log_{10}$ of the tolerances, the relationship between the transformed variables mentioned above is approximately linear.

4. This relationship allows estimation of linear regression parameters, using an iterative approach.

5. The estimated LC50 and associated confidence interval are calculated from the estimated linear regression parameters.

11.2.5.2 Requirements

1. To obtain a reasonably precise estimate of the LC50 with the Probit Method, the observed proportion mortalities must bracket 0.5.

2. The $\log_{10}$ of the tolerance is assumed to be normally distributed.

3. To calculate the LC50 estimate and associated 95% confidence interval, two or more of the observed proportion mortalities must be between zero and one.

11.2.5.3 General Procedure

1. Due to the intensive nature of the calculations for the estimated LC50 and associated 95% confidence interval using the Probit Method, it is recommended that the data be analyzed by a computer program.

2. A machine-readable, compiled, version of a computer program to estimate the LC1 and LC50 and associated 95% confidence intervals using the Probit Method can be obtained from EMSL-Cincinnati by sending a diskette with a written request to the Environmental Monitoring Systems Laboratory, 26 W. Martin Luther King Drive, Cincinnati, OH 45268.

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```
TRIMMED SPEARMAN-KARBER METHOD, VERSION 1.5

DATE:       08/18/93          TEST NUMBER: 1          DURATION:   96 H
TOXICANT:   Effluent
SPECIES:    Fathead minnow


RAW DATA:   Concentration       Number        Mortalities
--- ----    (%)                 Exposed


              .00                20              1
             6.25                20              0
            12.50                20              2
            25.00                20              0
            50.00                20              0
           100.00                20             16


SPEARMAN-KARBER TRIM:   20.51%

SPEARMAN-KARBER ESTIMATES:                  LC50:  77.11
                           95% Lower Confidence:  69.74
                           95% Upper Confidence:  85.26

NOTE:  MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
       ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

WOULD YOU LIKE TO HAVE A COPY SENT TO THE PRINTER(Y/N)?
```

Figure 9. Example of output from computer program for Trimmed Spearman-Karber Method.

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11.2.5.4 Example Using the Computer Program

1. Data from Table 20 are used to illustrate the operation of the Probit program for calculating the LC50 and the associated 95% confidence interval.

2. The program begins with a request for the following initial input (see Figure 10):
   a. Desired output of abbreviated (A) or full (F) output?
   b. Output designation (P = printer, D = disk file).
   c. Title for the output.
   d. Control data.
   c. The number of exposure concentrations
   d. Data for each toxicant concentration

3. The program output includes the following (see Figure 11):
   a. A table of the observed proportion responding, and the proportion responding adjusted for controls.
   b. The calculated chi-squared statistic for heterogeneity and the tabular value. This test is one indicator of how well the data fit the model. The program will issue a warning when the test indicates that the data do not fit the model.
   c. The estimated LC50 and 95% confidence limits.
   d. A plot of the fitted regression line with observed data overlaid on the plot.

4. The results of the data analysis for this example are as follows:
   a. The observed proportion mortalities were not adjusted for mortality in the control.
   b. The test for heterogeneity was not significant (the calculated Chi-square was less than the tabular value), thus the Probit Method appears to be appropriate for this data.
   c. The estimate of the LC50 is 22.9% with a 95% confidence interval of (18.8%, 27.8%).

11.3 DETERMINATION OF NO-OBSERVED-ADVERSE-EFFECT CONCENTRATION (NOAEC) FROM MULTI-CONCENTRATION TESTS, AND DETERMINATION OF PASS OR FAIL (PASS/FAIL) FOR SINGLE-CONCENTRATION (PAIRED) TESTS

11.3.1 Determination of the No-Observed-Adverse-Effect Concentration (NOAEC), for multi-concentration toxicity tests, and pass or fail (Pass/Fail) for single-concentration toxicity tests is accomplished using hypothesis testing. The NOAEC is the lowest concentration at which survival is not significantly different from the control. In Pass/Fail tests, the objective is to determine if the survival in the single treatment (effluent or receiving water) is significantly different from the control survival.

11.3.2 The first step in these analyses is to transform the responses, expressed as the proportion surviving, by the arc-sine-square-root transformation (Figures 12 and 13). The arc-sine-square-root transformation is commonly used on proportionality data to stabilize the variance and satisfy the normality requirement. Shapiro Wilk's test may be used to test the normality assumption.

11.3.3 If the data do not meet the assumption of normality and there are four or more replicates per group, then the non-parametric test, Wilcoxon Rank Sum Test, can be used to analyze the data.

11.3.4 If the data meet the assumption of normality, the F test for equality of variances is used to test the homogeneity of variance assumption. Failure of the homogeneity of variance assumption leads to the use of a modified t test, where the pooled variance estimate is adjusted for unequal variance, and the degrees of freedom for the test are adjusted.

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```
EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5
```

```
Do you wish abbreviated (A) of full (F) output? A
Output to printer or disk file (P / D)? P
Title ? PROBIT EXAMPLE

Number of responders in the control group = ? 0
Number of exposure concentrations, exclusive of controls ? 5

Input data starting with the lowest exposure concentration

Concentration = ? 6.25
Number responding = ? 0
Number exposed = ? 20

Concentration = ? 12.5
Number responding = ? 3
Number exposed = ? 20

Concentration = ? 25
Number responding = ? 9
Number exposed = ? 20

Concentration = ? 50
Number responding = ? 20
Number exposed = ? 20

Concentration = ? 100
Number responding = ? 20
Number exposed = ? 20
```

Number	Conc.	Number Resp.	Number Exposed
1	6.2500	0	20
2	12.5000	3	20
3	25.0000	9	20
4	50.0000	20	20
5	100.0000	20	20

```
Do you wish to modify your data ? n
The control response rate =  0
Do you wish to modify it? n
```

Figure 10. Example of input for computer program for Probit Method.

ED_001324_00000042-00093

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

PROBIT EXAMPLE

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
6.2500	20	0	0.000	0.000
12.5000	20	3	0.1500	0.1500
25.0000	20	9	0.4500	0.4500
50.0000	20	20	1.0000	1.0000
100.0000	20	20	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 3.076
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 7.815

PROBIT EXAMPLE

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
LC/EC 1.00	7.924	4.147	10.959
LC/EC 50.00	22.872	18.787	27.846

Figure 11. Example of output for computer program for Probit Method.

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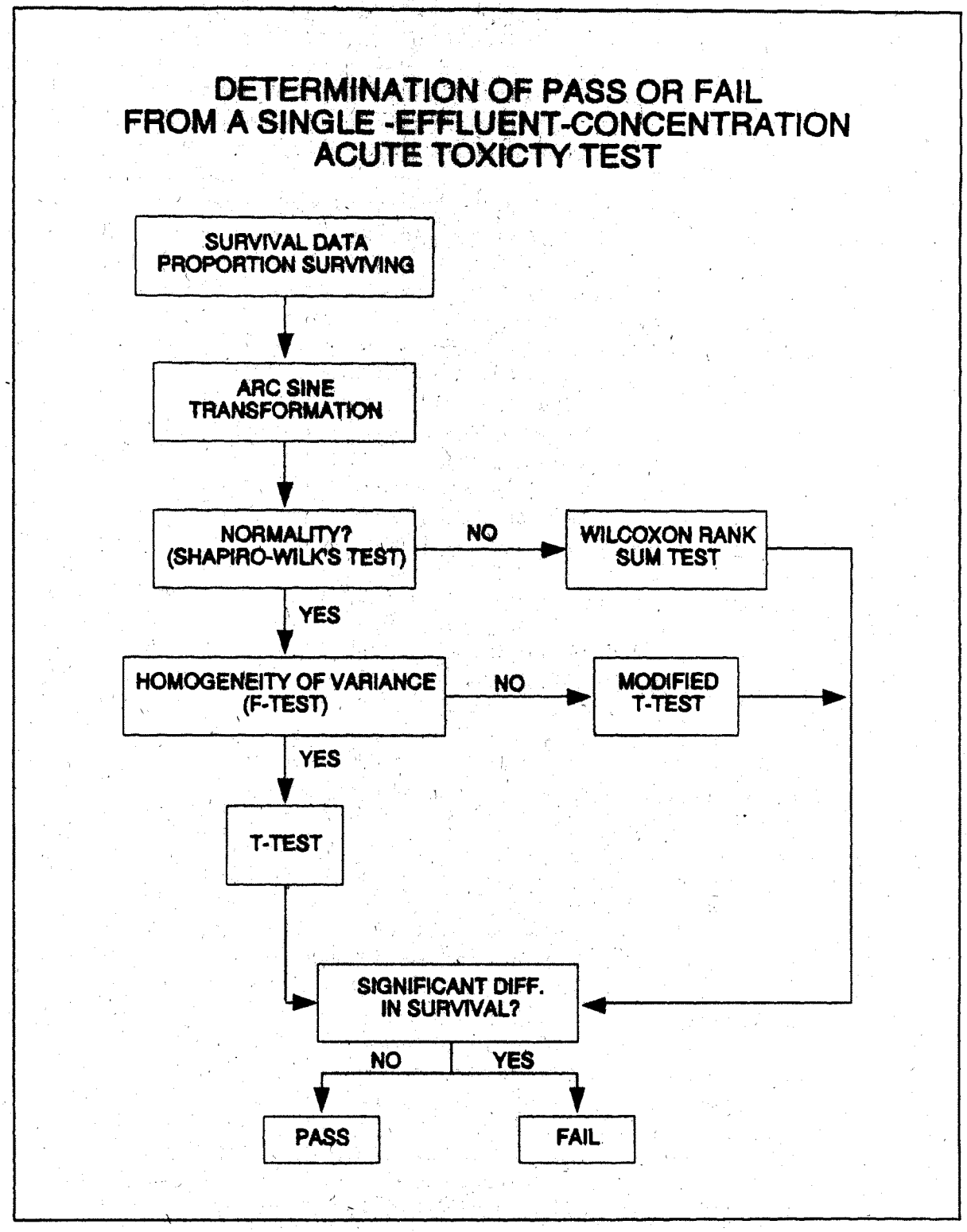

Figure 12. Flowchart for analysis of single-effluent concentration test data.

ED_001324_00000042-00095

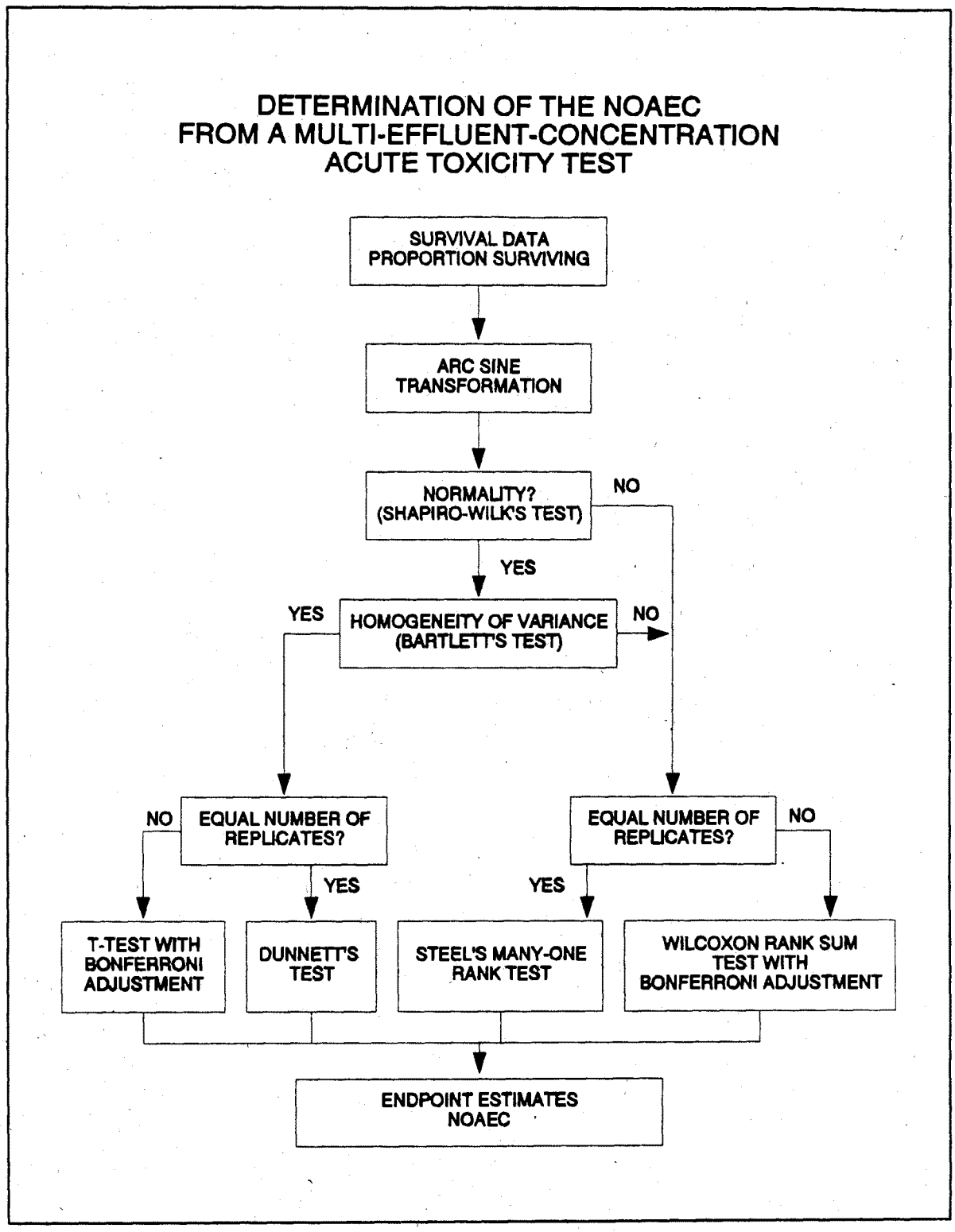

Figure 13. Flowchart for analysis of multi-effluent-concentration test data.

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11.3.5 GENERAL PROCEDURE

11.3.5.1 Arc Sine Square Root Transformation

11.3.5.1.1 The arc sine square root transformation consists of determining the angle (in radians) represented by a sine value. In this transformation, the proportion surviving is taken as the sine value, the square root of the sine value is calculated, and the angle (in radians) for the square root of the sine value is determined. Whenever the proportion surviving is 0 or 1, a special modification of the transformation must be used (Bartlett, 1937). Illustrations of the arc sine square root transformation and modification are provided below.

1. Calculate the response proportion (RP) for each replicate within a group, where:

   RP = (number of surviving organisms)/(number exposed)

2. Transform each RP to arc sine, as follows.

   a. For RPs greater than zero or less than one:

   $$Angle(in\ radians)\ '\ arc\ sine\sqrt{(RP)}$$

   b. Modification of the arc sine when RP = 0.

   $$Angle(in\ radians)\ '\ arc\ sine\sqrt{\frac{1}{4n}}$$

   where n = number animals/treatment rep.

   c. Modification of the arc sine when RP = 1.0.

   $$Angle\ '\ 1.5708\ radians\ \&\ (radians\ for\ RP\ '\ 0)$$

11.3.5.2 Shapiro Wilk's Test

11.3.5.2.1 After the data have been transformed, test the assumption of normality using Shapiro Wilk's test. The test statistic, W, is obtained by dividing the square of an appropriate linear combination of the sample order statistics by the usual symmetric estimate of variance (D). The calculated W must be greater than zero and less than or equal to one. This test is recommended for a sample size of 50 or less, and there must be more than two replicates per concentration for the test to be valid.

1. To calculate W, first center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration.

2. Calculate the denominator, D, of the test statistic:

   $$D\ '\ \mathop{j}_{i\ '\ 1}^{n}\ (X_i\&\overline{X})^2$$

   where: X_i = the ith centered observation

   $\overline{X}$ = the overall mean of the centered observations.

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3. Order the centered observations from smallest to largest.

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(i)}$$

where: $X^{(i)}$ denotes the ith ordered observation.

4. From Table 21, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$, where k is n/2 if n is even, and (n - 1)/2 if n is odd.

5. Compute the test statistic, W, as follows:

$$W = \frac{1}{D} \left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)} \right) \right]^2$$

11.3.5.2.2 The decision rule for the test is to compare the critical value from Table 22 to the computed W. If the computed value is less than the critical value, conclude that the data are not normally distributed.

11.3.5.3 F Test

11.3.5.3.1 The F test for equality of variances is used to test the homogeneity of variance assumption. When conducting the F test, the alternative hypothesis of interest is that the variances are not equal.

11.3.5.3.2 To make the two-tailed F test at the 0.01 level of significance, put the larger of the two sample variances in the numerator of F.

$$F = \frac{S_1^2}{S_2^2} \text{ where } S_1^2 > S_2^2$$

11.3.5.3.3 Compare the calculated F with the 0.005 level of a tabulated F value with n_1-1 and n_2-1 degrees of freedom, where n_1 and n_2 are the number of replicates for each of the two groups (Snedecor and Cochran, 1980). If the calculated F value is less than or equal to the tabulated F, conclude that the variances of the two groups are equal.

11.3.5.4 T Test

11.3.5.4.1 If the variances for the two groups are found to be statistically equivalent, then the equal variance t test is the appropriate test.

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TABLE 21. COEFFICIENTS FOR THE SHAPIRO WILK'S TEST (CONOVER, 1980)

i\n	2	3	4	5	6	7	8	9	10
1	0.7071	0.7071	0.6872	0.6646	0.6431	0.6233	0.6052	0.5888	0.5739
2	-	0.0000	0.1667	0.2413	0.2806	0.3031	0.3164	0.3244	0.3291
3	-	-	-	0.0000	0.0875	0.1401	0.1743	0.1976	0.2141
4	-	-	-	-	-	0.0000	0.0561	0.0947	0.1224
5	-	-	-	-	-	-	-	0.0000	0.0399

i\n	11	12	13	14	15	16	17	18	19	20
1	0.5601	0.5475	0.5359	0.5251	0.5150	0.5056	0.4968	0.4886	0.4808	0.4734
2	0.3315	0.3325	0.3325	0.3318	0.3306	0.3290	0.3273	0.3253	0.3232	0.3211
3	0.2260	0.2347	0.2412	0.2460	0.2495	0.2521	0.2540	0.2553	0.2561	0.2565
4	0.1429	0.1586	0.1707	0.1802	0.1878	0.1939	0.1988	0.2027	0.2059	0.2085
5	0.0695	0.0922	0.1099	0.1240	0.1353	0.1447	0.1524	0.1587	0.1641	0.1686
6	0.0000	0.0303	0.0539	0.0727	0.0880	0.1005	0.1109	0.1197	0.1271	0.1334
7	-	-	0.0000	0.0240	0.0433	0.0593	0.0725	0.0837	0.0932	0.1013
8	-	-	-	-	0.0000	0.0196	0.0359	0.0496	0.0612	0.0711
9	-	-	-	-	-	-	0.0000	0.0163	0.0303	0.0422
10	-	-	-	-	-	-	-	-	0.0000	0.0140

i\n	21	22	23	24	25	26	27	28	29	30
1	0.4643	0.4590	0.4542	0.4493	0.4450	0.4407	0.4366	0.4328	0.4291	0.4254
2	0.3185	0.3156	0.3126	0.3098	0.3069	0.3043	0.3018	0.2992	0.2968	0.2944
3	0.2578	0.2571	0.2563	0.2554	0.2543	0.2533	0.2522	0.2510	0.2499	0.2487
4	0.2119	0.2131	0.2139	0.2145	0.2148	0.2151	0.2152	0.2151	0.2150	0.2148
5	0.1736	0.1764	0.1787	0.1807	0.1822	0.1836	0.1848	0.1857	0.1864	0.1870
6	0.1399	0.1443	0.1480	0.1512	0.1539	0.1563	0.1584	0.1601	0.1616	0.1630
7	0.1092	0.1150	0.1201	0.1245	0.1283	0.1316	0.1346	0.1372	0.1395	0.1415
8	0.0804	0.0878	0.0941	0.0997	0.1046	0.1089	0.1128	0.1162	0.1192	0.1219
9	0.0530	0.0618	0.0696	0.0764	0.0823	0.0876	0.0923	0.0965	0.1002	0.1036
10	0.0263	0.0368	0.0459	0.0539	0.0610	0.0672	0.0728	0.0778	0.0822	0.0862
11	0.0000	0.0122	0.0228	0.0321	0.0403	0.0476	0.0540	0.0598	0.0650	0.0697
12	-	-	0.0000	0.0107	0.0200	0.0284	0.0358	0.0424	0.0483	0.0537
13	-	-	-	-	0.0000	0.0094	0.0178	0.0253	0.0320	0.0381
14	-	-	-	-	-	-	0.0000	0.0084	0.0159	0.0227
15	-	-	-	-	-	-	-	-	0.0000	0.0076

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TABLE 21. COEFFICIENTS FOR THE SHAPIRO WILK'S TEST (CONTINUED)

i\n	31	32	33	34	35	36	37	38	39	40
1	0.4220	0.4188	0.4156	0.4127	0.4096	0.4068	0.4040	0.4015	0.3989	0.3964
2	0.2921	0.2898	0.2876	0.2854	0.2834	0.2813	0.2794	0.2774	0.2755	0.2737
3	0.2475	0.2462	0.2451	0.2439	0.2427	0.2415	0.2403	0.2391	0.2380	0.2368
4	0.2145	0.2141	0.2137	0.2132	0.2127	0.2121	0.2116	0.2110	0.2104	0.2098
5	0.1874	0.1878	0.1880	0.1882	0.1883	0.1883	0.1883	0.1881	0.1880	0.1878
6	0.1641	0.1651	0.1660	0.1667	0.1673	0.1678	0.1683	0.1686	0.1689	0.1691
7	0.1433	0.1449	0.1463	0.1475	0.1487	0.1496	0.1505	0.1513	0.1520	0.1526
8	0.1243	0.1265	0.1284	0.1301	0.1317	0.1331	0.1344	0.1356	0.1366	0.1376
9	0.1066	0.1093	0.1118	0.1140	0.1160	0.1179	0.1196	0.1211	0.1225	0.1237
10	0.0899	0.0931	0.0961	0.0988	0.1013	0.1036	0.1056	0.1075	0.1092	0.1108
11	0.0739	0.0777	0.0812	0.0844	0.0873	0.0900	0.0924	0.0947	0.0967	0.0986
12	0.0585	0.0629	0.0669	0.0706	0.0739	0.0770	0.0798	0.0824	0.0848	0.0870
13	0.0435	0.0485	0.0530	0.0572	0.0610	0.0645	0.0677	0.0706	0.0733	0.0759
14	0.0289	0.0344	0.0395	0.0441	0.0484	0.0523	0.0559	0.0592	0.0622	0.0651
15	0.0144	0.0206	0.0262	0.0314	0.0361	0.0404	0.0444	0.0481	0.0515	0.0546
16	0.0000	0.0068	0.0131	0.0187	0.0239	0.0287	0.0331	0.0372	0.0409	0.0444
17	-	-	0.0000	0.0062	0.0119	0.0172	0.0220	0.0264	0.0305	0.0343
18	-	-	-	-	0.0000	0.0057	0.0110	0.0158	0.0203	0.0244
19	-	-	-	-	-	-	0.0000	0.0053	0.0101	0.0146
20	-	-	-	-	-	-	-	-	0.0000	0.0049

i\n	41	42	43	44	45	46	47	48	49	50
1	0.3940	0.3917	0.3894	0.3872	0.3850	0.3830	0.3808	0.3789	0.3770	0.3751
2	0.2719	0.2701	0.2684	0.2667	0.2651	0.2635	0.2620	0.2604	0.2589	0.2574
3	0.2357	0.2345	0.2334	0.2323	0.2313	0.2302	0.2291	0.2281	0.2271	0.2260
4	0.2091	0.2085	0.2078	0.2072	0.2065	0.2058	0.2052	0.2045	0.2038	0.2032
5	0.1876	0.1874	0.1871	0.1868	0.1865	0.1862	0.1859	0.1855	0.1851	0.1847
6	0.1693	0.1694	0.1695	0.1695	0.1695	0.1695	0.1695	0.1693	0.1692	0.1691
7	0.1531	0.1535	0.1539	0.1542	0.1545	0.1548	0.1550	0.1551	0.1553	0.1554
8	0.1384	0.1392	0.1398	0.1405	0.1410	0.1415	0.1420	0.1423	0.1427	0.1430
9	0.1249	0.1259	0.1269	0.1278	0.1286	0.1293	0.1300	0.1306	0.1312	0.1317
10	0.1123	0.1136	0.1149	0.1160	0.1170	0.1180	0.1189	0.1197	0.1205	0.1212
11	0.1004	0.1020	0.1035	0.1049	0.1062	0.1073	0.1085	0.1095	0.1105	0.1113
12	0.0891	0.0909	0.0927	0.0943	0.0959	0.0972	0.0986	0.0998	0.1010	0.1020
13	0.0782	0.0804	0.0824	0.0842	0.0860	0.0876	0.0892	0.0906	0.0919	0.0932
14	0.0677	0.0701	0.0724	0.0745	0.0765	0.0783	0.0801	0.0817	0.0832	0.0846
15	0.0575	0.0602	0.0628	0.0651	0.0673	0.0694	0.0713	0.0731	0.0748	0.0764
16	0.0476	0.0506	0.0534	0.0560	0.0584	0.0607	0.0628	0.0648	0.0667	0.0685
17	0.0379	0.0411	0.0442	0.0471	0.0497	0.0522	0.0546	0.0568	0.0588	0.0608
18	0.0283	0.0318	0.0352	0.0383	0.0412	0.0439	0.0465	0.0489	0.0511	0.0532
19	0.0188	0.0227	0.0263	0.0296	0.0328	0.0357	0.0385	0.0411	0.0436	0.0459
20	0.0094	0.0136	0.0175	0.0211	0.0245	0.0277	0.0307	0.0335	0.0361	0.0386
21	0.0000	0.0045	0.0087	0.0126	0.0163	0.0197	0.0229	0.0259	0.0288	0.0314
22	-	-	0.0000	0.0042	0.0081	0.0118	0.0153	0.0185	0.0215	0.0244
23	-	-	-	-	0.0000	0.0039	0.0076	0.0111	0.0143	0.0174
24	-	-	-	-	-	-	0.0000	0.0037	0.0071	0.0104
25	-	-	-	-	-	-	-	-	0.0000	0.0035

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TABLE 22. QUANTILES OF THE SHAPIRO WILK'S TEST STATISTIC[1] (CONOVER, 1980)

n	0.01	0.02	0.05	0.10	0.50	0.90	0.95	0.98	0.99
3	0.753	0.756	0.767	0.789	0.959	0.998	0.999	1.000	1.000
4	0.687	0.707	0.748	0.792	0.935	0.987	0.992	0.996	0.997
5	0.686	0.715	0.762	0.806	0.927	0.979	0.986	0.991	0.993
6	0.713	0.743	0.788	0.826	0.927	0.974	0.981	0.986	0.989
7	0.730	0.760	0.803	0.838	0.928	0.972	0.979	0.985	0.988
8	0.749	0.778	0.818	0.851	0.932	0.972	0.978	0.984	0.987
9	0.764	0.791	0.829	0.859	0.935	0.972	0.978	0.984	0.986
10	0.781	0.806	0.842	0.869	0.938	0.972	0.978	0.983	0.986
11	0.792	0.817	0.850	0.876	0.940	0.973	0.979	0.984	0.986
12	0.805	0.828	0.859	0.883	0.943	0.973	0.979	0.984	0.986
13	0.814	0.837	0.866	0.889	0.945	0.974	0.979	0.984	0.986
14	0.825	0.846	0.874	0.895	0.947	0.975	0.980	0.984	0.986
15	0.835	0.855	0.881	0.901	0.950	0.975	0.980	0.984	0.987
16	0.844	0.863	0.887	0.906	0.952	0.976	0.981	0.985	0.987
17	0.851	0.869	0.892	0.910	0.954	0.977	0.981	0.985	0.987
18	0.858	0.874	0.897	0.914	0.956	0.978	0.982	0.986	0.988
19	0.863	0.879	0.901	0.917	0.957	0.978	0.982	0.986	0.988
20	0.868	0.884	0.905	0.920	0.959	0.979	0.983	0.986	0.988
21	0.873	0.888	0.908	0.923	0.960	0.980	0.983	0.987	0.989
22	0.878	0.892	0.911	0.926	0.961	0.980	0.984	0.987	0.989
23	0.881	0.895	0.914	0.928	0.962	0.981	0.984	0.987	0.989
24	0.884	0.898	0.916	0.930	0.963	0.981	0.984	0.987	0.989
25	0.888	0.901	0.918	0.931	0.964	0.981	0.985	0.988	0.989
26	0.891	0.904	0.920	0.933	0.965	0.982	0.985	0.988	0.989
27	0.894	0.906	0.923	0.935	0.965	0.982	0.985	0.988	0.990
28	0.896	0.908	0.924	0.936	0.966	0.982	0.985	0.988	0.990
29	0.898	0.910	0.926	0.937	0.966	0.982	0.985	0.988	0.990
30	0.900	0.912	0.927	0.939	0.967	0.983	0.985	0.988	0.990
31	0.902	0.914	0.929	0.940	0.967	0.983	0.986	0.988	0.990
32	0.904	0.915	0.930	0.941	0.968	0.983	0.986	0.988	0.990
33	0.906	0.917	0.931	0.942	0.968	0.983	0.986	0.989	0.990
34	0.908	0.919	0.933	0.943	0.969	0.983	0.986	0.989	0.990
35	0.910	0.920	0.934	0.944	0.969	0.984	0.986	0.989	0.990
36	0.912	0.922	0.935	0.945	0.970	0.984	0.986	0.989	0.990
37	0.914	0.924	0.936	0.946	0.970	0.984	0.987	0.989	0.990
38	0.916	0.925	0.938	0.947	0.971	0.984	0.987	0.989	0.990
39	0.917	0.927	0.939	0.948	0.971	0.984	0.987	0.989	0.991
40	0.919	0.928	0.940	0.949	0.972	0.985	0.987	0.989	0.991
41	0.920	0.929	0.941	0.950	0.972	0.985	0.987	0.989	0.991
42	0.922	0.930	0.942	0.951	0.972	0.985	0.987	0.989	0.991
43	0.923	0.932	0.943	0.951	0.973	0.985	0.987	0.990	0.991
44	0.924	0.933	0.944	0.952	0.973	0.985	0.987	0.990	0.991
45	0.926	0.934	0.945	0.953	0.973	0.985	0.988	0.990	0.991
46	0.927	0.935	0.945	0.953	0.974	0.985	0.988	0.990	0.991
47	0.928	0.936	0.946	0.954	0.974	0.985	0.988	0.990	0.991
48	0.929	0.937	0.947	0.954	0.974	0.985	0.988	0.990	0.991
49	0.929	0.937	0.947	0.955	0.974	0.985	0.988	0.990	0.991
50	0.930	0.938	0.947	0.955	0.974	0.985	0.988	0.990	0.991

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11.3.5.4.2 Calculate the following test statistic:

$$t_i = \frac{\bar{X}_1 - \bar{X}_2}{S_p\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where: $\bar{X}_1$ = Mean for the control

$\bar{X}_2$ = Mean for the effluent concentration

$$S_p = \frac{\sqrt{(n_1-1)S_1^2 + (n_2-1)S_2^2}}{n_1 + n_2 - 2}$$

S_1^2 = Estimate of the variance for the control

S_2^2 = Estimate of the variance for the effluent concentration

n_1 = Number of replicates for the control

n_2 = Number of replicates for the effluent concentration

11.3.5.4.3 Since we are concerned with a decrease in survival from the control, a one-tailed test is appropriate. Thus, compare the calculated t with a critical t, where the critical t is at the 5% level of significance with n_1+n_2-2 degrees of freedom. If the calculated t exceeds the critical t, the mean responses are declared different.

11.3.5.5 Modified T Test

11.3.5.5.1 If the F test for equality of variance fails, the t test is still a valid test. However, the denominator and the degrees of freedom for the test are modified.

11.3.5.5.2 The t statistic, with the modification for the denominator, is calculated as follows:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

where: $\bar{X}_1$ = Mean for the control

$\bar{X}_2$ = Mean for the effluent concentration

S_1^2 = Estimate of the variance for the control

S_2^2 = Estimate of the variance for the effluent concentration

n_1 = Number of replicates for the control

n_2 = Number of replicates for the effluent concentration

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11.3.5.5.3 Additionally, the degrees of freedom for the test are adjusted using the following formula:

$$df^{)} = \frac{(n_1 - 1)(n_2 - 1)}{(n_2 - 1)C^2 + (1 - C)^2(n_1 - 1)}$$

$$C = \frac{\frac{S_1^2}{n_1}}{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

11.3.5.5.4 The modified degrees of freedom is usually not an integer. Common practice is to round down to the nearest integer.

11.3.5.5.5 The modified t test is then performed in the same way as the equal variance t test. The calculated t is compared to the critical t at the 0.05 significance level with modified degrees of freedom. If the calculated t exceeds the critical t, the mean responses are found to be statistically different.

11.3.5.6 Wilcoxon Rank Sum Test

11.3.5.6.1 If the data fail the test for normality and there are four or more replicates per group, the non-parametric Wilcoxon Rank Sum Test may be used to analyze the data. If less than four replicates were used, a non-parametric alternative is not available.

11.3.5.6.2 The Wilcoxon Rank Sum Test consists of jointly ranking the data and calculating the rank sum for the effluent concentration. The rank sum is then compared to a critical value to determine acceptance or rejection of the null hypothesis.

11.3.5.6.3 To carry out the test, combine the data for the control and the effluent concentration and arrange the values in order of size from smallest to largest. Assign ranks to the ordered observations, a rank of 1 to the smallest, 2 to the next smallest, etc. If ties in rank occur, assign the average rank to each tied observation. Sum the ranks for the effluent concentration.

11.3.5.6.4 If the survival in the effluent concentration is significantly less than that of the control, the rank sum for the effluent concentration would be lower than the rank sum of the control. Thus, we are only concerned with comparing the rank sum for the effluent concentration with some "minimum" or critical rank sum, at or below which the effluent concentration survival would be considered to be significantly lower than the mortality in the control. For a test at the 5% level of significance, the critical rank sum can be found in Table 23.

TABLE 23. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST FIVE PERCENT CRITICAL LEVEL

No. of Replicates in Control	No. of Replicates per Effluent Concentration							
	3	4	5	6	7	8	9	10
3		10	16	23	30	39	49	59
4	6	11	17	24	32	41	51	62
5	7	12	19	26	34	44	54	66
6	8	13	20	28	36	46	57	69
7	8	14	21	29	39	49	60	72
8	9	15	23	31	41	51	63	72
9	10	16	24	33	43	54	66	79
10	10	17	26	35	45	56	69	82

11.3.6 SINGLE CONCENTRATION TEST

11.3.6.1 Data from an acute effluent toxicity test with *Ceriodaphnia* are provided in Table 24. The proportion surviving in each replicate is transformed by the arc sine square root transformation prior to statistical analysis of the data (Figure 12).

TABLE 24. DATA FROM AN ACUTE SINGLE-CONCENTRATION TOXICITY TEST WITH *CERIODAPHNIA*

		Proportion Surviving	
	Replicate	Control	100% Effluent Concentration
RAW DATA	A	1.00	0.40
	B	1.00	0.30
	C	0.90	0.40
	D	0.90	0.20
ARC SINE TRANSFORMED DATA	A	1.412	0.685
	B	1.412	0.580
	C	1.249	0.685
	D	1.249	0.464
	$\bar{X}$	1.330	0.604
	S^2	0.0088	0.0111

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TABLE 25. EXAMPLE OF SHAPIRO WILK'S TEST: CENTERED OBSERVATIONS

Treatment	Replicate			
	A	B	C	D
Control	0.082	0.082	-0.081	-0.081
100% Effluent	0.081	-0.024	0.081	-0.140

11.3.6.2 After the data have been transformed, test the assumption of normality via the Shapiro Wilk's test.

11.3.6.2.1 The first step in the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 25.

11.3.6.2.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{8} (X_i - \bar{X})^2$$

For this set of data, $\bar{X} = 0$ and D = 0.060.

11.3.6.2.3 Order the centered observations from smallest to largest. The ordered observations are listed in Table 26.

11.3.6.2.4 From Table 21, for n = 8 and k = n/2 = 4, obtain the coefficients $a_1, a_2, ..., a_k$. The a_i values are listed in Table 27.

11.3.6.2.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{0.060} \times (0.2200)^2 = 0.0807$$

The differences, $X^{(n-i+1)}-X^{(i)}$, are listed in Table 27.

11.3.6.2.6 From Table 22, the critical W value for n = 8 and a significance level of 0.01, is 0.749. Since the calculated W, 0.807, is not less than the critical value the conclusion of the test is that the data are normally distributed.

TABLE 26. EXAMPLE OF SHAPIRO WILK'S TEST: ORDERED OBSERVATIONS

i	$X^{(i)}$
1	-0.140
2	-0.081
3	-0.081
4	-0.024
5	0.081
6	0.081
7	0.082
8	0.082

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TABLE 27. EXAMPLE OF SHAPIRO WILK'S TEST: TABLE OF COEFFICIENTS AND DIFFERENCES

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.6052	0.222	$X^{(8)} - X^{(1)}$
2	0.3164	0.163	$X^{(7)} - X^{(2)}$
3	0.1743	0.162	$X^{(6)} - X^{(3)}$
4	0.0561	0.105	$X^{(5)} - X^{(4)}$

11.3.6.3 The F test for equality of variances is used to test the homogeneity of variance assumption.

11.3.6.3.1 From Table 24, obtain the sample variances for the control and the 100% effluent. Since the variability of the 100% effluent is greater than the variability of the control, S^2 for the 100% effluent concentration is placed in the numerator of the F statistic and S^2 for the control is placed in the denominator.

$$F = \frac{0.0111}{0.0088} = 1.2614$$

11.3.6.3.2 There are four replicates for the control and four replicates for the 100% effluent concentration. Thus there are three degrees of freedom for the numerator and the denominator. For a two-tailed test at the 0.01 level of significance, the critical F value is 47.467. The calculated F, 1.2614, is less than the critical F, 47.467, thus the conclusion is that the variances of the control and 100% effluent are equal.

11.3.6.4 The assumptions of normality and homogeneity of variance have been met for this data set. An equal variance t test will be used to compare the mean responses of the control and 100% effluent.

11.3.6.4.1 To perform the t test, obtain the values for X_1, X_2, S_1^2, and S_2^2 from Table 24. Calculate the t statistic as follows:

$$t = \frac{1.330 - 0.604}{0.0997\sqrt{\frac{1}{4} + \frac{1}{4}}}$$

where:

$$S_p = \frac{\sqrt{(4-1)0.0088 + (4-1)(0.0111)}}{4+4-2}$$

11.3.6.4.2 For a one-tailed test at the 0.05 level of significance with 6 degrees of freedom, the critical t value is 1.9432. Since the calculated t, 10.298, is greater than the critical t, the conclusion is that the survival in the 100% effluent concentration is significantly less than the survival in the control.

11.3.6.5 If the data had failed the normality assumption, the appropriate analysis would have been the Wilcoxon Rank Sum Test. To provide an example of this test, the survival data from the t test example will be reanalyzed by the nonparametric procedure.

11.3.6.5.1 The first step in the Wilcoxon Rank Sum Test is to combine the data from the control and the 100% effluent concentration and arrange the values in order of size, from smallest to largest.

11.3.6.5.2 Assign ranks to the ordered observations, a rank of 1 to the smallest, 2 to the next smallest, etc. The combined data with ranks assigned is presented in Table 28.

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TABLE 28. EXAMPLE OF WILCOXON'S RANK SUM TEST: ASSIGNING RANKS TO THE CONTROL AND 100% EFFLUENT CONCENTRATIONS

Rank	Proportion Surviving	Control or 100% Effluent
1	0.20	100% EFFLUENT
2	0.30	100% EFFLUENT
3.5	0.40	100% EFFLUENT
3.5	0.40	100% EFFLUENT
5.5	0.90	CONTROL
5.5	0.90	CONTROL
7.5	1.00	CONTROL
7.5	1.00	CONTROL

11.3.6.5.3 Sum the ranks for the 100% effluent concentration.

11.3.6.5.4 For this set of data, the test is for a significant reduction in survival in the 100% effluent concentration as compared to the control. The critical value, from Table 23, for four replicates in each group and a significance level of 0.05 is 11. The rank sum for the 100% effluent concentration is 10 which is less than the critical value of 11. Thus the conclusion is that survival in the effluent concentration is significantly less than the control survival.

11.3.7 MULTI-CONCENTRATION TEST

11.3.7.1 Formal statistical analysis of the survival data is outlined in Figure 13. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Concentrations at which there is no survival in any of the test chambers are excluded from statistical determination of the NOAEC.

11.3.7.2 For the case of equal numbers of replicates across all concentrations and the control, the determination of the NOAEC endpoint is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro Wilk's Test, and Bartlett's Test is used to test for the homogeneity of variance. If either of these tests fail, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOAEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

11.3.7.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t-test with a Bonferroni adjustment. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

11.3.7.4 Example of Analysis of Survival Data

11.3.7.4.1 This example uses survival data from a fathead minnow test. The proportion surviving in each replicate must first be transformed by the arc sine square root transformation procedure. The raw and transformed data, means and standard deviations of the transformed observations at each toxicant concentration and control are listed in Table 29. A plot of the survival proportions is provided in Figure 14.

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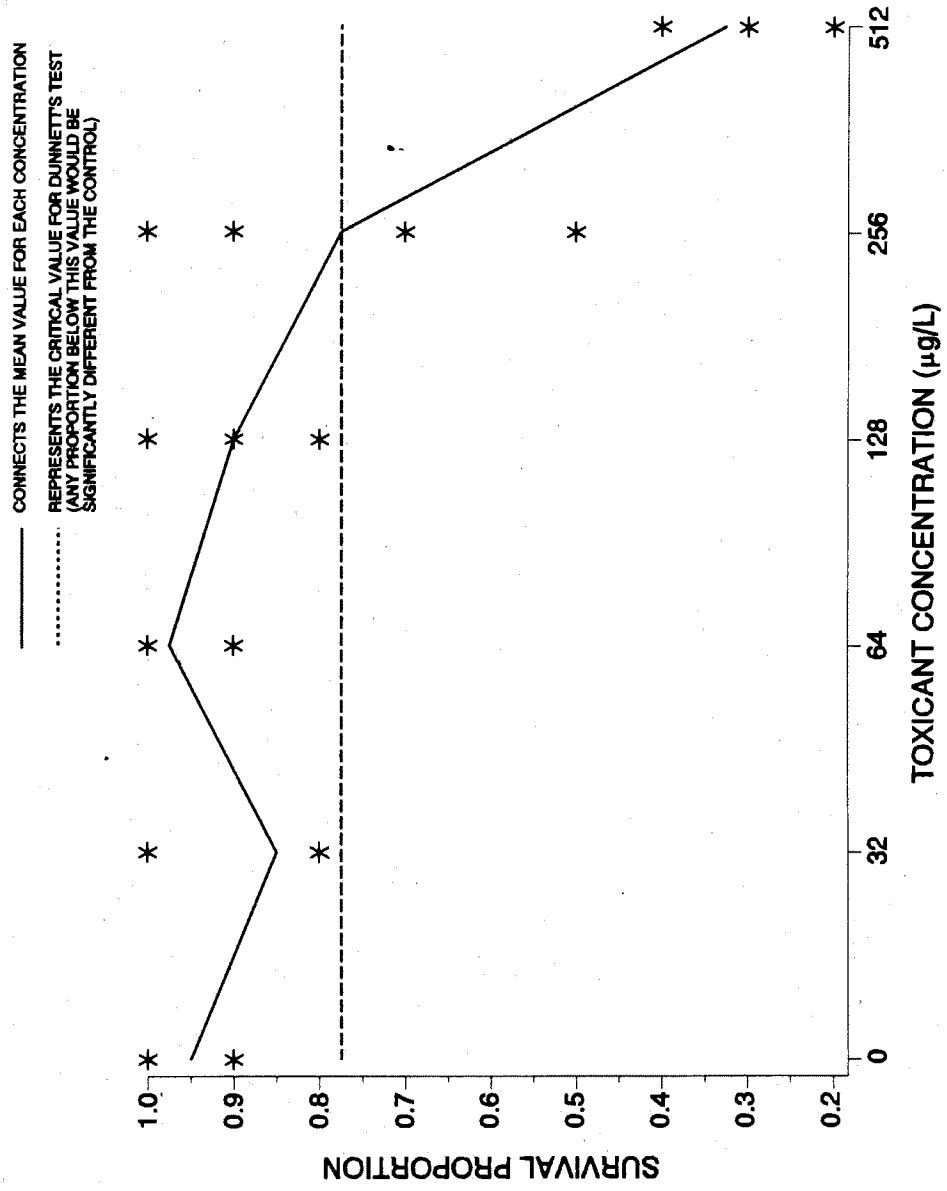

Figure 14. Plot of mean survival proportion data in Table 29.

11.3.7.4.2 Test for Normality

1. The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 30.

TABLE 29. FATHEAD MINNOW SURVIVAL DATA

			Toxicant Concentration (µg/L)				
	Replicate	Control	32	64	128	256	512
	A	1.0	0.8	0.9	0.9	0.7	0.4
RAW	B	1.0	0.8	1.0	0.9	0.9	0.3
	C	0.9	1.0	1.0	0.8	1.0	0.4
	D	0.9	0.8	1.0	1.0	0.5	0.2
ARC SINE	A	1.412	1.107	1.249	1.249	0.991	0.685
TRANS-	B	1.412	1.107	1.412	1.249	1.249	0.580
FORMED	C	1.249	1.412	1.412	1.107	1.412	0.685
	D	1.249	1.107	1.412	1.412	0.785	0.464
MEAN($\overline{Y}_i$)		1.330	1.183	1.371	1.254	1.109	0.604
S_i^2		0.0088	0.0232	0.0066	0.0155	0.0768	0.0111
i		1	2	3	4	5	6

TABLE 30. CENTERED OBSERVATIONS FOR SHAPIRO WILK'S EXAMPLE

		Toxicant Concentration (µg/L)				
Replicate	Control	32	64	128	256	512
A	0.082	-0.076	-0.122	-0.005	-0.118	0.081
B	0.082	-0.076	0.041	-0.005	0.140	-0.024
C	-0.081	0.229	0.041	-0.147	0.303	0.081
D	-0.081	-0.076	0.041	0.158	-0.324	-0.140

2. Calculate the denominator, D, of the statistic:

$$D' \sum_{i=1}^{n} (X_i - \overline{X})^2$$

where: X_i = the ith centered observation
$\overline{X}$ = the overall mean of the centered observations
n = the total number of centered observations

3. For this set of data: n = 24 (number of observations)

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$$\bar{X} = \frac{1}{24}(0.000) = 0.000$$

$$D = 0.4265$$

4. Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where: $X^{(i)}$ denotes the ith ordered observation.

The ordered observations for this example are listed in Table 31.

TABLE 31. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.324	13	-0.005
2	-0.147	14	0.041
3	-0.140	15	0.041
4	-0.122	16	0.041
5	-0.118	17	0.081
6	-0.081	18	0.081
7	-0.081	19	0.082
8	-0.076	20	0.082
9	-0.076	21	0.140
10	-0.076	22	0.158
11	-0.024	23	0.229
12	-0.005	24	0.303

5. From Table 21, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots a_k$, where k is approximately n/2 if n is even; (n-1)/2 if n is odd. For the data in this example, n=24 and k=12. The a_i values are listed in Table 32.

6. Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)}-X^{(i)}$ are listed in Table 32. For the data in this example,

$$W = \frac{1}{0.4265}(0.6444)^2 = 0.974$$

7. The decision rule for this test is to compare W as calculated in #6 to a critical value found in Table 23. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.974 is greater than the critical value, conclude that the data are normally distributed.

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TABLE 32. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.627	$X^{(24)} - X^{(1)}$
2	0.3098	0.376	$X^{(23)} - X^{(2)}$
3	0.2554	0.298	$X^{(22)} - X^{(3)}$
4	0.2145	0.262	$X^{(21)} - X^{(4)}$
5	0.1807	0.200	$X^{(20)} - X^{(5)}$
6	0.1512	0.163	$X^{(19)} - X^{(6)}$
7	0.1245	0.162	$X^{(18)} - X^{(7)}$
8	0.0997	0.157	$X^{(17)} - X^{(8)}$
9	0.0764	0.117	$X^{(16)} - X^{(9)}$
10	0.0539	0.117	$X^{(15)} - X^{(10)}$
11	0.0321	0.065	$X^{(14)} - X^{(11)}$
12	0.0107	0.0	$X^{(13)} - X^{(12)}$

11.3.7.4.3 Test for Homogeneity of Variance

1. The test used to examine whether the variation in mean proportion surviving is the same across all toxicant concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i) \ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

where: V_i = degrees of freedom for each toxicant concentration and control, $V_i = (n_i - 1)$
n_i = the number of replicates for concentration i.
$\ln = \log_e$
i = 1, 2, ..., p where p is the number of concentrations including the control

$$\bar{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

2. For the data in this example, (See Table 29) all toxicant concentrations including the control have the same number of replicates (n_i = 4 for all i). Thus, V_i = 3 for all i.

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3. Bartlett's statistic is therefore:

$$B = [(18)\ln(0.0236) - 3\sum_{i=1}^{P} \ln(S_i^2)]/1.1296$$

$$= [18(-3.7465) - 3(-24.7516)]/1.1296$$

$$= 6.8178/1.1296$$

$$= 6.036$$

4. B is approximately distributed as chi square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with five degrees of freedom, is 15.086. Since B = 6.036 is less than the critical value of 15.086, conclude that the variances are not different.

11.3.7.4.4 Dunnett's Procedure

1. To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table (Table 33).

TABLE 33. ANOVA TABLE

Source	DF	Sum of Squares (SS)	Mean Square (MS) (SS/DF)
BETWEEN	P - 1	SSB	S_B^2 = SSB/(P-1)
WITHIN	N - P	SSW	S_W^2 = SSW/(N-P)
Total	N - 1	SST	

where: p = number toxicant concentrations including the control
N = total number of observations $n_1 + n_2 ... + n_p$
n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

SSW = SST - SSB Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration "i"
Y_{ij} = the jth observation for concentration "i" (represents the proportion surviving for toxicant concentration i in test chamber j)

2. For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 4$

$N = 24$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 5.322$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 4.733$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 5.485$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 5.017$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 4.437$
$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} = 2.414$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 27.408$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$

$$= \frac{1}{4}(131.495) - \frac{(27.408)^2}{24} = 1.574$$

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 33.300 - \frac{(27.408)^2}{24} = 2.000$$

SSW = SST - SSB = 2.000 - 1.574 = 0.4260

S_B2 = SSB/(p - 1) = 1.574/(6 - 1) = 0.3150

S_W^2 = SSW/(N - p) = 0.426/(24 - 6) = 0.024

3. Summarize these calculations in the ANOVA table (Table 34).

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TABLE 34. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	DF	Sum of Squares (SS)	Mean Square (MS) (SS/DF)
BETWEEN	5	1.574	0.315
WITHIN	18	0.426	0.024
Total	23	2.002	

4. To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1)+(1/n_i)}}$$

where:
$\bar{Y}_i$ = mean proportion surviving for concentration i
$\bar{Y}_1$ = mean proportion surviving for the control
S_W = square root of within mean square
n_1 = number of replicates for control
n_i = number of replicates for concentration i.

5. Table 35 includes the calculated t values for each concentration and control combination. In this example, comparing the 32 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.330 - 1.183)}{[0.155\sqrt{(1/4) + (1/4)}]} = 1.341$$

6. Since the purpose of this test is to detect a significant reduction in proportion surviving, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 36. For an overall alpha level of 0.05, 18 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.41. The mean proportion surviving for concentration "i" is considered significantly less than the mean proportion surviving for the control if t_i is greater than the critical value. Since t is greater than 2.41, the 512 µg/L concentration has significantly lower survival than the control. Hence the NOAEC for survival is 256 µg/L.

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TABLE 35. CALCULATED T VALUES

Toxicant Concentration (µg/L)	i	t_i
32	2	1.341
64	3	-0.374
128	4	0.693
256	5	2.016
512	6	6.624

7. To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD \;'\; d\,S_w\sqrt{(1/n_1)\ \%\ (1/n)}$$

where: d = the critical value for the Dunnett's procedure
S_W = the square root of the within mean square
n = the common number of replicates at each concentration (this assumes equal replication at each concentration)
n_i = the number of replicates in the control.

8. In this example:

$$MSD \;'\; 2.41(0.155)\sqrt{(1/4)\ \%\ (1/4)}$$

$$= 2.41\ (0.155)(0.707)$$

$$= 0.264$$

9. The MSD (0.264) is in transformed units. To determine the MSD in terms of percent survival, carry out the following conversion.

(1) Subtract the MSD from the transformed control mean.

$$1.330 - 0.264 = 1.066$$

(2) Obtain the untransformed values for the control mean and the difference calculated in 1.

$$[\text{Sine}\ (\ 1.330)\ ]^2 = 0.943$$

$$[\text{Sine}\ (\ 1.066)\ ]^2 = 0.766$$

(3) The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from 2.

$$MSD_u = 0.943 - 0.766 = 0.177$$

10. Therefore, for this set of data, the minimum difference in mean proportion surviving between the control and any toxicant concentration that can be detected as statistically significant is 0.177.

11. This represents a decrease in survival of 19% from the control.

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TABLE 36. DUNNETT'S "T" VALUES (MILLER, 1981)

(One-tailed)d^{α}_{k}									
					$\alpha = .05$				
	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.58	2.85	2.98	3.08	3.16	3.24	3.30
6	1.94	2.34	2.56	2.71	2.83	2.92	3.00	3.07	3.12
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01
8	1.86	2.22	2.42	2.55	2.66	2.74	2.81	2.87	2.92
9	1.83	2.18	2.37	2.50	2.60	2.68	2.75	2.81	2.86
10	1.81	2.15	2.34	2.47	2.56	2.64	2.70	2.76	2.81
11	1.80	2.13	2.31	2.44	2.53	2.60	2.67	2.72	2.77
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.69	2.74
13	1.77	2.09	2.27	2.39	2.48	2.55	2.61	2.68	2.71
14	1.76	2.08	2.25	2.37	2.46	2.53	2.59	2.64	2.69
15	1.75	2.07	2.24	2.36	2.44	2.51	2.57	2.62	2.67
16	1.75	2.06	2.23	2.34	2.43	2.50	2.56	2.61	2.65
17	1.74	2.05	2.22	2.33	2.42	2.49	2.54	2.59	2.64
18	1.73	2.04	2.21	2.32	2.41	2.48	2.53	2.58	2.62
19	1.73	2.03	2.20	2.31	2.40	2.47	2.52	2.57	2.61
20	1.72	2.03	2.19	2.30	2.38	2.46	2.51	2.56	2.60
24	1.71	2.01	2.17	2.28	2.36	2.43	2.48	2.53	2.57
30	1.70	1.99	2.15	2.25	2.33	2.40	2.45	2.50	2.54
40	1.68	1.97	2.13	2.23	2.31	2.37	2.42	2.47	2.51
60	1.67	1.95	2.10	2.21	2.28	2.35	2.39	2.44	2.48
120	1.86	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45
α	1.64	1.92	2.06	2.16	2.23	2.29	2.34	2.33	2.42

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TABLE 36. DUNNETT'S "T" VALUES (MILLER, 1981) (CONTINUED)

(One-tailed)d^{α}_{k}									
					$\alpha = 0.1$				
	1	2	3	4	5	6	7	8	9
5	3.37	3.90	4.21	4.43	4.50	4.73	4.85	4.94	5.03
6	3.14	3.61	4.88	4.07	4.21	4.33	4.43	4.51	4.39
7	3.00	3.42	3.56	3.83	3.96	4.07	4.15	4.23	4.30
8	2.90	3.20	3.51	3.67	3.79	3.88	3.96	4.03	4.09
9	2.82	3.19	3.40	3.55	3.64	3.75	3.82	3.89	3.94
10	2.76	3.11	3.31	3.45	3.56	3.64	3.71	3.78	3.83
11	2.72	3.06	3.25	3.38	3.46	3.56	3.63	3.69	3.74
12	2.68	3.01	3.19	3.32	3.42	3.50	3.56	3.62	3.67
13	2.65	2.97	3.15	3.27	3.37	3.44	3.91	3.56	3.61
14	2.62	2.94	3.11	3.23	3.32	3.40	3.46	3.51	3.56
15	2.60	2.91	3.08	3.20	3.29	3.36	3.42	3.47	3.52
16	2.58	2.38	3.05	3.17	3.28	3.33	3.39	3.44	3.48
17	2.57	2.86	3.03	3.14	3.23	3.30	3.36	3.41	3.45
18	2.55	2.84	3.01	3.12	3.21	3.27	3.33	3.38	3.42
19	2.54	2.83	2.99	3.10	3.18	3.25	3.31	3.36	3.40
20	2.53	2.81	2.97	3.08	3.17	3.23	3.29	3.34	3.38
24	2.40	2.77	2.92	3.03	3.11	3.17	3.22	3.27	3.31
30	2.46	2.72	2.87	2.97	3.05	3.11	3.16	3.21	3.24
40	2.42	2.68	2.82	2.92	2.99	3.06	3.10	3.14	3.18
60	2.39	2.64	2.78	2.87	2.94	3.08	3.04	3.06	3.12
120	2.36	2.60	2.73	2.82	2.90	2.94	2.90	3.03	3.06
α	2.33	2.56	2.68	2.77	2.34	2.90	2.83	2.97	3.00

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SECTION 12

REPORT PREPARATION AND TEST REVIEW

12.1 REPORT PREPARATION

The following general format and content are recommended for the report:

12.1.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contractor (if contracted)
   a. Name of firm
   b. Phone number
   c. Address
6. Objective of test

12.1.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of discharge (MGD, CFS, GPM)
8. Design flow of treatment facility at time of sampling

12.1.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
   a. Sampling point (including latitude and longitude)
   b. Sample collection method
   c. Collection dates and times
   d. Mean daily discharge on sample collection date
   e. Lapsed time from sample collection to delivery
   f. Sample temperature when received at the laboratory
   g. Physical and chemical data

2. Receiving Water Samples
   a. Sampling point (including latitude and longitude)
   b. Sample collection method
   c. Collection dates and times
   d. Streamflow (at time of sampling)
   e. Lapsed time from sample collection to delivery
   f. Sample temperature when received at the laboratory
   g. Physical and chemical data

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3. Dilution Water Samples
   a. Source
   b. Collection date(s) and time(s) (where applicable)
   c. Pretreatment
   d. Physical and chemical characteristics (pH, hardness, salinity, etc.)

12.1.4 TEST CONDITIONS

1. Toxicity test method used (title, number, source)
2. Endpoint(s) of test
3. Deviations from reference method, if any, and reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type and volume of test chambers
7. Volume of solution used per chamber
8. Number of organisms per test chamber
9. Number of replicate test chambers per treatment
10. Feeding frequency, and amount and type of food
11. Acclimation temperature of test organisms (mean and range)
12. Test temperature (mean and range)

12.1.5 TEST ORGANISMS

1. Scientific name
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)

12.1.6 QUALITY ASSURANCE

1. Reference toxicant used routinely; source; date received; lot no.
2. Date and time of most recent reference toxicant test; test results and current cusum chart
3. Dilution water used in reference toxicant test
4. Physical and chemical methods used

12.1.7 RESULTS

1. Provide raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls) and replicate, and in graphical form (plots of toxicity data)
2. Provide table of endpoints: LC50, NOAEC, Pass/Fail (as required in the applicable NPDES permit)
3. Indicate statistical methods used to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data

12.1.8 CONCLUSIONS AND RECOMMENDATIONS

1. Relationship between test endpoints and permit limits.
2. Action to be taken.

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12.2 TEST REVIEW

12.2.1 Test review is an important part of an overall quality assurance program (Section 4) and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the regulatory authority.

12.2.2 SAMPLING AND HANDLING

12.2.2.1 The collection and handling of samples are reviewed to verify that the sampling and handling procedures given in Section 8 were followed. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times (Subsection 8.5.4). Any deviations from the procedures given in Section 8 should be documented and described in the data report (Subsection 12.1).

12.2.3 TEST ACCEPTABILITY CRITERIA

12.2.3.1 Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. Any test not meeting the minimum test acceptability criteria is considered invalid. All invalid tests must be repeated with a newly collected sample.

12.2.4 TEST CONDITIONS

12.2.4.1 Test conditions are reviewed and compared to the specifications listed in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) also are reviewed and compared to specified ranges. Any deviations from specifications should be documented and described in the data report (Subsection 12.1).

12.2.4.2 The summary of test condition tables presented for each method identify test conditions as required or recommended. For WET test data submitted under NPDES permits, all required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. Deviations from recommended test conditions must be evaluated on a case-by-case basis to determine the validity of test results. Deviations from recommended test conditions may or may not invalidate a test result depending on the degree of the departure and the objective of the test. The reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result as valid. For example, if dissolved oxygen is measured below 4.0 mg/L in one test chamber, the reviewer should consider whether any observed mortality in that test chamber corresponded with the drop in dissolved oxygen.

12.2.4.3 Whereas slight deviations in test conditions may not invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicate the need for improved quality control in that laboratory.

12.2.5 STATISTICAL METHODS

12.2.5.1 The statistical methods used for analyzing test data are reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report. Statistical methods other than those recommended in the statistical flowcharts may be appropriate (see Subsection 11.1.4), however, the laboratory must document the use of and provide the rationale for the use of any alternate statistical method. In all cases (flowchart recommended methods or alternate methods), reviewers should verify that the necessary assumptions are met for the statistical method used.

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12.2.6 CONCENTRATION-RESPONSE RELATIONSHIPS

12.2.6.1 The concept of a concentration-response, or more classically, a dose-response relationship is "the most fundamental and pervasive one in toxicology" (Casarett and Doull, 1975). This concept assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response. A response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant. The classical concentration-response relationship is depicted as a sigmoidal shaped curve, however, the particular shape of the concentration-response curve may differ for each coupled toxicant and response pair. In general, more severe responses (such as acute effects) occur at higher concentrations of the toxicant, and less severe responses (such as chronic effects) occur at lower concentrations. A single toxicant also may produce multiple responses, each characterized by a concentration-response relationship. A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results.

12.2.6.2 The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. USEPA (2000a) provides guidance on evaluating concentration-response relationships to assist in determining the validity of WET test results. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to USEPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a). This guidance provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations: that calculated effect concentrations are reliable and should be reported, that calculated effect concentrations are anomalous and should be explained, or that the test was inconclusive and the test should be repeated with a newly collected sample. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting.

12.2.7 REFERENCE TOXICANT TESTING

12.2.7.1 Test review of a given effluent or receiving water test should include review of the associated reference toxicant test and current control chart. Reference toxicant testing and control charting is required for documenting the quality of test organisms (Subsection 4.7) and ongoing laboratory performance (Subsection 4.15). The reviewer should verify that a quality control reference toxicant test was conducted according to the specified frequency required by the permitting authority or recommended by the method (e.g., monthly). The test acceptability criteria, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant test are reviewed to verify that the reference toxicant test conducted was a valid test. The results of the reference toxicant test are then plotted on a control chart (see Subsection 4.15) and compared to the current control chart limits (± 2 standard deviations).

12.2.7.2 Reference toxicant tests that fall outside of recommended control chart limits are evaluated to determine the validity of associated effluent and receiving water tests (see Subsection 4.15). An out of control reference toxicant test result does not necessarily invalidate associated test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increasing test organism sensitivity or toward decreasing test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test. More frequent and/or concurrent reference toxicant testing may be advantageous if recent problems (e.g., invalid tests, reference toxicant test results outside of control chart limits, reduced health of organism cultures, or increased within-test variability) have been identified in testing.

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12.2.8 TEST VARIABILITY

12.2.8.1 The within-test variability of individual tests should be reviewed. Excessive within-test variability may invalidate a test result and warrant retesting. For evaluating within-test variability, reviewers should consult EPA guidance on upper and lower percent minimum significant difference (PMSD) bounds (USEPA, 2000b).

12.2.8.2 USEPA guidance on WET variability recommends incorporating upper and lower bounds using the PMSD to control and minimize within-test method variability and increase test sensitivity (USEPA, 2000b). The minimum significant difference (MSD) is the smallest difference between the control and another test treatment that can be determined as statistically significant in a given test, and the PMSD is the MSD represented as a percentage of the control response. The equation and examples of MSD calculations are shown in Subsection 11.3.7.4.4.

12.2.8.3 To assist in reviewing within-test variability, EPA recommends maintaining control charts of PMSDs calculated for successive effluent tests (USEPA, 2000b). A control chart of PMSD values characterizes the range of variability observed within a given laboratory, and allows comparison of individual test PMSDs with the laboratory's typical range of variability. Control charts of other variability and test performance measures, such as the MSD, standard deviation or CV of control responses, or average c ontrol response, also may be useful for reviewing tests and minimizing variability.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.1. *CERIODAPHNIA DUBIA*

1. SYSTEMATICS

1.1 MORPHOLOGY AND TAXONOMY

1.1.1 *Ceriodaphnia* are closely related and morphologically similar to *Daphnia*, but are smaller and have a shorter generation time (USEPA, 1986). They are generally more rotund, lack the prominent rostral projection typical of *Daphnia*, and do not develop the dorsal helmets and long posterior spines often observed in *Daphnia*.

1.1.2 With *Ceriodaphnia dubia*, the female has a heavy, setulated pecten on the postabdominal claw (Figure 1A), and the male was long antennules (Figure 1C), in contrast to the closely related *C. reticulata*, where the female has heavy, triangular denticles in the pecten of the postabdominal claw (Figure 2A), and the male has very short antennules (Figure 2C). Some clones having intermediate characters may be hybrids or phenotypic variants of *C. dubia* (USEPA, 1986). Detailed descriptions of the males and females of both species and the variant were given by USEPA (1986).

1.1.3 Although males are very similar to females, they can be recognized by their rapid, erratic swimming habit, smaller size, denser coloration, extended antennules and claspers, and rostrum morphology.

2. ECOLOGY AND LIFE HISTORY

2.1 DISTRIBUTION

2.1.1 *C. dubia*, has been reported from littoral areas of lakes, ponds, and marshes throughout most of the world, but it is difficult to ascertain its true distribution because it has been reported in the literature under several other names (*C. affinis, C. quadrangula*, and *C. reticulata)*. It has also been suggested that reports of *C. dubia* in New Zealand and parts of Asia may be yet another unnamed species (Berner, personal communication).

2.2 ECOLOGY

2.2.1 *Ceriodaphnia* ecology and life history are very similar to those of other daphnids. Specific information on the ecology and life history of *Ceriodaphnia dubia* is either not available or is widely scattered throughout the literature. However, it is known to be a pond and lake dwelling species that is usually common among the vegetation in littoral areas (Fairchild, 1981). In the Lake of Velence, Hungary, *C. dubia* was most common in regions where "grey" and "dark brown" waters merged (Pal, 1980). In Par Pond (Savannah River Plant, Aiken, SC) the *Ceriodaphnia* were much more abundant in the heated water (effluent from the nuclear reactor) than in the ambient area (Vigerstad and Tilly, 1977), and in a reservoir in Russia, animals from the heated water were larger and heavier than those living under normal water temperatures (Kititsyna and Sergeeva, 1976). In Iran they are common in warmer, montane, oligotrophic lakes (Smagowicz, 1976).

2.2.2 In Lake Kinneret, Israel, *Ceriodaphnia reticulata* are abundant only between March and June, with a peak in May when the temperature ranges between 20 and 22°C. When summer temperatures reached 27-28°C, the *Ceriodaphnia* were reduced in size and egg production became significantly less, leading to a progressive decline of the population (Gophen, 1976). In Lake Parvin, France, the period of development was from June to September (Devaux, 1980).

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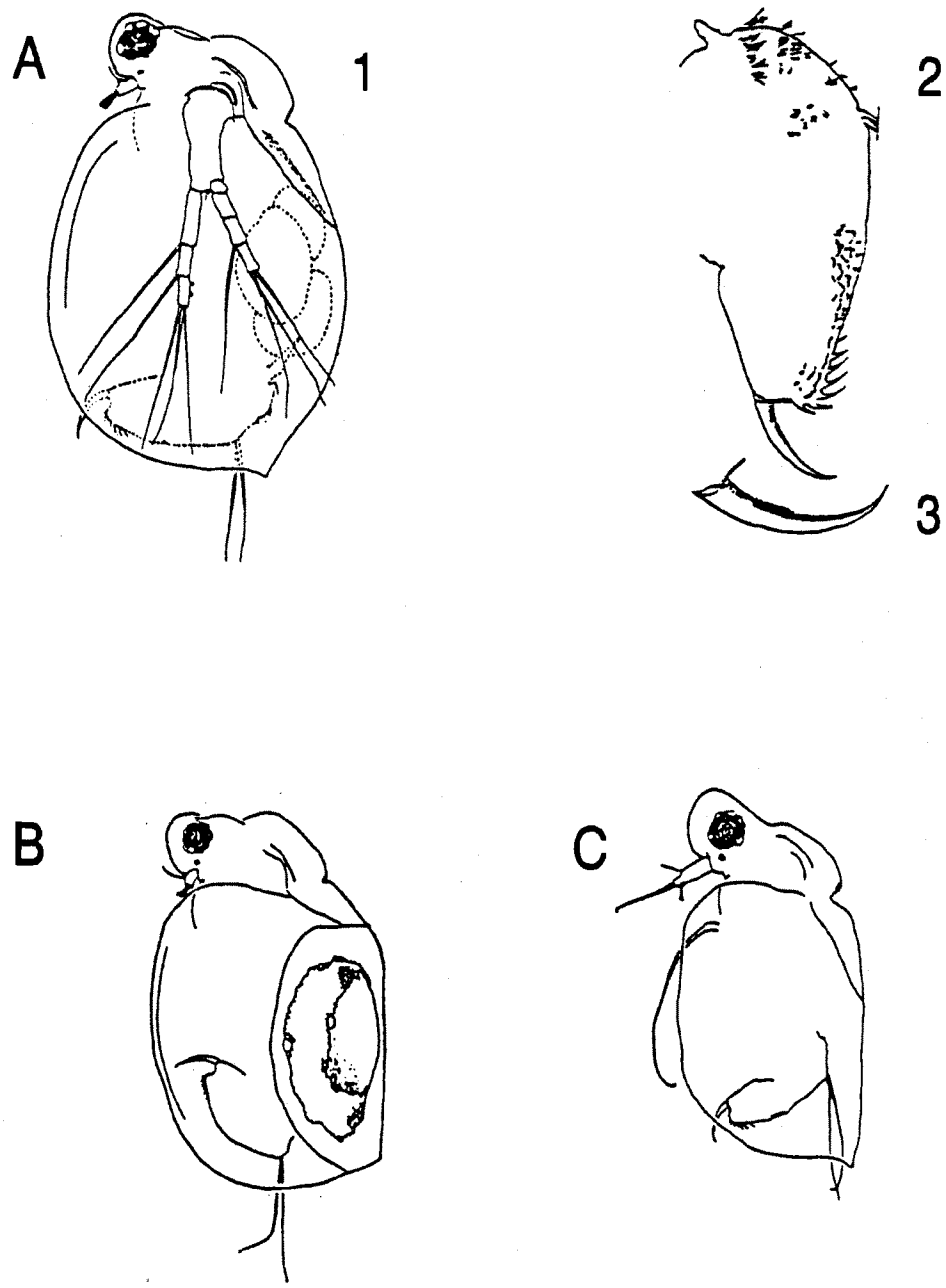

Figure 1. *Ceriodaphnia dubia*. A. (1) parthenogenetic female, (2) postabdomen, and (3) claw; B. ephippial female; C. Male. (From USEPA, 1986)

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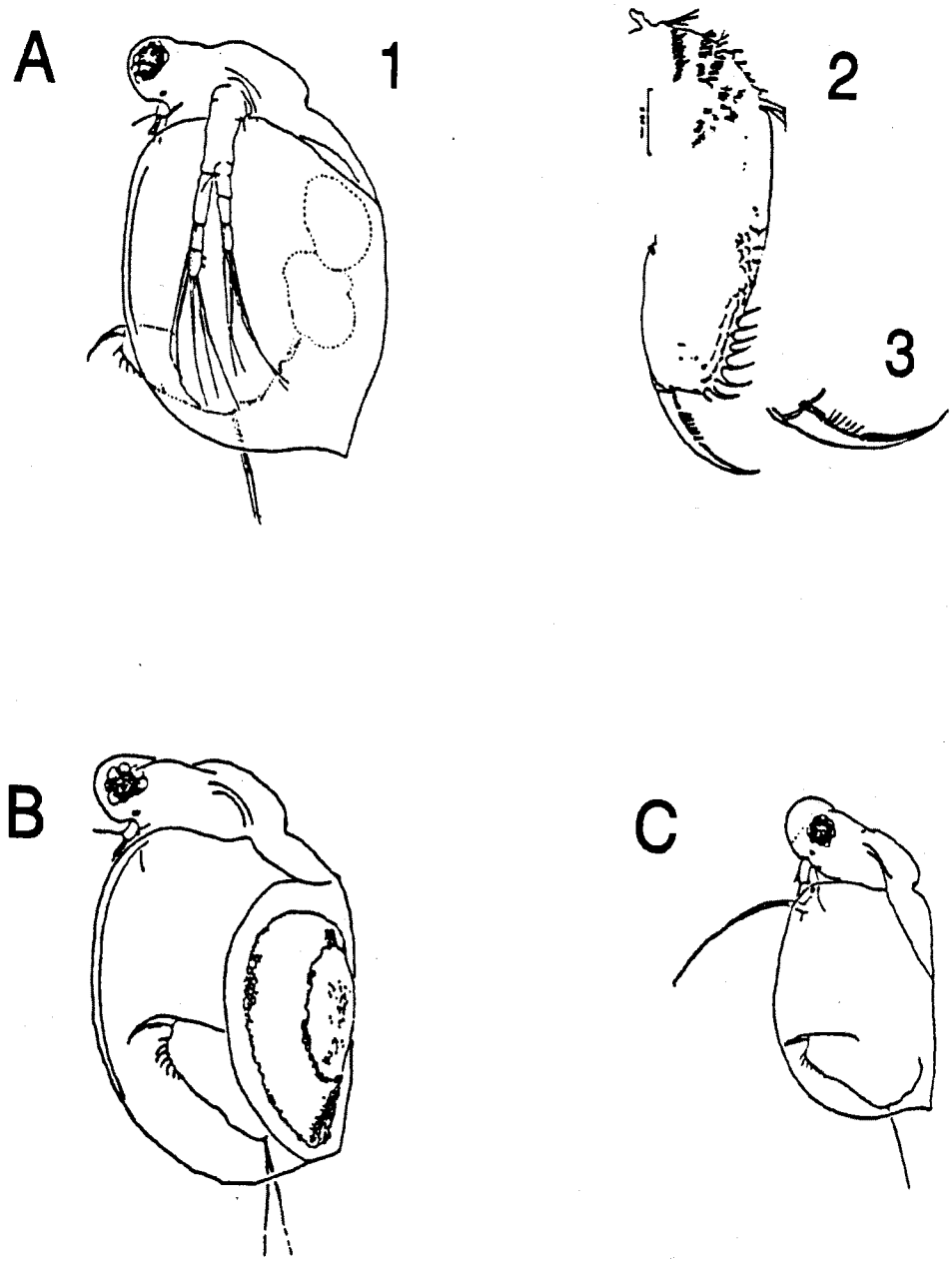

Figure 2. *Ceriodaphnia reticulata*. A. (1) parthenogenetic female, (2) postabdomen, (3) and claw; B. ephippial female; C. Male. (From USEPA, 1986)

2.2.3 *Ceriodaphnia* typically swim with an erratic, jerking motion for a period of time, and hang motionless in the water between swimming bouts. This swimming behavior results in a mean speed of 1.5-2.5 mm/s. When approached by a predator, however, it flees by swimming away quickly along a straight path (Wong, 1981).

2.2.4 During most of the year, populations of *Ceriodaphnia* consist almost entirely of females; the males appearing principally in autumn. Production of males appears to be induced primarily by low water temperatures, high population densities, and/or a decrease in available food. As far as is presently known, *C. dubia* reproduce only by cyclic parthenogenesis in which the males contribute to the genetic makeup of the young during the sexual stage of reproduction.

2.2.5 The females tend to aggregate during sexual reproductive activity, when ephippia are produced (Brandl and Fernando, 1971). Ephippia are embryos encased in a tough covering, and are resistent to drying. They can be stored for long periods and shipped through the mail in envelopes, like seeds. When placed in water at the proper temperature, ephippia hatch in a few days producing a new parthenogenetic population.

2.2.6 *Ceriodaphnia* have many predators, including fish, the mysid *Mysis relicta, Chaoborus* larvae, and copepods. As with *Daphnia*, it also reacts to intense predation with defensive strategies. *Ceriodaphnia reticulata* (possibly *C.dubia*) in a Minnesota lake, reacted to the copepod, *Cyclops vernalis*, by producing large offspring and growing to a large size at the expense of early reproduction (Lynch, 1979). They reacted to fish predators by producing smaller offspring in larger numbers.

2.3 FOOD AND FEEDING

2.3.1 Cladocera are polyphagous feeders and find their food in the seston. Daphnids, including the *Ceriodaphnia*, are classified as fine mesh filter feeders by Geller and Mueller (1981). These fine mesh filter feeders are most abundant in eutrophic lakes during summer phytoplankton blooms when suspended bacteria are available as food only for filter-feeding species with fine mesh.

2.3.2 Lynch (1978) examined the gut contents of *Ceriodaphnia reticulata* (possibly *C. dubia*) from a Minnesota pond and found bacteria, detritus and partially digested algae. In this pond, *Ceriodaphnia* and *Daphnia pulex* shared the same resource base and had very similar diets, but the *Ceriodaphnia* fed more intensively on diatoms. The *Ceriodaphnia* were considered to be less sensitive to low food levels than *Daphnia*, because of their high rate of population growth during periods of low food levels in late summer.

2.4 LIFE CYCLE

2.4.1 Four distinct periods may be recognized in the life cycle of *Ceriodaphnia*: (1) egg, (2) juvenile, (3) adolescent, and (4) adult. The life span of *Ceriodaphnia*, from the release of the egg into the brood chamber until the death of the adult, is highly variable depending on the temperature and other environmental conditions. Generally the life span increases as temperature decreases, due to lowered metabolic activity. For example, the average life span of *Ceriodaphnia dubia* is about 30 days at 25°C, and 50 days at 20°C. One female was reported to have lived 125 days and produced 29 broods at 20°C (Cowgill et al., 1985).

2.4.2 Typically, a clutch of 4 to 10 eggs is released into the brood chamber, but clutches with as many as 20 eggs are common. The eggs hatch in the brood chamber and the juveniles, which are already similar in form to the adults, are released in approximately 38 h, when the female molts (casts off her exoskeleton or carapace). The total number of young produced per female varies with temperature and other environmental conditions. The most young are produced in the range of 18-25°C (124 young per female in a 28-day life span at 24°C) (113 young per female in a 77-day life span at 18°C) but production falls off sharply below 18°C (13 young per female in a 24-day life span at 12°C) (McNaught and Mount, 1985).

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2.4.3 The time required to reach maturity (produce their first offspring) in *C.dubia* varies from three to five days and appears to be dependent on body size and environmental conditions. A study of the growth and development of parthenogenetic eggs by Shuba and Costa (1972) revealed that at 24°C the embryos matured to free-swimming juveniles in approximately 38 h. The eggs that did not develop fully usually were aborted after 12 hours.

2.4.4 The growth rate of the organism is greatest during its juvenile stages (early instars), and the body size may double during each of these stages. Each instar stage is terminated by a molt. Growth occurs immediately after each molt while the new carapace is still elastic.

2.4.5 Following the juvenile stages, the adolescent period is very short, and consists of a single instar. It is during the adolescent instar that the first clutch of eggs reaches full development in the ovary. Generally, eggs are deposited in the brood chamber within minutes after molting, and the young which develop are released just before the next molt.

2.4.6 In general, the duration of instars increases with age, but also depends on environmental conditions. A given instar usually lasts approximately 24 h under favorable conditions. However, when conditions are unfavorable, it may last as long as a week. Four events take place in a matter of a few minutes at the end of each adult instar: (1) release of young from the brood chamber to the outside, (2) molting, (3) increase in size, and (4) release of a new clutch of eggs into the brood chamber. The number of young per brood is highly variable, depending primarily on food availability and environmental conditions. *C. dubia* may produce as many as 25 young in a single brood, but more commonly the number is six to ten. The number of young released during the adult instars reaches a maximum at about the fourth instar, after which there is a gradual decrease.

3. CULTURING METHODS

3.1 *Ceriodaphnia* are available from commercial biological supply houses. Guidance on the source of culture animals to be used by a permittee for self-monitoring effluent toxicity tests should be obtained from the permitting authority. Only a small number of organisms (20-30) are needed to start a culture. Before test organisms are taken from a culture, the culture should be maintained for at least two generations using the same food, water, and temperature as will be used in the toxicity tests.

3.2 Cultures of test organisms should be started at least three weeks before the brood animals are needed, to ensure an adequate supply of neonates for the test. Only a few individuals are needed to start a culture because of their prolific reproduction.

3.3 Starter animals may be obtained from an outside source by shipping in polyethylene bottles. Approximately 20-30 animals and 3 mL of food (see below) are placed in a l-L bottle filled full with culture water. Animals received from an outside source should be transferred to new culture media gradually over a period of 1-2 days to avoid mass mortality.

3.4 It is best to start the cultures with one animal, which is sacrificed after producing young, embedded, and retained as a permanent microscope slide mount to facilitate identification and permit future reference. The species identification of the stock culture should be verified by a taxonomic authority. The following procedure is recommended for making slide mounts of *Ceriodaphnia* (Beckett and Lewis, 1982):

1. Pipet the animal onto a watch glass.
2. Reduce the water volume by withdrawing excess water with the pipet.
3. Add a few drops of carbonated water (club soda or seltzer water) or 70% ethanol to relax the specimen so that the post-abdomen is extended. (Optional: with practice, extension of the postabdomen may be accomplished by putting pressure on the cover slip).
4. Place a small amount (one to three drops) of mounting medium on a glass microscope slide. The recommended mounting medium is CMCP-9/9AF Medium, prepared by mixing two parts of CMCP-9 with

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one part of CMCP-9AF. For more viscosity and faster drying, CMC-10 stained with acid fuchsin may be used.

5. Using a forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a cover slip and exert minimum pressure to remove any air bubbles trapped under the cover slip. Slightly more pressure will extend the postabdomen.
7. Allow mounting medium to dry.
8. Make slide permanent by placing CMC-10 around the edges of the coverslip.
9. Identify to species (see Pennak, 1989, and USEPA, 1986).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

3.5 CULTURE MEDIA

3.5.1 Although *Ceriodaphnia* stock cultures can be successfully maintained in some tap waters, well waters, and surface waters, use of synthetic water as the culture medium is recommended because (1) it is easily prepared, (2) it is of known quality, (3) it yields reproducible results, and (4) allows adequate growth and reproduction. Culturing may be successfully done in hard, moderately hard or soft reconstituted water, depending on the hardness of the water in which the test will be conducted. The quality of the dilution water is extremely important in *Ceriodaphnia* culture. The use of MILLIPORE MILLI-Q® or SUPER-Q®, or equivalent, to prepare reconstituted water is highly recommended. The use of diluted mineral water (DMW) for culturing and testing is widespread due to the ease of preparation.

3.5.2 The chemicals used and instructions for preparation of reconstituted water are given in Section 7, Dilution Water. The compounds are dissolved in distilled or deionized water and the media are vigorously aerated for several hours before using. The initial pH of the media is between 7.0 and 8.0, but it will rise as much as 0.5 unit after the test is underway.

3.6 MASS CULTURE

3.6.1 Mass cultures are used only as a "backup" reservoir of organisms. Neonates from mass cultures are not to be used directly in toxicity tests.

3.6.2 One-liter or 2L glass beakers, crystallization dishes, "battery jars," or aquaria may be used as culture vessels. Vessels are commonly filled to three-fourths capacity. Cultures are fed daily. Four or more cultures are maintained in separate vessels and with overlapping ages to serve as back-up in case one culture is lost due to accident or other unanticipated problems, such as low DO concentrations or poor quality of food or laboratory water.

3.6.3 Mass cultures which will serve as a source of brood organisms for individual culture should be maintained in good condition by frequent renewal of the medium and brood organisms. Cultures are started by adding 40-50 neonates per liter of medium. The stocked organisms should be transferred to new culture medium at least twice a week for two weeks. After two weeks, the culture is discarded and re-started with neonates in fresh medium. Using this schedule, 1-L cultures will produce 500 to 1000 neonate *Ceriodaphnia* each week.

3.6.6 Reserve cultures also may be maintained in large (80-L) aquaria or other large tanks.

3.7 INDIVIDUAL CULTURE

3.7.1 Individual cultures are used as the immediate source of neonates for toxicity tests.

3.7.2 Individual organisms are cultured in 15 mL of culture medium in 30-mL (1 oz) plastic cups or 30-mL glass beakers. One neonate is placed in each cup. It is convenient to place the cups in the same type of board used for toxicity tests.

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3.7.3 Organisms are fed daily and are transferred to fresh medium a minimum of three times a week, typically on Monday, Wednesday, and Friday. On the transfer days, food is added to the new medium immediately before or after the organisms are transferred.

3.7.4 To provide cultures of overlapping ages, new boards are started weekly, using neonates from adults which produce at least eight young in their third or fourth brood. These adults can be used as sources of neonates until 14 days of age. A minimum of two boards are maintained concurrently to provide backup supplies of organisms in case of problems.

3.7.5 Cultures which are properly maintained should produce at least 20 young per adult in three broods (seven days or less at 25°C). Typically, 60 adult females (one board) will produce more than the minimum number of neonates (120) required for two tests.

3.7.6 Records should be maintained on the survival of brood organisms and number of offspring at each renewal. Greater than 20% mortality of adults or less than an average of 20 young per adult on a board at 25°C during a one-week period would indicate problems, such as poor quality of culture media or food. Organisms on that board should not be used as a source of test organisms.

3.8 CULTURE MEDIUM

3.8.1 Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW is recommended as a standard culture medium (see Section 7, Dilution Water).

3.9 CULTURE CONDITIONS

3.9.1 *Ceriodaphnia* should be cultured at the temperature at which they will be used in the toxicity tests (20°C or 25°C $\pm$ 2°C).

3.9.2 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A 16-h/8-h day/night cycle is recommended.

3.9.3 Clear, double-strength safety glass or 6 mm plastic panels are placed on the culture vessels to exclude dust and dirt, and reduce evaporation.

3.9.4 The organisms are delicate and should be handled as carefully and as little as possible so that they are not unnecessarily stressed. They are transferred with a pipet of approximately 2-mm bore, taking care to release the animals under the surface of the water. Any organism that is injured during handling should be discarded.

3.10 FOOD PREPARATION AND FEEDING

3.10.1 Feeding the proper amount of the right food is extremely important in *Ceriodaphnia* culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may reduce the toxicity of the test solutions, clog the animal's filtering apparatus, or greatly decrease the DO concentration and increase mortality. A combination of Yeast, CEROPHYLL®, and Trout chow (YCT) or flake food, along with the unicellular green alga, *Selenastrum capricornutum*, will provide suitable nutrition if fed daily.

3.10.2 The YCT and algae are prepared as follows:

3.10.2.1 Digested trout chow (or flake food):

1. Preparation of trout chow requires one week. Use starter or No. 1 pellets prepared according to

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current U.S. Fish and Wildlife Service specifications, or flake food.

2. Add 5.0 g of trout chow pellets or flake food to 1 L of MILLI-Q® water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.

3. At the end of digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (i.e., NITEX® 110 mesh). Combine with equal volumes of supernatant from CEROPHYLL® and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the sediment.

3.10.2.2 Yeast:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S® to 1 L of MILLI-Q ® water.
2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.
3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and CEROPHYLL® preparations (below). Discard excess material.

3.10.2.3 CEROPHYLL® (Dried, Powdered, Cereal Leaves):

1. Place 5.0 g of dried, powdered, cereal leaves in a blender. Dried, powdered, alfalfa leaves obtained from health food stores have been found to be a satisfactory substitute for cereal leaves.
2. Add 1 L of MILLI-Q® water.
3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.
4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

3.10.2.4 Combined YCT Food:

1. Mix equal (approximately 300 mL) volumes of the three foods as described above.
2. Place aliquots of the mixture in small (50-mL to 100-mL) screw-cap plastic bottles and freeze until needed.
3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of two weeks.
4. It is advisable to measure the dry weight of solids (dry 24 h at 105°C) in each batch of YCT before use. The food should contain 1.7 - 1.9 g solids/L. Cultures or test solutions should contain 12-13 mg solids/L.

3.10.3 Algal (*Selenastrum*) Food

3.10.3.1 Algal Culture Medium

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.
2. Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L and mix well. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.

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TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 ML MILLI-Q® WATER
1. MACRONUTRIENTS		
A.	$MgCl_2 \cdot 6H_2O$	6.08 g
	$CaCl_2 \cdot 2H_2O$	2.20 g
	$NaNO_3$	12.75 g
B.	$MgSO_4 \cdot 7H_2O$	7.35 g
C.	K_2HPO_4	0.522 g
D.	$NaHCO_3$	7.50 g
2. MICRONUTRIENTS:		
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg[a]
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg[b]
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg[c]
	$CuCl_2 \cdot 2H_2O$	0.006 mg[d]
	$Na_2EDTA \cdot 2H_2O$	150.0 mg
	Na_2SeO_4	1.196 mg[e]

[a]$ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #2.

[b]$CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #2.

[c]$Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock #2.

[d]$CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock #2.

[e]Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #2.

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TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRONUTRIENT	CONCENTRATION (MG/L)	ELEMENT	CONCENTRATION (MG/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRONUTRIENT	CONCENTRATION (µG/L)	ELEMENT	CONCENTRATION (µG/L)
H_3BO_3	185	B	32.5
$MnCl_2 \cdot 4H_2O$	416	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300	--	----
Na_2SeO_4	2.39	Se	1.00

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3. Immediately filter the medium through a 0.45 μm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.
4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.
5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

3.10.3.2 Algal Cultures

3.10.3.2.1 Two types of algal cultures are maintained: (1) stock cultures, and, (2) "food" cultures.

3.10.3.2.2 Establishing and Maintaining Stock Cultures of Algae

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.
2. The stock cultures are used as a source of algae to initiate "food" cultures for *Ceriodaphnia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Ceriodaphnia* cultures and tests. Stock culture volume may be rapidly "scaled up" to several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.
3. Culture temperature is not critical. Stock cultures may be maintained in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
4. Cultures are mixed twice daily by hand or stirred continuously.
5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5×10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.
6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4°C. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms every four to six months.

3.10.3.2.3 Establishing and Maintaining "Food" Cultures of Algae

1. "Food" cultures are started seven days prior to use for *Ceriodaphnia* cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5×10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and the excess stored in the refrigerator.
2. Food cultures may be maintained at 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent

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lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).

3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3°C.

3.10.3.3 Preparing Algal Concentrate for Use as *Ceriodaphnia* Food

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for approximately two-to-three weeks and siphoning off the supernatant.
2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer, and used to determine the concentration required to achieve a final cell count of 3.0 to 3.5 X 10^7 cells/mL.
3. Assuming a cell density of approximately 1.5 X 10^6 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^9 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate (1500 feedings at 0.1 mL/feeding) for use as food. This would be enough algal food for four *Ceriodaphnia* tests.
4. Algal concentrate may be stored in the refrigerator for one month.

3.11 FEEDING

3.11.1 Cultures should be fed daily to maintain the organisms in optimum condition so as to provide maximum reproduction. Stock cultures which are stressed because they are not adequately fed may produce low numbers of young, large number of males, and ephippial females. Also, their offspring may produce few young when used in toxicity tests.

1. If YCT is frozen, remove a bottle of food from the freezer 1 h before feeding time, and allow to thaw.
2. Mass cultures are fed daily at the rate of 7 mL YCT and 7 mL algae concentrate/L culture.
3. Individual cultures are fed at the rate of 0.1 mL YCT and 0.1 mL algae concentrate per 15 mL culture.
4. YCT and algal concentrate should be thoroughly mixed by shaking before dispensing.
5. Return unused YCT food mixture and algae concentrate to the refrigerator. Do not re-freeze YCT. Discard unused portion after one week.

3.12 FOOD QUALITY

3.12.1 The quality of food prepared with newly acquired supplies of yeast, trout chow, dried cereal leaves, or algae, should be determined in side-by-side comparisons of *Ceriodaphnia* survival and reproduction, using the new food and food of known, acceptable quality, over a seven-day period in control medium.

4. TEST ORGANISMS

4.1 Neonates, or first instar *Ceriodaphnia* less than 24 hours old, taken from the 3rd or 4th brood, are used in toxicity tests. To obtain the necessary number of young for an acute toxicity test, it is recommended that the animals be cultured in individual 30 mL beakers or plastic cups for seven days prior to the beginning of the test. Neonates are used from broods of at least eight young. Fifty adults in individual cultures will usually supply enough neonates for one toxicity test.

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4.2 Use a disposable, widemouth pipette to transfer *Ceriodaphnia*. The diameter of the opening should be approximately 4 mm. The tip of the pipette should be kept under the surface of the water when the *Ceriodaphnia* are released to prevent air from being trapped under the carapace. Liquid containing adult *Ceriodaphnia* can be poured from one container to another without risk of injuring the animals.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.2. *DAPHNIA (D. MAGNA* AND *D. PULEX*)

1. **SYSTEMATICS**

1.1 MORPHOLOGY AND TAXONOMY

1.1.1 The generalized anatomy of a parthenogenetic female is shown in Figure 1. *Daphnia pulex* is an extremely variable species consisting of several reproductively isolated clonal groups and is often not distinguishable from other species (such as *D. obtusa*) that have large teeth on the middle pecten of the postabdomenal claw (Figure 2C) (Lynch, 1985; Dodson, 1981). Probably the most distinctive feature of the parthenogenetic female *D. pulex* is the long second abdominal process of the abreptor (postabdomen) that extends beyond the base of the anal setae (Figure 2A).

1.1.2 *D. pulex* is a wide ranging species that shows little variation throughout its range. Two of its most distinctive characteristics are the deeply sinuate posterior margin of the abreptor (Figures 3A and 3D) and the ridges on the head which run parallel to the mid-dorsal line (Figure 3B).

1.1.3 *D. pulex* is much smaller than *D. magna*, attaining a length of up to 3.5 mm compared to 5.0 or 6.0 mm for *D. magna*. Although the two species can often be separated by size, they can be differentiated with certainty only by examining the postabdominal claws for size and number of spines using a compound microscope. *D. pulex* has 5-7 stout teeth on the middle pecten (Figure 2C) while *D. magna* has a uniform row of 20 or more small teeth (Figure 3E). Another characteristic for separating the neonates of the two species is the location of the nuchal organ which is higher up on the posterior margin of the head in *D. magna* than in *D. pulex* (Schwartz and Hebert, 1984). For a more complete taxonomic discussion of the two species see Brooks (1957).

2. **DISTRIBUTION**

2.1 *D. magna* has a worldwide distribution in the northern hemisphere. In North America it appears to be absent from the eastern United States (except for Northern New England) and Alaska (Figure 4). *D. pulex* occurs over most of North America except the tropics and high arctic (Figure 5), and probably occurs in Europe and South America as well. Both species often occur in the same pools but *D. pulex* usually out-competes *D. magna* in mixed populations and takes over as the sole inhabitant by summer's end (Modlin, 1982; Lynch, 1983).

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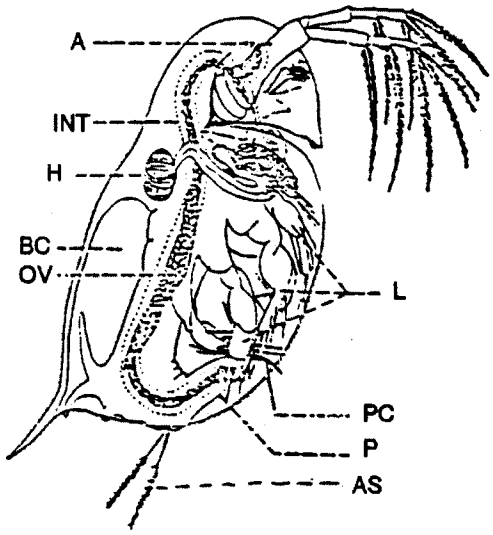

Figure 1. Generalized anatomy of a female *Daphnia*, X70; A, antenna; AS, anal setae; BC, brood chamber; H, heart; INT, intestine; L, legs; OV, ovary; P, postabdomen; PC, posbdominal claw. (From Pennak, 1989).

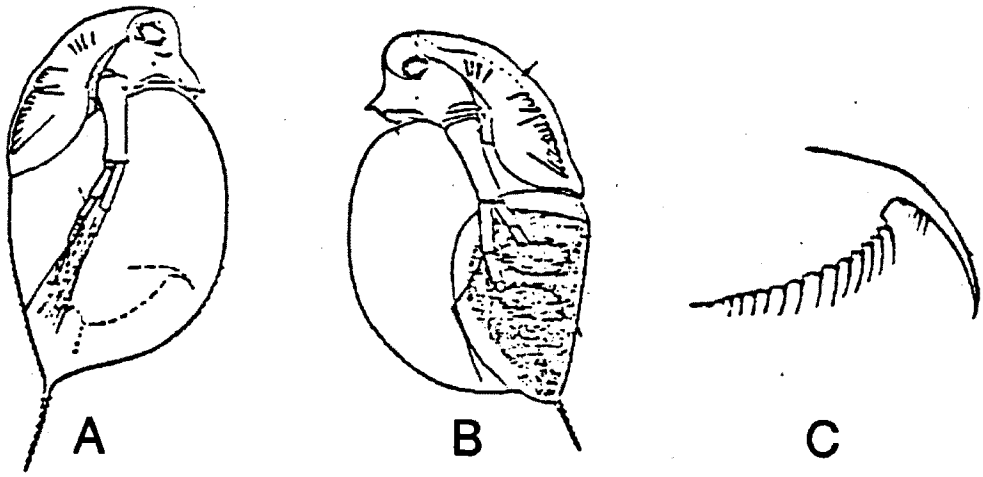

Figure 2. Female *Daphnia pulex*. A, lateral aspect (note smoothly rounded posterior margin of postabdomen); B, ephippial female; C, postabdomen showing large spines on the claw. (From Brooks, 1957)

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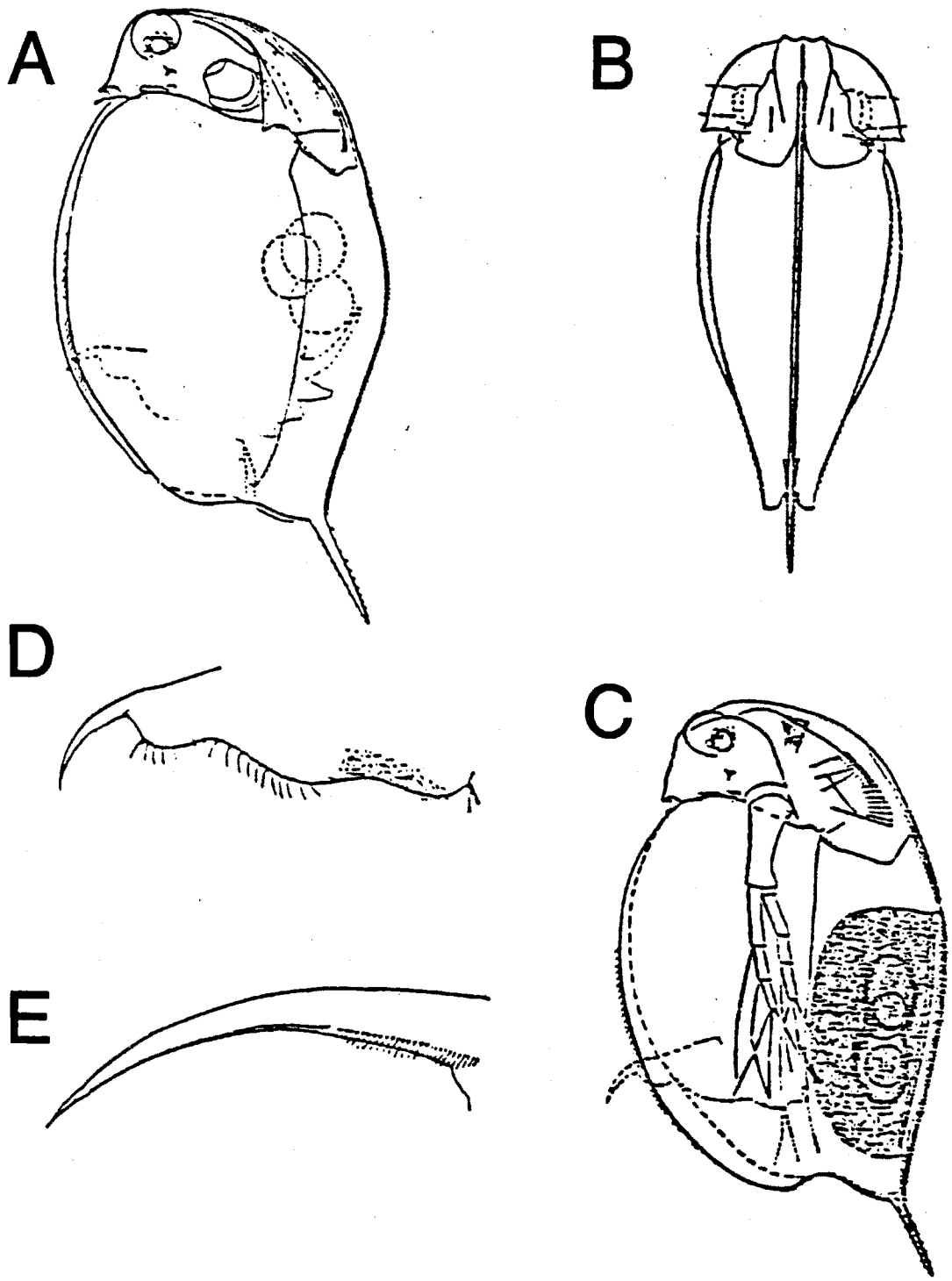

Figure 3. Female *D. Magna.* A. lateral aspect; B. dorsal aspect; C. ephippial female; D. postabdomen showing sinuate posterior margin; E. postabdominal claw. (From Brooks, 1957)

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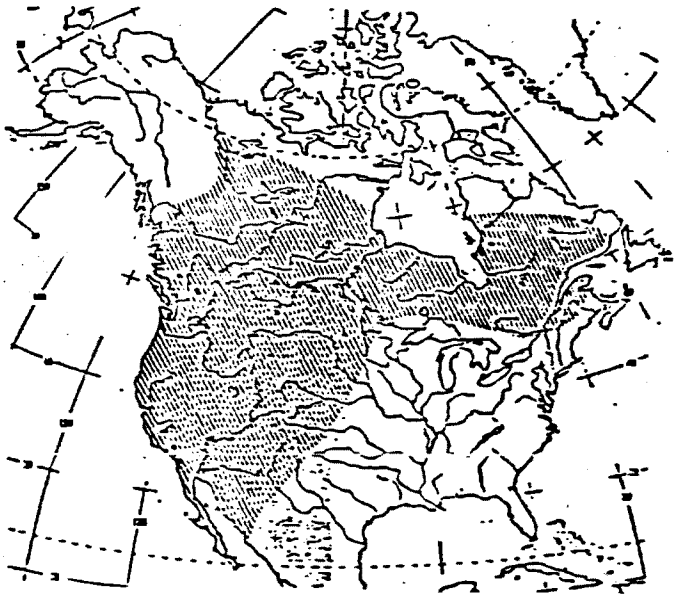

Figure 4. Map showing the North American distribution of *D. magna.*

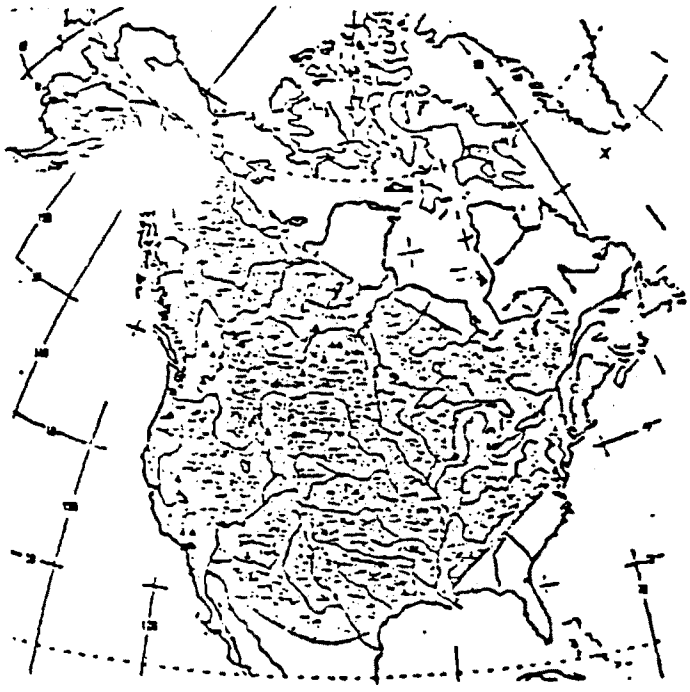

Figure 5. Map showing the North American distribution of *D. pulex.*

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3. ECOLOGY AND LIFE HISTORY

3.1 GENERAL ECOLOGY

3.1.1 *D. magna* is principally a lake dweller and is restricted to waters in northern and western North America exceeding a hardness of 150 mg/L (as $CaCO_3$) (Pennak, 1989). In the Netherlands, *D. magna* are found in shallow ponds with muddy bottoms rich in organic matter and with low oxygen demand (3 to 4 mg/L). *D. pulex* is principally a pond dweller where the oxygen content is higher, but is also found in lakes. It is generally considered a clean water species being dominant in nature during periods of low turbidity. However, Scholtz, et al. (1988) found that high turbidity had little effect on survival and reproduction in laboratory studies.

3.1.2 *Daphnia* populations are generally sparse in winter and early spring, but as water temperatures reach 6°C to 12°C, they increase in abundance and subsequently may reach population densities as high as 200 to 500 individuals/L (Pennak, 1989). Populations in ponds decline to very low numbers during the summer months. In autumn there may be a second population pulse, followed by a decline to winter lows.

3.1.3 During most of the year, populations of *Daphnia* consist almost entirely of females, the males being abundant only in spring or autumn when up to 56% of the offspring of *D. magna* may be males (Barker and Hebert, 1986). Males are distinguished from females by their smaller size, larger antennules, modified postabdomen, and first legs, which are armed with a stout hook used in clasping. Production of males appears to be induced principally by low temperatures or high densities and subsequent accumulation of excretory products, and/or a decrease in available food. These conditions may induce the appearance of sexual (resting) eggs (embryos) in cases called ephippia (Figures 2B and 3C), which are cast off during the next molt. It appears that the shift toward male and sexual egg production is related to the metabolic rate of the parent. Any factor which tends to lower metabolism may be responsible. Ruvinsky et al. (1978) suggested that the genome of the animal has two developmental programs based on identical sets of chromosomes. The female program consistently functions under a wide range of conditions, whereas the male program is turned on by specific ecological stimuli. The eggs from which the males and females develop have identical chromosome sets. Sex determination is based on changes in chromatin structure when the mother receives a specific signal that sexual reproduction is needed for adaptation to extreme conditions.

3.1.4 *D. magna* reproduce only by cyclic parthenogenesis in which males contribute to the genetic makeup of the young during the sexual stage of reproduction, whereas *D. pulex* may reproduce either by cyclic or obligate parthenogenesis in which the zygotes develop within the ephippium by ameoitic parthenogenesis with no genetic contribution from the males. Thus, the ephippial and live-born offspring are genetically identical to their mothers. Both forms may be present in the same population resulting in cyclic populations exhibiting considerable genetic variation early in the year and an obligate population with a low range of genotypic values. After 25 generations of asexual reproduction the variation in the cyclic parthenogenesis group becomes about the same as that in the obligate group (Lynch, 1984). These populations exhibiting a low range of genotypic values are much more vulnerable to perturbations such as nutrient introduction or toxic discharges. The clonal makeup of a *Daphnia* population is effected by food, oxygen, temperature and predation (Weider, 1985; Brookfield, 1984).

3.1.5 Ephippia are small and lightweight and can be dried and stored for long periods making them easy to transport. They may be shipped in envelopes like seeds. Upon arrival at the new location the ephippia can be hatched in a few days when placed in water at the proper temperature (Schwartz and Hebert, 1987).

3.1.6 *Daphnia* are preyed upon by many predators and have developed behavioral and morphological antipredator defenses to make themselves more difficult to catch and consume. Dodson (1988) showed that *D. pulex* responded to a possible chemical stimuli released by the predator which resulted in the daphnids retreating from the vicinity of the predators. Certain clones of *D. pulex* may develop morphological changes when predators are present but not when they are absent from the pond. Some of these changes are of such magnitude that they have been described as separate species. *D. minnehaha* is a morphological variation of *D. pulex* which develops spines in response to the

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stimuli of predators (Krueger and Dodson, 1981). Different genotypes of *D. pulex* react in different ways to the predator (*Chaoborus*) factor and to temperature (Havel, 1985).

3.2 FOOD AND FEEDING

3.2.1. Both *D. pulex* and *D. magna* are well adapted to live in algal blooms, which are high in proteins and carbohydrates, where they feed on algae and bacteria. *D. magna* prefers bacteria to algae as food (Ganf, 1983; Hadas et al., 1983) while *D. pulex* uses bacteria as food only when algal biomass declines (Borsheim and Olsen, 1984). Food type and abundance affect the sensitivity of *Daphnia* to pollutants and their reproduction rate. Keating and Dagbusan (1986) showed that both *D. pulex* and *D. magna* fed diatoms were more tolerant of pollutants than those fed only green algae. Lipid reserves are a good indication of the nutritional condition of the animals (Holm and Shapiro, 1984; Tessier and Goulden, 1982).

3.3 LIFE HISTORY

3.3.1 Four distinct periods may be recognized in the life history of *Daphnia*: (1) egg, (2) juvenile, (3) adolescence, and (4) adult (Pennak, 1989). The life span of *Daphnia*, from the release of the egg into the brood chamber until the death of the adult, is highly variable depending on the species and environmental conditions (Pennak, 1989). Generally the life span increases as temperature decreases, due to lowered metabolic activity. The average life span of *D. magna* is about 40 days at 25°C, and about 56 days at 20°C. The average life span of *D. pulex* at 20°C is approximately 50 days. Typically, a clutch of 6 to 10 eggs is released into the brood chamber, but as many as 57 have been reported. The eggs hatch in the brood chamber and the juveniles, which are already similar in form to the adults, are released in approximately two days when the female molts (casts off her exoskeleton or carapace). The time required to reach maturity (produce their first offspring) in *D. pulex* varies from six to 10 days (mean = 7.78 days) and also appears to be dependent on body size. The growth rate of the organism is greatest during its juvenile stages (early instars), and the body size may double during each of these stages. *D. pulex* has three to four juvenile instars, whereas *D. magna* has three to five instars. Each instar stage is terminated by a molt. Growth occurs immediately after each molt while the new carapace is still elastic.

3.3.2 Following the juvenile stages, the adolescent period is very short, and consists of a single instar. It is during the adolescent instar that the first clutch of eggs reaches full development in the ovary. Generally, eggs are deposited in the brood chamber within minutes after molting, and the young which develop are released just before the next molt.

3.3.3 *D. magna* usually has 6-22 adult instars, and *D. pulex* has 18-25. In general, the duration of instars increases with age, but also depends on environmental conditions. A given instar generally lasts approximately two days under favorable conditions, but when conditions are unfavorable, may last as long as a week.

3.3.4 Four events take place in a matter of a few minutes at the end of each adult instar: (1) release of young from the brood chamber to the outside, (2) molting, (3) increase in size, and (4) release of a new clutch of eggs into the brood chamber. The number of young per brood is highly variable for *Daphnia*, depending primarily on food availability and environmental conditions. *D. magna* and *D. pulex* may both produce as many as 30 young during each adult instar, but more commonly the number is six to 10. The number of young released during the adult instars of *D. pulex* reaches a maximum at the tenth instar, after which there is a gradual decrease (Anderson and Zupancic, 1937). Scholtz et al. (1988) reported that nearly all of the eggs that are oviposited by *D. pulex* became neonates, indicating a highly successful hatching rate. The maximum number of young produced by *D. magna* occurs at the fifth adult instar, after which it decreases (Anderson and Jenkins, 1942).

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4. **CULTURING METHODS**

4.1 SOURCES OF ORGANISMS

4.1.1 *Daphnia* are available from commercial biological supply houses. Only a small number of organisms (20-30) are needed to start a culture. *D. pulex* is preferred over *D. magna* by some biologists because it is more widely distributed, is tolerant of a wider range of environmental conditions, and is easier to culture. However, the neonates are smaller, swim faster and are more difficult to count, and produce more "floaters" than *D. magna* and, therefore, are somewhat more difficult to use in toxicity tests. Guidance on the source and species of *Daphnia* to be used by a permittee for effluent toxicity tests should be obtained from the permitting authority.

4.1.2 Cultures of test organisms should be started at least three weeks before the brood animals are needed, to ensure an adequate supply of neonates for the test.

4.1.3 Starter animals may be obtained from an outside source by shipping in polyethylene bottles. Approximately 20-30 animals and 3 mL of food (see below) are placed in a 1-L bottle filled full with culture water. Animals received from an outside source should be transferred to new culture media gradually over a period of 1-2 days to avoid mass mortality.

4.1.4 It is best to start the cultures with one animal, which is sacrificed after producing young, embedded, and retained as a permanent microscope slide mount to facilitate identification and permit future reference. The species identification of the stock culture should be verified by a taxonomic authority. The following procedure is recommended for making slide mounts of *Daphnia* (Beckett and Lewis, 1982):

1. Pipet the animal onto a watch glass.
2. Reduce the water volume by withdrawing excess water with the pipet.
3. Add a few drops of carbonated water (club soda or seltzer water) or 70% ethanol to relax the specimen so that the post-abdomen is extended. (Optional: with practice, extension of the postabdomen may be accomplished by putting pressure on the cover slip).
4. Place a small amount (one to three drops) of mounting medium on a glass microscope slide. The recommended mounting medium is CMCP-9/9AF Medium, prepared by mixing two parts of CMCP-9 with one part of CMCP-9AF. For more viscosity and faster drying, CMC-l0 stained with acid fuchsin may be used.
5. Using a forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a cover slip and exert minimum pressure to remove any air bubbles trapped under the cover slip. Slightly more pressure will extend the postabdomen.
7. Allow mounting medium to dry.
8. Make slide permanent by placing CMC-10 around the edges of the coverslip.
9. Identify to species (see Pennak, 1989).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

4.2 CULTURE MEDIA

4.2.1 Although *Daphnia* stock cultures can be successfully maintained in some tap waters, well waters, and surface waters, use of synthetic water as the culture medium is recommended because (1) it is easily prepared, (2) it is of known quality, (3) it yields reproducible results, and (4) allows adequate growth and reproduction. Reconstituted hard water (total hardness of 160 -180 mg/L as $CaCO_3$) is recommended for *D. magna* culturing, and reconstituted moderately hard water (total hardness of 80-90 mg/L $CaCO_3$) is recommended for *D. pulex* culturing. The quality of the dilution water is important in *Daphnia* culture. The use of MILLIPORE MILLI-Q® or SUPER-Q®, or equivalent, to prepare reconstituted water is highly recommended. The use of diluted mineral water (DMW) for culturing and testing is widespread due to the ease of preparation.

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4.2.2 The chemicals used and instructions for preparation of reconstituted water are given in Section 7, Dilution Water. The compounds are dissolved in distilled or deionized water and the media are vigorously aerated for several hours before using. The initial pH of the media is between 7.0 and 8.0, but it will rise as much as 0.5 unit after the test is underway.

4.3 CULTURE CONDITIONS

4.3.1 *Daphnia* can be cultured successfully over a wide range of temperatures, but should be protected from sudden changes in temperature, which may cause death. The optimum temperature is approximately 20°C, and if ambient laboratory temperatures remain in the range of 18-26°C, normal growth and reproduction of *Daphnia* can be maintained without special temperature control equipment. *D. magna* can survive when the DO concentration is as low as 3 mg/L but *D. pulex* does best when the DO concentration is above 5 mg/L. Therefore it is recommended that the DO concentration in the culture be maintained at 5 mg/L or above. Unless the cultures are too crowded or overfed, aeration is usually not necessary.

4.3.2 Illumination

4.3.2.1 The variations in ambient light intensities (10-20 $\mu E/m^2/s$, or 50-100 ft-c) and prevailing day/night cycles in most laboratories do not seem to affect *Daphnia* growth and reproduction significantly. However, a minimum of 16 h of illumination should be provided each day.

4.3.3 Culture Vessels

4.3.3.1 Culture vessels of clear glass are recommended since they allow easy observation of the *Daphnia*. A practical culture vessel is an ordinary 4-L glass beaker, which can be filled with approximately 3 L medium (reconstituted water). Maintain several (at least five) culture vessels, rather than only one. This will ensure back-up cultures so that in the event of a population "crash" in one or several chambers, the entire *Daphnia* population will not be lost. If a vessel is stocked with 30 adult *Daphnia*, it will provide approximately 300 young each week.

4.3.3.2 Initially, all culture vessels should be washed well (see Section 5, Facilities and Equipment). After the culture is established, clean each chamber weekly with distilled or deionized water and wipe with a clean sponge to rid the vessel of accumulated food and dead *Daphnia* (see section on culture maintenance below). Once per month, wash each vessel with detergent during medium replacement. Rinse three times with tap water and then with culture medium to remove all traces of detergent.

4.3.4 Weekly Culture Media Replacement

4.3.4.1 Careful culture maintenance is essential. The medium in each stock culture vessel should be replaced three times each week with fresh medium.

This is best accomplished by changing solutions Monday, Wednesday, and Friday, as follows:

1. Place about 300 mL of the old media in a temporary holding vessel.
2. Transfer about 25 or 30 adults from the old culture vessel to the holding vessel using a wide bore pipette.
3. Discard the remaining *Daphnia* along with the media.
4. Clean the culture vessel as described above.
5. Fill the newly-cleaned vessel with fresh medium.
6. Gently transfer (by pouring) the contents of the temporary holding vessel (old medium with the *Daphnia*) into the vessel containing the new medium making sure that none of the animals stick to the sides of the vessel.

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7. Feed the animals

4.3.4.2 If the medium is not replaced three times weekly, waste products will accumulate, which could cause a population crash or the production of males and/or sexual eggs.

4.3.4.3 *Daphnia* cultures should be thinned whenever the population exceeds 200 individuals per stock vessel to prevent over-crowding, which may cause a population crash, or the production of males and/or ephippia. A good time to thin the populations is on Monday, Wednesday, and Friday, before feeding. To transfer *Daphnia*, use a 15-cm disposable, jumbo bulb pipette, or 10-mL "serum" pipette which has had the delivery tip cut off and fire polished. The diameter of the opening should be approximately 5 mm. A serum pipette, a pipette bulb, such as a PROPIPETTE®, or (MOPET®) portable, motorized pipettor, will provide the controlled suction needed when selectively collecting *Daphnia*.

4.3.4.4 Liquid containing adult *D. pulex* and *D. magna* can be poured from one container to another without risk of air becoming trapped under their carapaces. However, the very young *Daphnia* are much more susceptible to air entrapment and for this reason should be transferred from one container to another using a pipette. The tip of the pipette should be kept under the surface of the liquid when the *Daphnia* are released.

4.3.4.5 Each culture vessel should be covered with a clear plastic sheet or glass plate to exclude dust and dirt, and minimize evaporation.

4.4 FOOD PREPARATION AND FEEDING

4.4.1 Feeding the proper amount of the right food is extremely important in *Daphnia* culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may reduce the toxicity of the test solutions, clog the animal's filtering apparatus, or greatly decrease the DO concentration and increase mortality. YCT, a combination of Yeast, CEROPHYLL®, and Trout chow (or flake food), along with the unicellular green alga, *Selenastrum capricornutum*, will provide suitable nutrition if fed daily.

4.4.2 The YCT and algae are prepared as follows:

4.4.2.1 Digested trout chow (or flake food):

1. The preparation requires one week. Use starter or No. 1 pellets prepared according to current U.S. Fish and Wildlife Service specifications, or flake food.
2. Add 5.0 g of trout chow pellets or flake food to 1 L of MILLI-Q® water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.
3. At the end of digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (i.e., NITEX® 110 mesh). Combine with equal volumes of supernatant from CEROPHYLL® and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the sediment.

4.4.2.2 Yeast:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S® to 1 L of MILLI-Q® water.
2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.
3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and CEROPHYLL® preparations (below). Discard excess

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material.

4.4.2.3 CEROPHYLL® (Dried, Powdered, Cereal Leaves):

1. Place 5.0 g of dried, powdered, cereal leaves in a blender. Dried, powdered, alfalfa leaves obtained from health food stores have been found to be a satisfactory substitute for cereal leaves.
2. Add 1 L of MILLI-Q® water.
3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.
4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

4.4.2.4 Combined YCT Food:

1. Mix equal (approximately 300 mL) volumes of the three foods as described above.
2. Place aliquots of the mixture in small (50-mL to 100-mL) screw-cap plastic bottles and freeze until needed.
3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of one week.
4. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1.7 - 1.9 g solids/L. Cultures or test solutions should contain 12-13 mg solids/L.

4.4.3 Algal (*Selenastrum*) Food

4.4.3.1 Algal Culture Medium

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.
2. Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L and mix well. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.
3. Immediately filter the medium through a 0.45μm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.
4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.
5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

4.4.3.2 Algal Cultures

4.4.3.2.1 Two types of algal cultures are maintained: (1) stock cultures, and, (2) "food" cultures.

4.4.3.2.2 Establishing and Maintaining Stock Cultures of Algae

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.
2. The stock cultures are used as a source of algae to initiate "food" cultures for *Daphnia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Daphnia* cultures and tests. Stock culture volume may be rapidly "scaled up" to

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several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.

3. Culture temperature is not critical. Stock cultures may be maintained in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
4. Cultures are mixed twice daily by hand or stirred continuously.
5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5×10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 1,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.
6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4°C. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms every four to six months.

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TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 ML MILLI-Q® WATER
1. MACRONUTRIENTS		
A.	$MgCl_2 \cdot 6H_2O$	6.08 g
	$CaCl_2 \cdot 2H_2O$	2.20 g
	$NaNO_3$	12.75 g
B.	$MgSO_4 \cdot 7H_2O$	7.35 g
C.	K_2HPO_4	0.522 g
D.	$NaHCO_3$	7.50 g
2. MICRONUTRIENTS:		
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg[a]
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg[b]
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg[c]
	$CuCl_2 \cdot 2H_2O$	0.006 mg[d]
	$Na_2EDTA \cdot 2H_2O$	150.0 mg
	Na_2SeO_4	1.196 mg[e]

[a]$ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #2.

[b]$CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #2.

[c]$Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock #2.

[d]$CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock #2.

[e]Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #2.

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TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRO NUTRIENT	CONCENTRATION (MG/L)	ELEMENT	CONCENTRATION (MG/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRO NUTRIENT	CONCENTRATION (μG/L)	ELEMENT	CONCENTRATION (μG/L)
H_3BO_3	185	B	32.5
$MnCl_2 \cdot 4H_2O$	416	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300	--	----
Na_2SeO_4	2.39	Se	1.00

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4.4.3.2.3 Establishing and Maintaining "Food" Cultures of Algae

1. "Food" cultures are started seven days prior to use for *Daphnia* cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5 X 10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and the excess stored in the refrigerator.
2. Food cultures may be maintained at 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3°C.

4.4.3.3 Preparing Algal Concentrate for Use as *Daphnia* Food

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for approximately two-to-three weeks and siphoning off the supernatant.
2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer, and used to determine the concentration required to achieve a final cell count of 3.0 to 3.5 X 10^7/mL.
3. Assuming a cell density of approximately 1.5 X 10^6 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^9 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate.
4. Algal concentrate may be stored in the refrigerator for one month.

4.5 FEEDING

4.5.1 Feeding rate and frequency are important in maintaining the organisms in optimal condition so that they achieve maximum reproduction. Stock cultures which are stressed because they are not adequately fed may produce low numbers of young, large numbers of males, and ephippial females. When the young taken from these inadequately fed *Daphnia* cultures are used in toxicity tests, they may show higher than acceptable mortality in controls and greater than normal sensitivity to toxicants. Steps to follow when feeding the YCT and algal diet are as follows:

1. If YCT is frozen, remove a bottle of the food from the freezer at least 1 h before feeding time, and allow to thaw.
2. Mass cultures are fed Monday, Wednesday, and Friday at the rate of 4.5 mL YCT and 2 mL of algae concentrate per 3-L culture.
3. On Tuesday and Thursday the culture water is stirred to re-suspend the settled algae and another 2 mL of algal concentrate is added.
4. The YCT and algal concentrate is thoroughly mixed by shaking before dispensing.
5. Return unused YCT food mixture and algal concentrate to the refrigerator. Do not re-freeze the YCT. Discard unused portion of YCT after one week.

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4.5.2 The quality of food prepared with newly acquired supplies of yeast, trout chow, and dried cereal leaves, or algae, should be determined in side-by-side comparisons of *Daphnia* survival and reproduction tests, using the new food and food of known, acceptable quality, over a seven-day period in control medium.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.3. MYSIDS (*MYSIDOPSIS BAHIA* AND *HOLMESIMYSIS COSTATA*)

1. DISTRIBUTION

1.1 Mysids (Figure 1) are small shrimp-like crustaceans found in both the marine and freshwater environments. The mysid(s) that currently are of primary interest in the NPDES program are the estuarine species, *Mysidopsis bahia* (now identified as *Americamysis bahia*; Price *et al.*, 1994) and the marine species, *Holmesimysis costata*. It occurs primarily at salinities above 15‰; Stuck et al. (1979a) and Price (1982) found greatest abundances at salinities near 30‰. Three sympatric species of *Mysidopsis, M. almyra, M. bahia*, and *M. bigelowi*, have been cultured and used in toxicity testing. The distribution of *Mysidopsis* species has been reported by Stuck et al. (1979b), Price (1982), and Heard et al. (1987).

1.2 *M. bahia* occurs primarily at salinities above 15‰; Stuck *et al.* (1979a) and Price (1982) found greatest abundances at salinities near 30‰. Three sympatric species of *Mysidopsis, M. almyra, M. bahia*, and *M. bigelowi*, have been cultured and used in toxicity testing. The distribution of *Mysidopsis* species has been reported by Stuck *et al.* (1979b), Price (1982), and Heard *et al.* (1987).

1.3 *H. costata* (Holmes 1900; previously referred to as *Acanthomysis sculpta*) is a west coast species that lives in the surface canopy of the giant kelp *Macrocystis pyrifera* where it feeds on zooplankters, kelp, epiphytes, and detritus. There are few references to the ecology of this mysid species (Holmquist, 1979; Clutter, 1967, 1969; Green, 1970; Turpen *et al.*, 1994). *H. costata* is numerically abundant in kelp forest habitats and is considered to be an important food source for kelp forest fish (Clark 1971, Mauchline 1980). *H. costata* eggs develop for about 20 days in their marsupium (abdominal pouch) before the young are released as juveniles; broods are released at night during molting. Females release their first brood at 55 to 70 days post-release (at 12°C), and may have multiple broods throughout their approximately 120-day life.

1.4 Other marine mysids that have been used in toxicity testing and held or cultured in the lab include *Metamysidopsis elongata, Neomysis americana, Neomysis awatschensis, Neomysis intermedia*, and recently for the Pacific coast, *Holmesimysis sculpta* and *Neomysis mercedis*. A freshwater species, *Mysis relicta*, presently not used in toxicity testing, but found in the same habitat as *Daphnia pulex*, might be considered in the future for toxicity testing.

2. *MYSIDOPSIS BAHIA*

2.1 LIFE CYCLE

2.1.1 In laboratory culture, *Mysidopsis bahia* reach sexual maturity in 12 to 20 days, depending on water temperature and diet (Nimmo et al., 1977). Normally, the female will have eggs in the ovary at approximately 12 days of age. The lamellae of the marsupium pouch have formed or are in the process of forming when the female is approximately 4 mm in length (Ward, 1993). Unlike *Daphnia*, the eggs will not develop unless fertilized. Mating takes place at night and lasts only a few minutes (Mauchline, 1980).

2.1.2 Brood pouches are normally fully formed at approximately 15 days (approximately 5 mm in body length), and young are released in 17 to 20 days (Ward, 1993). The number of eggs deposited in the brood and the number of young produced per brood are a direct function of body length as well as environmental conditions. Mature females have produced as many as 25 Stage I larvae (egg-shaped embryo) per brood (8-9 mm in body length) in natural and artificial seawater (FORTY FATHOMS®) but average 11 ± 6 Stage III larvae (final stage before larvae

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are released), with increasing numbers correlated with increasing body length (Ward, 1993). A new brood is produced every 4 to 7 days.

2.1.3 At time of emergence, juveniles are immobile, making them susceptible to predation by adult mysids. The juveniles are planktonic for the first 24-48 hours and then settle to the bottom, orient to the current, and actively pursue food organisms such as *Artemia*. Carr et al. (1980) reported that the stage in the life cycle of *M. almyra* most sensitive to drilling mud was the juvenile molt, which occurs between 24 and 48 hours after release from the brood pouch. Ward (1989) found a relationship between $CaCO_3$ level and growth and reproduction and that *M. bahia* were more sensitive to cadmium during molting (24-72 h post release) in high or low levels of $CaCO_3$. Work done by Lee and Buikema (1979) for *Daphnia pulex* also showed increased sensitivity during molting.

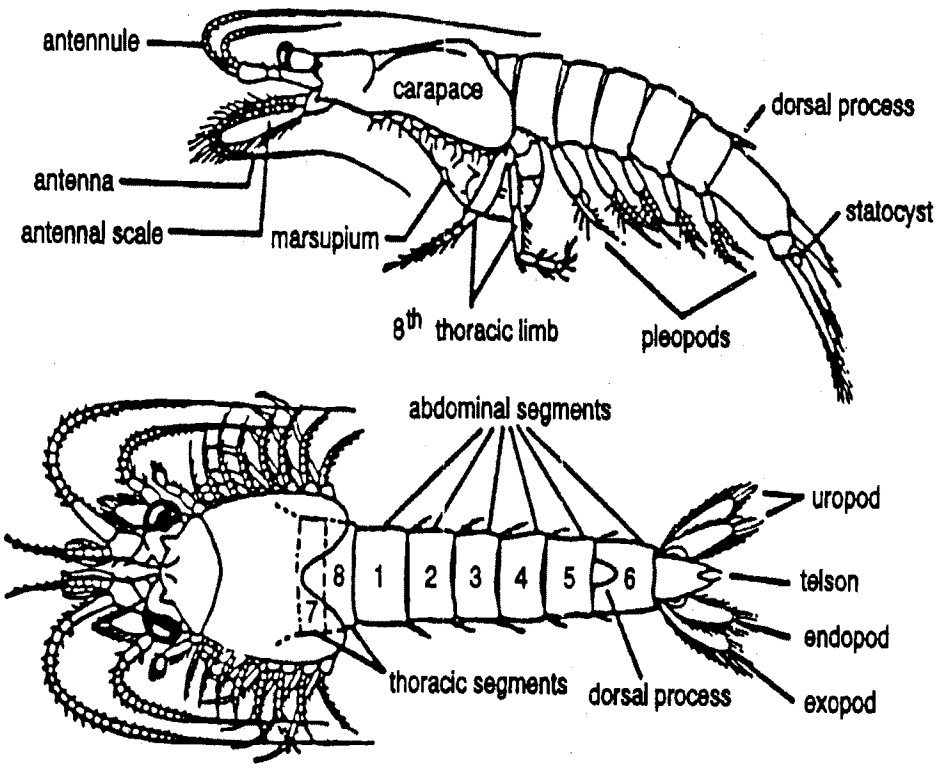

Figure 1. Lateral and dorsal view of a mysid with morphological features identified (From Stuck et al., 1979a).

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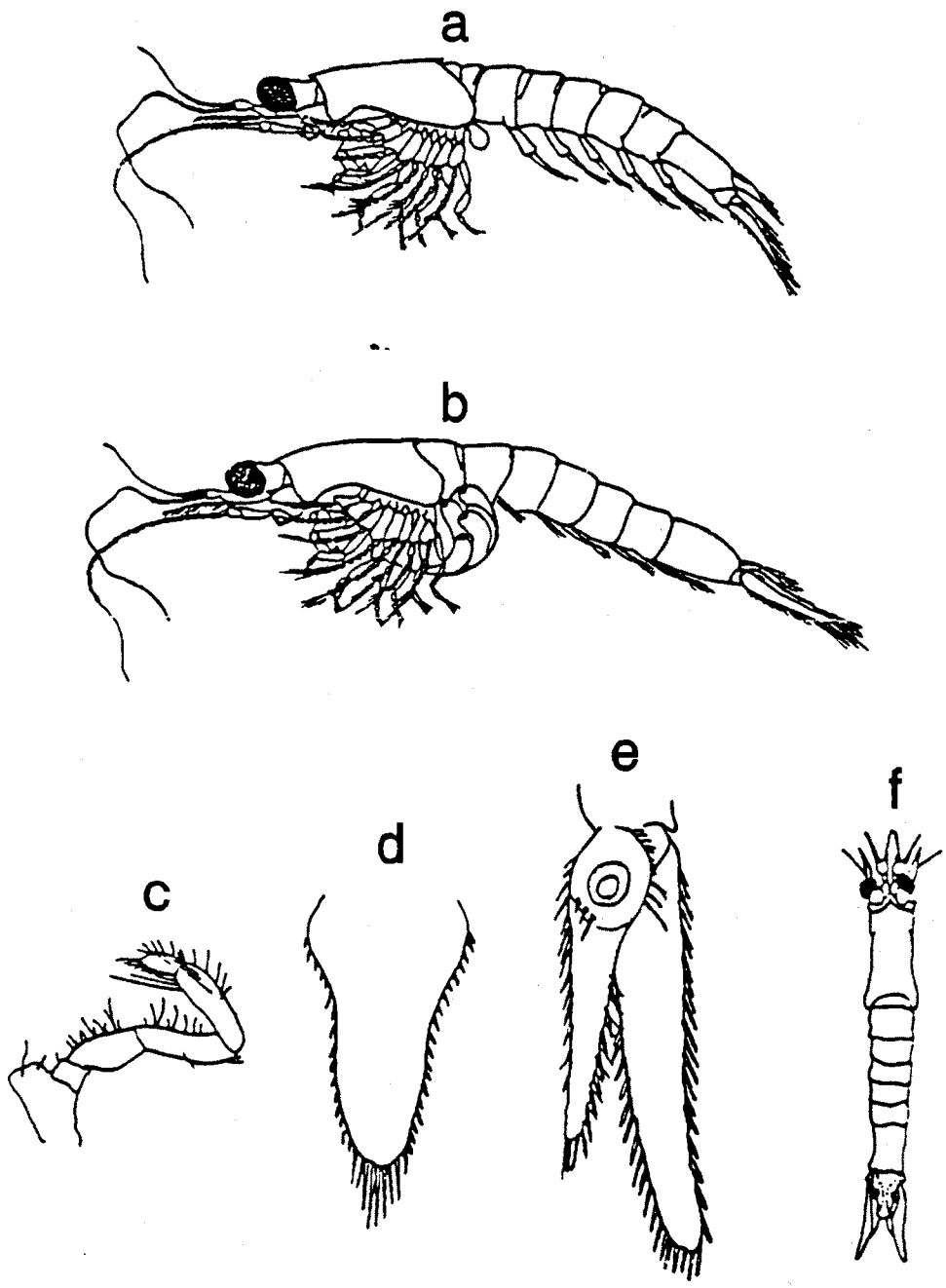

Figure 2. Morphological features most useful in identifying *Mysidopsis bahia*. a. male; b. female; c. thoracic leg 2; d. telson; e. right uropod, dorsal; f. male, dorsal (redrawn from Molenock, 1969; Heard et al., 1987). Note gonad in area where marsupium is located on female and length of male pleopods as compared to female. Also note the 3 spines on the endopod of the uropod (e).

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TABLE 1. GENUS *MYSIDOPSIS*: COMMON AND SPECIFIC CHARACTERS OF SPECIES KNOWN FROM U.S. ALTANTIC AND GULF OF MEXICO WATERS (HEARD, ET AL., 1987 AND MODIFIED IN PART FROM BRATTEGARD, 1969)

	M. Eclipes	M. Brattstroemi	M. Mortenseni	M. Furca	M. Almyra	M. Bahia	M. Bigelowi	M. sp. (Inshore)	M. sp. (Offshore)
Anterior dorsal margin of carapace	Triangular	Triangular	Triangular	Rounded	Rounded	Triangular	Triangular	Triangular	Triangular
Presence of distal segment on antennal	yes	yes	yes	yes	no	no	no	no	no
Length/breadth ratio of antennal scale	2-2.5	3-3.5	3.5-4	4	6	6	5.4	5.4	5.2
# segments in carpoporpodus of thoracic endopods 3-8	3	3	3 (2 in juv.)	3	2	2	2	2	2
# segments in exopod of male pleopod 4	?	6	6-7	7	7	7	7-8	7	7
# spines on uropodal endopod	8-9	10-20	18-31	20-40	1	1-4	5 (occ. 3-4)	3-4 (occ.2 or 5)	5 (occ. 4)
Length of terminal pair(s) of telson spines relative to lateral margin spines	Almost twice as long	slightly longer	Slightly longer	> twice as long	Gradually increasing	Gradually increasing	Abruptly increasing	Abruptly increasing	Abruptly increasing
# of pairs of apical telson spines	1	1	1	2	4-8	3-6	3	3-4	3
# setae on inner margin of segment 6 of second thoracic endopod	ca 3	ca 4	ca 3	ca 3	2-3	2-3	6-12	6-12	6-12
# setae on inner margin of segment 5 of second thoracic endopod	ca 5	ca 4	ca 2	ca 3	7-18	7-18	2	2	5-7

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2.2 MORPHOLOGY AND TAXONOMY

2.2.1 Since *Mysidopsis bahia* occur with two other species of *Mysidopsis*, an understanding of the taxonomy of *M. almyra, M. bahia*, and *M. bigelowi* is important for culturing and testing practices. The taxonomic key of Heard et al. (1987) is suggested (see Table 1 for morphological guide to *Mysidopsis*).

2.2.2 Adults of *M. bahia* range in length from 4.4 mm to 9.4 mm (Molenock, 1969), measured from the anterior margin of the carapace to the end of uropods. The mature females are normally larger than the males and the pleopods of the female are smaller than those of the male (Ward, 1993) (Figure 2). *Mysidopsis bahia* can be positively identified as male or female when they are 4 mm in body length (Ward, 1993). Living organisms are usually transparent, but may be tinted yellow, brown or black. *Mysidopsis bigelowi* can be readily distinguished from *M. almyra* and *M. bahia* by the morphology of the second thoracic leg. *Mysidopsis bigelowi* has a greatly enlarged endopod of the thoracic limb 2 ("first leg") and the limb has a distinctive row of 6 to 12 spiniform setae on the inner margin of the sixth segment (Heard et al., 1987). *Mysidopsis bahia* can also be distinguished from other species of *Mysidopsis* by the number of apical spines on the telson (4-5 pairs) and the number of spines on the inner uropods distal to the statocyst (normally 2-3) (Figure 2).

2.2.3 Heard et al. (1987) state that the most reliable character for separating adult *M. almyra* and *M. bahia* is the number of spines on the inner uropods (*M. almyra* will always have a single spine). Further, Price (1982) found that for all stages of development for both species, the shape of the anterior margin of the carapace (rostral plate) could be used to distinguish *M. almyra* (broadly rounded) from *M. bahia* (more produced). Figure 2 illustrates the morphological features most useful in identifying *M. bahia* (redrawn Molenock, 1969; Heard et al., 1987).

2.3 CULTURE METHODS

2.3.1 SOURCE OF ORGANISMS

2.3.1.1 Starter cultures of mysids can be obtained from commercial sources, particularly in the Gulf of Mexico region for *M. almyra* and *M. bahia*.

2.3.1.2 Mysids of different species can also be collected by plankton tows or dip nets (approximately 1.0 mm mesh size) in estuarine systems. Heard et al. (1987) have identified specimens of *M. bahia* along the eastern coast, however, it has been principally identified as a subtropical species found in the Gulf of Mexico and along the east coast of Florida. Since many species of mysids may be present at a given collection site, the identification of the organisms selected for culture should be verified by an experienced taxonomist. The permittee should consult the permitting authority for guidance on the source of test organisms (indigenous or laboratory reared) before use.

2.4 CULTURING SYSTEM

2.4.1 Stock cultures can be maintained in continuous-flow or closed recirculating systems. In laboratory culture of *M. bahia*, recirculating systems are probably the most common practice. During the past ten years, a number of closed recirculating systems have been described. Since no single recirculating technique is the best in all respects, the system adopted will depend on the facilities and equipment available and the objectives of the culturing activities. Two other species of mysid, *M. almyra* and *M. bigelowi*, have also been successfully reared in the system described in this section (Ward, 1991). Further, there now exist a number of review papers (Venables, 1987 and Lussier et al., 1988) that describe in detail techniques developed by others that will be very helpful in culturing *Mysidopsis*.

2.4.2 Closed recirculating systems are unique because the re-used seawater they contain develops an unusual set of characteristics caused primarily by metabolic waste produced by the mysids. The accumulation of waste products and suspended particles in the water column is prevented by passing the seawater through a biological filtration system, in which ammonia and nitrite are oxidized by nitrifying bacteria.

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2.5 CULTURE TANKS

2.5.1 Stock cultures of mysids are maintained in a closed recirculating system. The system should consist of four 200-L glass aquaria. However, smaller tanks, such as 80-L glass aquaria, can be used. When setting up a system, it is important to consider surface to volume ratio since this will determine how many mysids can be held in each aquarium. If smaller tanks must be used, the 20-gallon "high" form is recommended. Figure 3 (Ward, 1984; 1991) illustrates the main components of the biological filtration system. The flow rate through the filter is controlled by the water valve and is maintained between 4-5 L/min. This flow will be sufficient to establish a moderate current (from the filter return line) in the aquarium to allow the mysids (which are positively rheotactic) to align themselves with the current formed.

2.5.2 The filtration system consists of commercially-available under-gravel filter plates and external power filter. Each aquarium has two filter plates, forming a false bottom on each side of the tank, on which 2 cm of crushed coral are placed. The external power filter (Eheim, model 2017) canister is layered as shown in Figure 3 with a thin layer of filter fiber between each layer of carbon and crushed oyster shells. There has been some modification of the original filtration system (Ward, 1984), with crushed coral instead of oyster shells used on the filter bed, because crushed coral does not dissolve in seawater as readily as crushed oyster shells. If the system described above cannot be used, an acceptable alternative is an airlift pumping arrangement (Spotte, 1979). Crushed coral and oyster shells are commercially available and should be washed with deionized water and autoclaved before use.

2.6 CULTURE MEDIA

2.6.1 A clean source of filtered natural seawater (0.45 µm pore diameter) should be used to culture *Mysidopsis bahia*, however, artificial seasalts (FORTY FATHOMS®) have also been successfully used (Ward, 1993). A salinity range between 20 and 30‰ can be used (25‰ is suggested) to culture *M. bahia*. Leger and Sorgeloos (1982) reported success in culturing *M. bahia* in a formula following Dietrich and Kalle (Kalle, 1971), and still report continued use of this formula (Leger et al., 1987b). Other commercial brands have also been used (Reitsema and Neff, 1980; Nimmo and Iley, 1982; Nimmo et al., 1988) with varying degrees of success. The culture methods presented in Ward (1984; 1991) have been tried with a number of commercial brands of artificial seawater listed in Bidwell and Spotte (1985). Commercial brands of seasalts can be extremely variable in the amount of $NaHCO_3$ they provide, which, if not controlled, can affect growth and reproduction (Ward; 1989, 1991). In a comparative study, Ward (1993) found normal larval development within the marsupium using both natural seawater and FORTY FATHOMS® (i.e., Stage I - embryo; Stage II - eyeless larva; Stage III - eyed larva which is the final stage before release) and stressed the importance of proper preparation of the seasalts and monitoring of conditions in the tank.

2.6.2 The culture media should be aged to allow the build-up of nitrifying bacteria in the filter substrate. To expedite the aging process, 15 mL of a concentrated suspension of *Artemia* should be added daily. If using natural or artificial seawater, the carbonate alkalinity level should be maintained between 90 and 120 mg/L. It is also important to establish an algal community, *Spirulina subsalsa*, in the filter bed (Ward, 1984) and a healthy surface dwelling diatom community, *Nitzchia* sp., on the walls (Ward, 1991) in conjunction with the transfer of part of the biological filter from a healthy tank, when possible. After seven days, the suitability of the medium is checked by adding 20 adult mysids. If the organisms survive for 96 hours, the culture should be suitable for stocking.

2.6.3 If brine solutions are used, 100‰ salinity must not be exceeded. This corresponds to a carbonate alkalinity value of approximately 50 mg/L, which will allow relatively normal physiological mechanisms associated with $CaCO_3$ to occur during certain phases of the life cycle for *M. bahia* (Ward, 1989).

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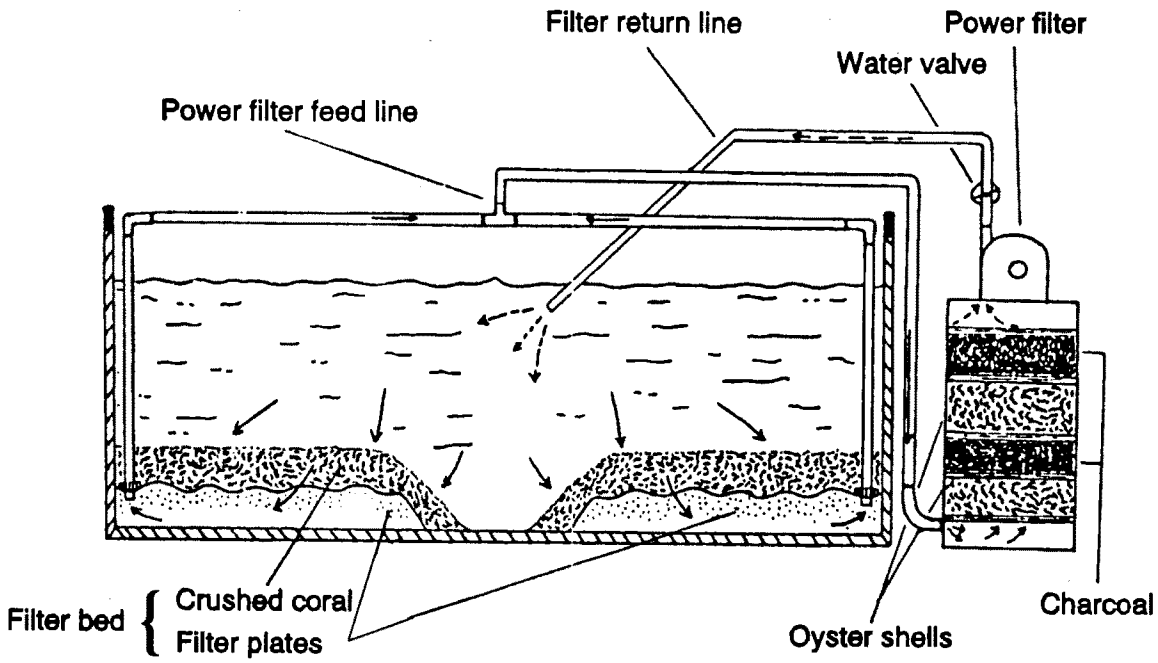

Figure 3. Closed recirculating system showing the two phases of the biological filtration system which consists of the filter bed and external power filter (from Ward, 1984; Ward, 1991).

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2.7 ENVIRONMENTAL FACTORS

2.7.1 Temperature must be maintained within a range of 24°C to 26°C. Twelve to sixteen hours illumination should be provided daily at 50 to 100 ft-c. The daily light cycle can be provided by combining overhead room lights, cool-white fluorescent bulbs (approx. 50 ft-c, 12L:12D), with individual Grow-lux fluorescent bulbs placed horizontally over each tank (approx. 65 ft-c, 10L:14D). This procedure will avoid acute illumination changes by allowing the room lights to turn on one hour before and one hour after the aquaria lights. A timing device, such as an electronic microprocessor-based timer (ChronTrol®, model CD, or equivalent) can be used to control the light cycle. These procedures are fully outlined in Ward (1984; 1991).

2.7.2 Good aeration ($ 60% saturation by vigorous aeration with an air stone), a 10-20 percent exchange of seawater per week, and carbonate in the filtration system are essential in helping to control pH drops caused by oxidation of NH_4-N and NO_2-N by bacteria.

2.7.3 The single most important environmental factor when culturing *Mysidopsis bahia* or other organisms in recirculators is the conversion of ammonia to nitrite, and nitrite to nitrate by nitrifying bacteria. Spotte (1979) has suggested upper limits of 0.1 mg total NH_4-N/L, 0.1 mg NO_2-N/L and 20 mg NO_3-N/L for good laboratory operation of recirculating systems. For the recirculating system and techniques described here for mysids, the levels of ammonia, nitrite and nitrate never exceeded 0.05 mg of total ammonia-N/L ($NH_{3(aq)}$and NH_4^+), 0.08 mg NO_2-N/L and 18 mg NO_3-N/L (Ward, 1991). The toxicity of ammonia is based primarily on unionized ammonia (NH_3) and the proportion of NH_3 species to NH_4^+ species is dependent on pH, ionic strength and temperature. It is strongly recommended that the concentrations of total ammonia, nitrite and nitrate do not exceed those reported here. The ammonia, nitrite, and nitrate levels can be checked by using color comparison test kits such as those made by LaMotte Chemical or equivalent methods.

2.7.4 Bacterial oxidation of excreted ammonia by two groups of autotrophic nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*), results in an increase of hydrogen ions, which causes a drop in pH and subsequent loss of buffering capacity. Typically, the culturist responds to the change in pH by adding Na_2CO_3 or $NaHCO_3$. However, such efforts to buffer against a drop in pH will result in an increase in alkalinity and the uncontrolled use of carbonates can affect reproduction, especially at higher alkalinity values (Ward; 1989, 1991). Therefore, when using carbonates to buffer against pH changes, alkalinity values should not exceed 120 mg/L, which is easily measured by using a titrator kit such as that available from LaMotte Chemical or equivalent methods.

2.7.5 Figure 4 (Ward, 1991) depicts juvenile production per aquarium, no buffer added, over a period of 24 weeks. A regression line was calculated for these data and the slope and correlation coefficient were analyzed by Student's t test. The data showed that even when the pH dropped as low of 7.5, there was a significant increase ($P < 0.001$) in juvenile production. However, the pH should be maintained above 7.8 by the controlled use of $NaHCO_3$ and frequent water exchanges.

2.8 FEEDING

2.8.1 Frequent feeding with live food is necessary to prevent cannibalism of the young by the adults. McKenny (1987) suggests feeding densities of 2-3 *Artemia* per mL of seawater and Lussier et al. (1988) suggest a feeding rate of 150 *Artemia* nauplii per mysid daily.

2.8.2 In the *M. bahia-Artemia* predator-prey relationship, it is also important to provide sufficient quantities of nutritionally viable free-swimming stage-I nauplii (Ward, 1987); final hatching from the membranous-sac (pre-nauplii) into stage-I nauplii does not always occur. *Artemia* cysts that have been incubated for 24 h should be periodically examined with a stereozoom microscope to enumerate free-swimming stage-I nauplii and prenauplii (membranous-sac stage).

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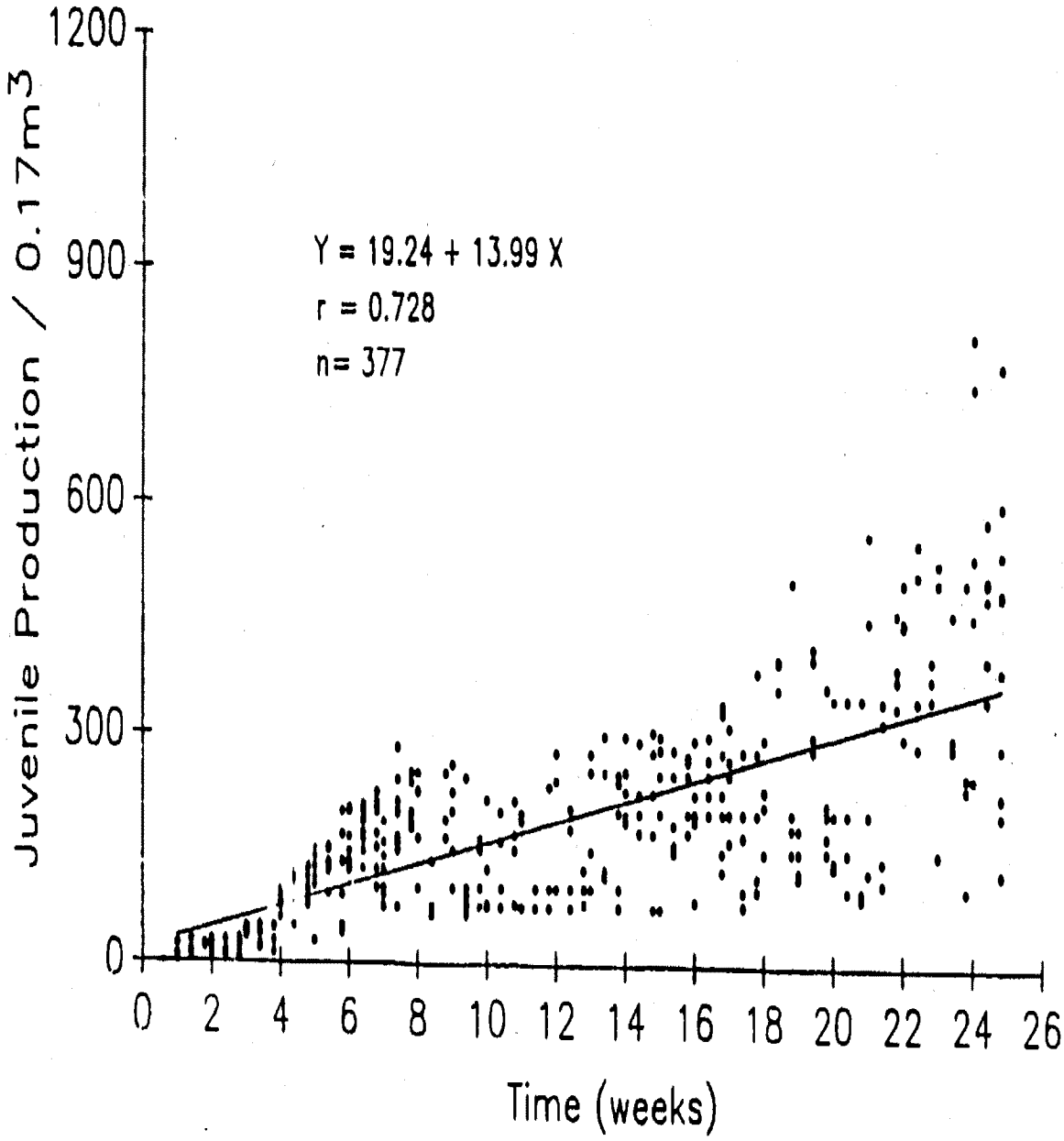

Figure 4. Juvenile production per aquarium over time (from Ward, 1991).

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2.8.3 It has also been found that heavy metals can affect the hatchability of *Artemia* (Rafiee et al., 1986; Liu and Chen, 1987), therefore, when using natural seawater the level of metals should always be checked.

2.8.4 Ward (1987; 1991) has tried different brands of *Artemia* from different geographic origins and lot numbers; many achieved stage I nauplii and still caused variability in production of mysids which suggests that they were nutritionally lacking. Leger et al. (1985; 1987a) have drawn attention to poor larval survival of *M. bahia* and low levels of certain polyunsaturated fatty acids found in the *Artemia* fed. The enhancement of *Artemia* has also been studied and there are numerous techniques that have been successful (Leger et al., 1986).

2.8.5 Ward (1987; 1991) has found that it is important to control the flow of seawater in recirculating systems (keep below 5 L/min) so that *Artemia* does not become limiting to the mysid. Newly hatched *Artemia* should be fed to mysids at least twice a day. To supply *Artemia* to the mysid population on the weekend and prevent cannibalism of newly released mysids, an automatic feeder such as described by Schimmel and Hansen (1975) or Ward (1984; 1991) could be used. Ward (1991) designed a system to hatch *Artemia* when personnel were not available to set up *Artemia* for the following morning and afternoon feeding, such as Monday. Cysts were placed in two 4-L Erlenmeyer flasks (dry), an airstone was placed in each flask, and two vessels overhead were filled with 3500 mL of 30‰ seawater each. The previously described timer (ChronTrol®, Model CD) was used to open the normally closed solenoids, allowing the seawater to gravity feed and hydrate the cysts.

2.8.6 It is possible that a surface dwelling diatom community acts as a secondary food that supplements deficient brands of *Artemia*, especially for newly released juveniles. Ward (1991) has observed that a strong fertilizing action is caused by the excretory products of the mysid population. As the concentration of nitrate increases (nitrification) to about 5 mg/L (in approximately 7-10 weeks in an aquarium), a bloom of surface dwelling diatoms, principally *Nitzschia*, but including *Amphora* and *Cocconeis*, occurs in natural or artificial seawater (Ward, 1993). It is interesting to note that, at the same time, there is a dramatic increase in the number of juveniles observed in the aquaria (Figure 4). The diatoms form layers on the walls of the aquarium and swarms of newly released juveniles have been found among them, possibly feeding upon them.

2.8.7 *Nitzschia* has been identified as a food source for the marine mud snail, *Ilyanassa obsoleta* (Collier, 1981), and the sea urchin, *Lytechinus pictus* (Hinegardner and Tuzzi, 1981). The diatom, *Skeletonema*, has also been used as a supplemental food for *M. bahia* (Venables, 1987). De Lisle and Roberts (1986) reported on the use of rotifers, *Branchionus plicatilis*, as a superior food for juvenile mysids. Rotifers are active swimmers, ranging in size from 100-175 µm as compared to 420-520 µm for *Artemia*, and would provide a good alternative food source if their fatty acid profile is adequate.

2.9 CULTURE MAINTENANCE

2.9.1 To avoid an excessive accumulation of algal growth on the internal surfaces of the aquaria, the walls and internal components should be scraped periodically and the shell substrate (coral or oyster) turned over weekly. Also, the filter plates must be completely covered so that the biological filter functions properly. After a culture tank has been in operation for approximately 2-3 months, detritus builds up on the bottom, which is removed with a fish net after first removing the mysids. The rate of water flow through the tanks should be maintained between 4-5 L/min, and 10-20% of the seawater in each aquarium should be exchanged weekly.

2.9.2 Some culturists have noted problems with hydrozoan pests in their cultures and there are procedures for their eradication, if necessary (Lawler and Shepard, 1978; Hutton et al., 1986).

2.10 PRODUCTION LEVEL

2.10.1 At least four aquaria should be maintained to insure a sufficient number of organisms on a continuing basis. If each 200-L aquarium is initially stocked with between 200 and 500 adults (do not exceed 500 adults), they will provide sufficient numbers of test organisms (Figure 4) each month. If the cultures are correctly

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maintained, at least 20 percent of the adult population should consist of gravid females (have a visible oostegite brood pouch with young). It is also advantageous to cull older mysids in the population every 4-6 weeks and to move mysids among the four aquaria to diversify the gene pool.

2.11 TEST ORGANISMS

2.11.1 Juvenile *Mysidopsis bahia*, one to five days old, are used in the acute toxicity test and the survival, growth and fecundity test (USEPA, 1994). To obtain the necessary number for a test, there are a number of techniques available. A mysid generator such as the one described by Reistsema and Neff (1980) has been successfully used. Another method to obtain juveniles is to take approximately 200 adult females (bearing embryos in their brood pouches) from the stock culture and place them in a large (10 cm X 15 cm) standard fish transfer net (2.0 to 3.0 mm openings) that is partially submerged in an 8-L aquarium containing 4 L of clean culture medium. As the juveniles are released from the brood pouches, they drop through the fish net into the aquarium. The adults and juveniles in the aquarium are fed twice daily 24-hour post hydrated *Artemia*. The adults are allowed to remain in the net for 48 h, and are then returned to the stock tanks. The juveniles that are produced in the small tank may be used in the toxicity tests over a five-day period. Another method for obtaining juveniles (Ward 1987; 1989) is simply to remove juveniles from the stock culture with a fine mesh net, place them in 2-L PYREX® crystalline dishes with media, positioned on a light table that has an attached viewing plate (2 mm squares), and remove juveniles less than 2 mm in length (approximately 24 h old).

3. ***HOLMESIMYSIS COSTATA***

3.1 MORPHOLOGY AND TAXONOMY

3.1.1 Laboratories unfamiliar with the test organism should collect preliminary samples to verify species identification. Refer to Holmquist (1979) or send samples of mysids and any similar co-occurring organisms to a qualified taxonomist. Request certification of species identification from any organism supplier. Records of verification should be maintained along with a few preserved specimens. A review by Holmquist (1979) considered previous references to *Acanthomysis sculpta* in California to be synonymous with *Holmesimysis costata* and this is considered definitive at this time.

3.2 SOURCE OF BROODSTOCK AND TRANSPORT

3.2.1 Broodstock of *H. costata* are collected by sweeping a small-mesh (0.5-1 mm) hand net through the water just under the surface canopy blades of giant kelp *Macrocystis pyrifera*. Although this method collects mysids of all sizes, attention should be paid to the number of gravid females collected because these are used to produce the juvenile mysids used in toxicity testing. Gravid females are identified by their large, extended marsupia filled with young. Mysids should be collected from waters remote from sources of pollution to minimize the possibility of physiological or genetic adaptation to toxicants.

3.2.2 Mysids can be transported for a short time (< 3 h) in tightly covered 20 L plastic buckets. The buckets should be filled to the top with seawater from the collection site, and should be gently aerated or oxygenated to maintain dissolved oxygen above 60% saturation. Transport temperatures should remain within 3°C of the temperature at the collection site.

3.2.3 For longer transport times of up to 36 h, mysids can be shipped in sealed plastic bags filled with seawater. The following transport procedure has been used successfully:

1) fill the plastic bag with one L of dilution water seawater,
2) saturate the seawater with oxygen by bubbling pure oxygen for at least 10 minutes,
3) place 25-30 adult mysids, or up to 100 juvenile mysids in each bag,
4) for adults add about 20 *Artemia* nauplii per mysid, for 100 juveniles add a pinch (10 to 20 mg) of

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ground Tetramin® flake food and 200 newly-hatched *Artemia* nauplii,
5) seal the bag securely, eliminating any airspace, and
6) place it within a second sealed bag in an ice chest.

Do not overfeed mysids in transport, as this may deplete dissolved oxygen, causing stress or mortality in transported mysids. A well-insulated ice chest should be cooled to approximately 15°C by adding one 1-L blue ice block for every five 1-L bags of mysids (organisms will tolerate the temperature range of 12 to 16°C). Wrap the ice in newspaper and a plastic bag to insulate it from the mysid bags. Pack the bags tightly to avoid shifting within the cooler.

3.3 HOLDING AND CULTURING

3.3.1 After collection, the mysids should be transported directly to the laboratory and placed in seawater tanks or aquaria equipped with flowing seawater or adequate aeration and filtration. Initial flow rates should be adjusted so that any temperature change occurs gradually (0.5°C per h). Broodstock will be collected and maintained at two temperatures as follows: 1) maintain the water temperature of 15 ± 1°C for mysids collected south of Pt. Conception, CA and 2) maintain the water temperatures of 13 ± 1°C for mysids collected north of Pt. Conception, CA. Mysids can be cultured in tanks ranging from 4 to 1000 L. Tanks should be equipped with gentle aeration and blades of *Macrocystis* to provide habitat. Static culture tanks can be used if there is constant aeration, temperature control, and frequent water changes (one half the water volume changed at least twice a week). Maintain culture density below 20 animals per L by culling out adult males or juveniles.

3.4 FEEDING

3.4.1 Adult mysids should be fed 100 *Artemia* nauplii per mysid per day. Juveniles should be fed 5 to 10 newly released *Artemia* nauplii per juvenile per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. Static chambers should be carefully monitored and rations adjusted to prevent overfeeding and fouling of culture water.

3.5 TEST ORGANISMS

3.5.1 Juvenile *Holmesimysis costata* three to four days old, are used in the acute toxicity test and the survival and growth test (Hunt *et al.*, 1997; USEPA, 1995). To obtain the necessary number for a test, there are a number of techniques available for the acute toxicity test and the survival and growth test (USEPA, 1995). Approximately 150 gravid female mysids will typically produce approximately 400 juveniles. Gravid females can be identified by their large, extended marsupia filled with (visible) eyed juveniles. Marsupia appear distended and gray when females are ready to release young, due to presence of the juveniles. Gravid females are easily isolated from other mysids using the following technique: 1) use a small dip net to capture about 100 mysids from the culture tank, 2) transfer the mysids to a screen-bottomed plastic tube (150 µm-mesh, 25-cm diameter) partly immersed in a water bath or bucket, 3) lift the screen-tube out of the water to immobilize mysids on the damp screen, 4) gently draw the gravid females off the screen with a suction bulb and fire-polished glass tube (5-mm I.D.), and 5) collect gravid females in a separate screen tube. Re-immerse the screen continuously during the isolation process; mysids should not be exposed to air for more than a few seconds at a time.

3.5.2 Four to five days before a toxicity test begins, transfer gravid females into a removable, 2-mm-mesh screened cradle suspended within an aerated 80-L aquarium. Before transfer, make sure there are no juveniles in with the adult females. Extraneous juveniles are excluded to avoid inadvertently mixing them with the soon-to-be released juveniles used in testing. Provide the gravid females with newly hatched *Artemia* nauplii (approximately 200 per mysid) to help stimulate juvenile release. *Artemia* can be provided continuously throughout the night from an aerated reservoir holding approximately 75,000 *Artemia*. Direct the flow from the feeder into the screened compartment with the females, and add a few blades of *Macrocystis* for habitat. The females are placed within the screened compartment so that as the juveniles are released, they can swim through the mesh into the bottom of the

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aquarium. Outflows on flow-through aquaria should be screened (150-µm-mesh) to retain juveniles and allow some *Artemia* to escape.

3.5.3 Juveniles are generally released at night, so it is important to turn off all lights at night to promote release. In the morning, the screened compartment containing the females should be removed and placed in a separate aquarium. Juveniles should be slowly siphoned through a wide-diameter hose into a 150-µm-mesh screen-bottom tube (25 cm diam.) immersed in a bucket filled with clean seawater. Once the release aquarium is emptied, it should be washed with hot fresh water to eliminate stray juveniles that might mix with the next cohort.

3.5.4 After collection, the number of juveniles should be estimated visually or by counting subsamples with a small beaker. If there are not enough juveniles, the juveniles from previous or subsequent releases can be combined so that the test is initiated with three and/or four-day old juveniles. Mysids 2-days old and younger have higher mortality rates, while mysids older than four days may vary in their toxicant sensitivity or survival rate (Hunt *et al.*, 1989; Martin *et al.*, 1989).

3.5.5 Test juveniles should be transferred to additional screen-tubes (or to 4-L static beakers if flowing seawater is unavailable). The screen-tubes are suspended in a 15-L bucket so that dilution water seawater (0.5 L/min) can flow into the tube, through the screen, and overflow from the bucket. Check water flow rates (< 1 L/min) to make sure that juveniles or *Artemia* nauplii are not forced down onto the screen. The height of the bucket determines the level of water in the screen tube. About 200 to 300 juveniles can be held in each screen-tube (200 juveniles per static 4-L beaker). Juveniles should be fed 40 newly hatched *Artemia* nauplii per mysid per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. A blade of *Macrocystis* (well rinsed in seawater) should be added to each chamber. Chambers should be gently aerated and temperature controlled at 15 ± 1°C (or 13 ± 1°C if collected north of Pt. Conception). Half of the seawater in static chambers should be changed at least once between isolation and test initiation.

3.5.6 The day juveniles are isolated is designated day 0 (the morning after their nighttime release). The toxicity test should begin on day three or four. For example, if juveniles are isolated on Friday, the toxicity test would begin on the following Monday or Tuesday. Pool all of the test juveniles into a 1-L beaker. Using a 10-mL wide-bore pipet or fire-polished glass tube (approximately 2-3 mm I.D), place one or two juveniles into as many plastic cups (one for each test chamber). These cups should contain enough clean dilution seawater to maintain water quality and temperature during the transfer process (approximately 50 mL per cup). When each of the cups contains one or two juveniles, repeat the process, adding mysids until each cup contains 10 organisms. Carefully pour or pipet off excess water in the cups, leaving less than 5 mL with the test mysids. This 5 mL volume can be estimated visually after initial measurements. Carefully pour or pipet the juveniles into the test chambers immediately after reducing the water volume. Gently rocking the water back and forth before pouring may help prevent juveniles from clinging to the walls of the randomization cups. Juveniles can become trapped in drops; have a squirt bottle ready to gently rinse down any trapped mysids. If more than 5 mL of water is added to the test solution with the juveniles, report the amount on the data sheet. Be sure that all water used in culture, transfer, and test solutions is within 1°C of the test temperature. Because of the small volumes involved in the transfer process, temperature control is best accomplished in a constant-temperature room.

3.5.7 Immobile mysids that do not respond to a stimulus are considered dead. The stimulus should be two or three gentle prods with a disposable pipet. Mysids that exhibit any response clearly visible to the naked eye are considered living. The most commonly observed movement in moribund mysids is a quick contraction of the abdomen. This or any other obvious movement qualifies a mysid as alive.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.4. BRINE SHRIMP (*ARTEMIA SALINA*)

1. **SYSTEMATICS**

1.1 MORPHOLOGY AND TAXONOMY

1.1.1 The taxonomic status of *Artemia* has long been controversial because there is considerable morphological variability over parts of its range. The present consensus is that there is a single cosmopolitan species, *Artemia salina*, which has numerous intergrading physiological and morphological varieties (Pennak, 1989). Brine shrimp belong to the subclass Branchiopoda which is characterized by many pairs of flattened appendages on the thorax (Figure 1), in contrast to other members of the Crustacea that have no more than six pairs. Probably the most distinctive feature of *Artemia salina* is the compressed, triangular, and blade-shaped distal segment of the second antenna of the male (Figure 2). The mature adult is 8-10 mm long with a stalked lateral eye, sensorial antennulae,a linear digestive tract and 11 pairs of thoracopods. In the male the antennae are transformed into muscular claspers used to secure the female during copulation.

2. **DISTRIBUTION**

2.1 *Artemia* are found nearly worldwide in saline lakes and pools. In North America, they have been reported throughout the western United States, in Nebraska and Connecticut and in Saskatchewan, Canada. They are probably more widely distributed than indicated because of limited effort in collecting from many areas of the country. They are absent from many suitable habitats, probably because of their limited dispersal methods.

3. **ECOLOGY AND LIFE HISTORY**

3.1 GENERAL ECOLOGY

3.1.1 The ecological conditions under which brine shrimp live are highly variable. The salinity can exceed 300‰, where most other life cannot survive. Favored by the absence of predators and food competitors in such places, *Artemia* develop very dense populations. Although not a marine species, they sometimes occur in bays and lagoons where brines are formed by evaporation of seawater (salt pans). They are more commonly found in highly saline lakes, such as the Great Salt Lake, where the shoreline may become ringed with brown layers of accumulated brine shrimp cysts. Brine shrimp are also common in evaporation basins used for the commercial production of salt.

3.1.2 The reproductive habits of different populations vary considerably. In parts of Europe parthenogenesis is the rule, males being rare or absent, but in North America most *Artemia* populations seem to be diploid with males common.

3.1.3 The principal mechanism of *Artemia* dispersion is transportation of the cysts by wind or waterfowl and by deliberate or accidental human inoculation.

3.1.4 Growth of brine shrimp is influenced by many factors and the tolerance of these factors is strain dependent. Optimum temperature for most strains ranges between 25 and 35EC but strains have been reported thriving at 40EC. Most geographical strains do not survive temperatures below 6EC except as cysts. These cysts are tolerant of temperatures from far below 0EC to near the boiling point of water. Although *Artemia* can survive and reproduce under a wide range of salinity, they are seldom found in nature in salinities below 45‰ or above 200‰. The pH tolerance of *Artemia* varies from neutral to highly alkaline but the cysts will hatch best at a pH of 8 or higher.

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3.1.5 Many predators including many zooplankton that populate natural salt waters, many salt water fish, several insect groups (odonates, hemipterans and beetles), and birds feed on brine shrimp in situations where they can tolerate the conditions.

3.2 FOOD AND FEEDING

3.2.1 Brine shrimp are typically filter-feeders that consume organic detritus, microscopic algae and bacteria. Blooms of microscopic algae are favorite habitats of *Artemia*, and large populations develop in such areas where they feed on the algae and heterotrophic bacteria that are produced by these blooms. Brine shrimp populations have done well in cultures when fed algae, rice bran (Sorgeloos et al., 1979), soybean meal or whey powder (Bossuyt and Sorgeloos, 1979). The nauplii do not need food for four days after hatching.

3.3 LIFE HISTORY

3.3.1 Most strains of *Artemia* produce cysts that float (cysts from the Mono Lake, California strain sink). These cysts remain in diapause as long as they are kept dry or under anaerobic conditions. Upon hydration, the embryo in the cyst becomes activated. After several hours the outer membrane bursts and the embryo emerges still encased in the hatching membrane. Soon the hatching membrane is ruptured and the free-swimming nauplius is born. The first instar is brownish-orange colored and has three pairs of appendages (Figure 3). The larva grows through about 15 molts and becomes differentiated into male or female after the tenth molt. Copulation is initiated when the male grasps the female with its modified antennae (Figure 4). The fertilized eggs develop either into free-swimming nauplii, or they are surrounded by a thick shell and deposited as cysts which are in diapause.

4. **METHODS FOR HATCHING ARTEMIA CYSTS**

4.1 SOURCES OF CYSTS

4.1.1 Brine shrimp cysts are available from many commercial sources, representing several geographical strains. The cysts from any source can vary from batch to batch in terms of nutritional quality for the test organisms. Therefore, it is recommended that each new batch purchased should be analyzed chemically, and that a side-by-side feeding test be performed on their nutritional suitability by comparing the response of the test organisms with the new cysts and cysts of known quality (ASTM, 1993). A list of sources of cysts is provided at the end of this chapter.

4.2 STORAGE OF CYSTS

4.2.1 Sealed cans of *Artemia* cysts can be stored for years at room temperature, but once opened, should be used up within two months. After each use, the can should be tightly covered with a plastic lid and stored in the refrigerator. If the entire contents of a can will not be used up in two months, it is recommended that the portion that is expected to be unused be placed in a tightly closed container and frozen until needed.

4.3 HATCHING OF CYSTS

4.3.1 A 2-L separatory funnel makes a convenient brine shrimp hatching vessel, but nearly any transparent or translucent (preferably colorless) conical shaped container that will hold water may be used. A satisfactory apparatus can be prepared by removing the bottom of a 2 L plastic soft drink bottle and inserting a rubber stopper with a flexible tube and pinch cock. The hatching chambers must be clean and free from toxic material. All detergents should be completely removed by rinsing well with deionized water.

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Figure 1. Drawing of male (A) and female (B) brine shrimp (From Kuenen and Bass-Becking, 1938).

Figure 2. Head of adult male showing triangular distal segment of antennae modified as claspers (From Persoone et al., 1980a).

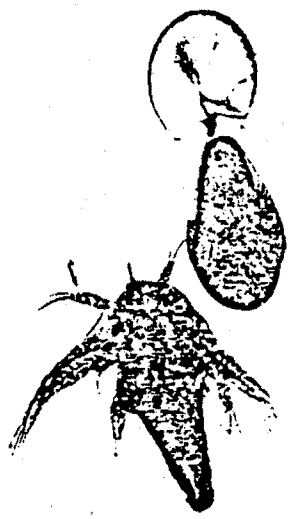

Figure 3. Pre-nauplius and freshly hatched first instar (From Persoone et al., 1980a).

Figure 4. Male and female brine shrimp nauplius preparing to copulate (From Persoone et al., 1980a).

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4.3.2 Salinity of the water used for hatching brine shrimp cysts should be between 25 and 35‰. Natural sea water or water made up from artificial sea salts may be used. The hatching medium can be prepared by placing 1800 mL of deionized water in the hatching chamber and adding 50-70 g non-iodized salt. After the salt is added, lower a 1 mL pipette or glass tube fitted to an air supply into the vessel, so that the tip rests on the bottom, and bubble air vigorously through it to dissolve the salt.

4.3.3 Add the desired quantity of cysts to the vessel. Approximately 15 mL of cysts in a 2-L hatching vessel will provide enough brine shrimp nauplii to feed three large stock cultures of mysids in 76-L aquaria, or 1000-1500 newly hatched fish in four to six 8-L tanks.

4.3.4 Continue the aeration to keep the cysts and newly hatched nauplii from settling to the bottom where the DO would quickly be depleted and the newly hatched animals would die.

4.3.5 The area in which the cysts are hatched should be provided with approximately 20 FE/m^2/s (100 ft-c) of illumination.

4.3.6 The cysts will hatch in about 24 h at a temperature of 25EC. Hatching time varies with incubation temperature and the geographic strain of *Artemia* used.

4.4 HARVESTING THE NAUPLII

4.4.1 When the brine shrimp nauplii first emerge from the cyst, they are enclosed in a membranous sac (Figure 3). To be taken as food by the test organisms, the pre-nauplii must emerge from the sac and swim about (Stage I or first instar nauplius).

4.4.2 The first instar (Stage I) nauplii do not feed. Their value as food for the test organisms decreases from birth until they begin feeding. Because they do not feed in the hatching vessels, it is important to harvest and use the nauplii soon after hatching. The nauplii can be easily harvested in the following manner:

1. After approximately 24 h at 25EC, remove the pipet supplying air and allow the nauplii to settle to the bottom of the hatching chamber. The empty egg shells will float to the top and the newly hatched nauplii and unhatched eggs will settle to the bottom. A light trained on the bottom of the separatory funnel will hasten the settling process.

2. After approximately 5 min, using the stopcock, drain off the nauplii into a 250 mL beaker.

3. After another 5 min, again drain the nauplii into the beaker.

4. The nauplii are further concentrated by pouring the suspension into a small cylinder which has one end closed with #20 plankton netting or they may be washed through a 150 µm net or screen.

5. The concentrate is resuspended in 50 mL of appropriate culture water, mixed well, and dispensed with a pipette. (Mysids require approximately 100-150 nauplii/mysid/day).

6. Discard the remaining contents of the hatching vessel and wash the vessel with hot soap and water.

7. Prepare fresh salt water for each new hatch.

4.4.3 To have a fresh supply of *Artemia* nauplii daily, at least two hatching vessels should be used, so that the newly-hatched can be harvested daily.

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4.5 FEEDING ASSAY

4.5.1 Before using brine shrimp nauplii from a new batch of cysts for routine feeding of cultures and test organisms, they should be tested for their ability to support life, growth, and reproduction of the test animals. Two treatments with four replicates each are required for this test. In Treatment (A), the test organisms are fed the nauplii from the new batch of *Artemia* cysts, and in Treatment (B), the test organisms are fed nauplii of known, good quality, such as from the reference *Artemia* cysts or from a batch of *Artemia* cysts that have been successfully used in culturing and testing.

4.5.2 If there is no significant difference in the survival, growth, and/or reproduction of the organisms in the two treatments at the end of a 7-day period, it is assumed that the new batch of *Artemia* cysts is satisfactory. If the survival, growth, and/or reproduction in treatment A is significantly less than the response in treatment B over a 7-day test period it is assumed that the new batch of brine shrimp cysts are unsuitable for use as a food source for the organisms tested.

4.5.3 Test chambers and all test conditions during the feeding assay should be similar to those planned for use in the subsequent toxicity tests.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.5 FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

1. MORPHOLOGICAL AND ANATOMICAL CHARACTERISTICS

1.1 Fathead minnows vary greatly in many characteristics throughout their wide geographic range. The morphology and characters for identification are taken from Clay (1962), Hubbs and Lagler (1964), Eddy and Hodson (1961), Scott and Crossman (1973), and Trautman (1981). Adults (Figure 1) are small fish, typically 43 mm to 102 mm, and averaging about 50 mm, in total length. The standard lengths are usually less than four and one-half times the body depth. The first rudimentary ray of the dorsal fin is more or less thickened and distinctly separated from the first well-developed ray by a membrane. The lateral line is usually incomplete, but may be complete in specimens from some geographic areas. The scales are cycloid and moderate in size. Andrews (1970), reporting on fish collected in Colorado, noted that no scales were found on fish smaller than 14 mm, and the average length for first scale formation was 16.3 mm. The scales in the lateral series number 41 to 54.

1.2 The mouth is terminal. The snout does not extend beyond the upper lip and is decidedly oblique. Nuptial tubercles occur on mature males only, are large and well-developed on the snout, and rarely extend beyond the nostrils. They occur in three main rows, with a few on the lower jaw. In addition to nuptial tubercles, the males have an elongate, fleshy, or spongy pad extending in a narrow band from the nape to the dorsal fin. The pad is wide anteriorly, and narrows to engulf the first dorsal ray. In addition, the sides of the body become almost black except for two wide vertical bars which are light in color. In contrast to the males, the mature females remain quite drab.

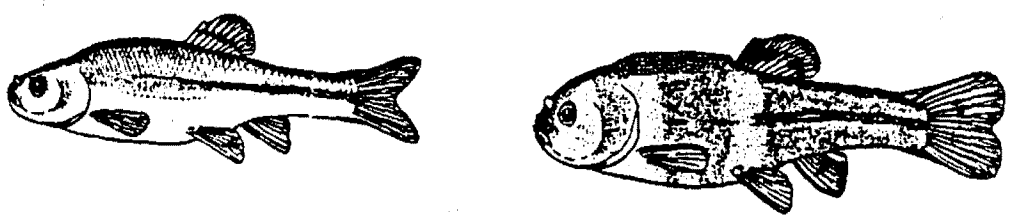

Figure 1. Fathead minnow: adult female (left) and breeding male (right). (From Eddy and Hodson, 1961).

1.3 The peritoneum is brownish-black, and the intestine is long and coiled one or more times.

1.4 Some external markings occur infrequently. Young occasionally have a dusky band on the snout and opercules. Other young and adults, from clear and weedy waters, have a distinct, lateral band across the body. The band may be absent in breeding males or, if present, becomes very diffuse anteriorly. This band is usually most apparent on preserved specimens. Dymond (1926), Trautman (1981), and others described the saddle-like pattern often associated with breeding males in which a light area develops just behind the head and another beneath the dorsal fin, the areas between producing a saddle affect. A dark spot is usually present in front of the dorsal fin in mature males, and a narrow, dark, vertical bar or spot is present at the base of the caudal fin, but often is not very distinct.

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2. **TAXONOMY**

2.1 The specific name (*Pimephales promelas*) appears to be incorrectly applied to this fish because the fathead minnow does not fit the description originally given by Rafinesque (1820) (Lee et al., 1980). Common names include "northern fathead minnow", and "blackhead minnow," in addition to fathead minnow. The holotype was collected near Lexington, Kentucky.

2.2 Some geographic variations have been noted in the morphology of the fathead minnow. Vandermeer (1966) indicated that the introduction of this species outside its native range may have resulted in some local deviations from the broad patterns of geographic variation in taxonomic characters. Some populations have been designated as subspecifically distinct: *Pimephales promelas promelas*, the northern form; *P. p. harveyensis*, the Harvey Lake form, from Isle Royal in Lake Superior and *P. p. confertus*, the southern form (Hubbs and Lagler, 1949, 1964). However, Taylor (1954), Vandermeer (1966), and others expressed doubt concerning the validity of assigning subspecific status to the variants and recommended against their recognition. Vandermeer (1966), in a statistical analysis of the geographic variations in taxonomic characters, stated that two of the three described subspecies intergrade clinally.

2.3 Of the eight characters measured, two showed a north-south trend; (1) eye diameter, with the northern fish having smaller eyes, and (2) completeness of the lateral line, with the northern fish having the least complete lateral line. However, Scott and Crossman (1973), indicated that some Canadian populations exhibit a nearly complete lateral line. The American Fisheries Society (1980) does not recognize any of the fathead minnow subspecies.

3. **DISTRIBUTION**

3.1 The fathead minnow is widely distributed in North America (Figure 2). It is a popular bait fish, and the ease with which it is propagated has led to its widespread introduction both within and outside the native range of the species. It has been so widely distributed in the eastern and southwestern United States by bait transportation that it is difficult to determine its original range. The presumed native distribution (Vandermeer, 1966; Scott and Crossman, 1973; Lee, et al., 1980) extended from the Great Slave Lake in the northwest to New Brunswick, in eastern Canada, southward throughout the Mississippi valley in the United States, to southern Chihuahua in Mexico. Distribution records for this species also now include Oregon (Andreasen, 1975), and the Central Valley (Kimsey and Fisk, 1964) and other locations in California (Andreasen, 1975), but there are no records for British Columbia.

3.2 This species is found in a wide range of habitats. It is most abundant in muddy brooks, streams, creeks, ponds, and small lakes, is uncommon or absent in streams of moderate and high gradients and in most of the larger and deeper impoundments, and is tolerant of high temperature and turbidity, and low oxygen concentrations.

3.3 Species associated with the fathead minnow seem to vary greatly throughout its range (Scott and Crossman, 1973; Trautman 1981). Trautman (1981) reported that fathead minnows and bluntnose minnows, *Pimephales notatus* (Rafinesque), were competitors, and that fathead minnows occurred in greatest numbers only where bluntnose minnows were absent or comparatively few in number. He also stated that the fathead minnow may hybridize with the bluntnose minnow.

3.4 The fathead minnow is primarily omnivorous, although Coyle (1930) reported algae to be one of its main foods in Ohio. Elsewhere in the United States, young fish have been reported to feed on organic detritus from bottom deposits, and unicellular and filamentous algae and planktonic organisms. Adults feed on aquatic insects, worms, small crustaceans, and other animals. Scott and Crossman (1973) and others regard the fathead minnow as a highly desirable forage fish, providing food for other fishes and birds.

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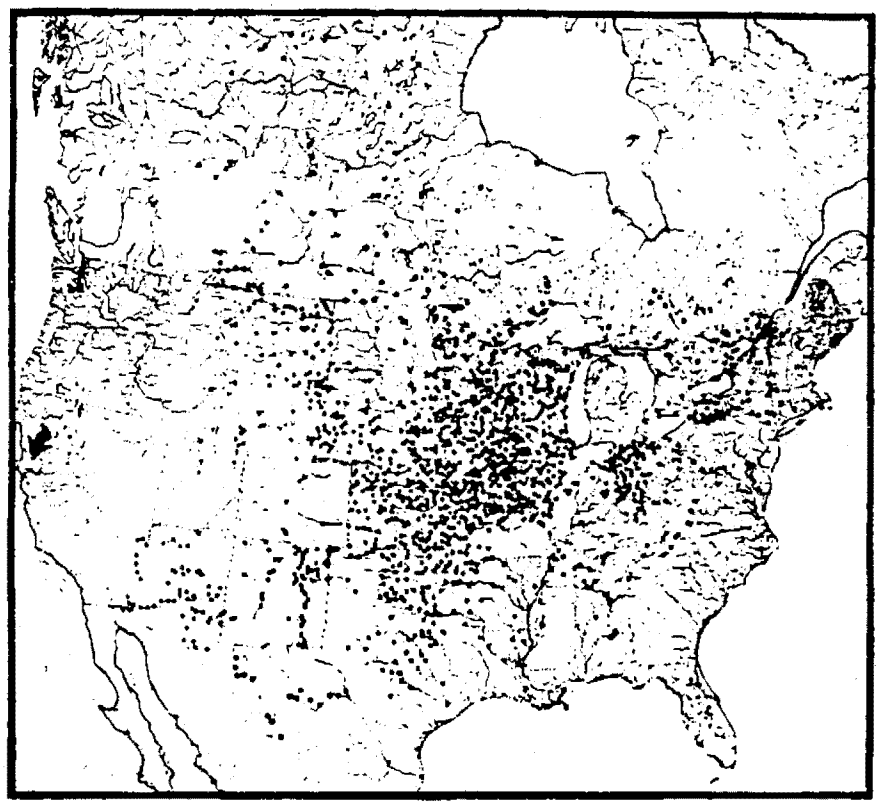

Figure 2. Map showing the distribution of the fathead minnow in North America. Open circles represent transplanted populations. Most Atlantic slope records are probably transplanted populations. (From Lee et al., 1980).

4. GENERAL LIFE HISTORY

4.1 The natural history and spawning behavior (Markus, 1934; Flickinger, 1973; Andrews and Flickinger, 1974; and others) of the fathead minnow are well known because of the early interest in raising the fish for bait and for feeding other pond fish, such as bass. Sexual dimorphism occurs at maturity. Breeding males develop a conspicuous, narrow, elongated, gray, fleshy pad of spongy tubercles on the back, anterior to the dorsal fin, and two or three rows of strong nuptial tubercles across the snout. The sides of the body become almost black except for two wide vertical bars which are light in color. In contrast, the females remain quite drab.

4.2 The initiation of spawning varies with temperature throughout its geographic range. Isaak (1961), Carlander (1969), and others reported that, in the wild, fathead minnows begin spawning in the spring, when the water temperature reaches 16-18EC, and continue to spawn throughout most of the summer. The minimum spawning temperature, however, may vary with population and latitude.

4.3 Markus (1934) reported that spawning always occurred at night, whereas Isaak (1961) observed spawning during the day, as well as at night. Gale and Buynak (1982) and others reported that spawning often began before dawn and usually was completed before noon. Observations of the fathead minnow cultures at EPA's Newtown Facility also indicate the majority of fathead minnows spawn in early morning.

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4.4 Breeding males are very territorial and select sites for spawning, such as the underside of a log or branch, rock, board, tin can, or almost any other solid inanimate object, usually in water from 7 cm to 1 m in depth. A receptive female is sought out and brought into position below the nest site. After circling below the nesting site, the female is nudged and lifted on the male's back until she lies on her side immediately below the undersurface of the spawning substrate, where she releases a small number of eggs (usually 100 to 150) at a time. The eggs are adhesive and attach to the underside of the spawning substrate. The females have a urogenital structure (ovipositor) to help deposit the eggs on the underside of objects. Flickinger (1966) indicated that the ovipositor is noticeable at least a month prior to spawning. The reported size of the eggs varies from 1.15 mm (Markus, 1934) to 1.3 mm in diameter (Wynne-Edwards, 1932).

4.5 Immediately after the eggs are laid, they are fertilized by the male, and the female is driven off. Once eggs are deposited in the nest, the male becomes very aggressive and will use the large tubercles on his snout to help drive off all intruding small fishes. In addition to fertilizing and guarding the eggs, the male agitates the water around the eggs, which ventilates them and keeps them free of detritus. Some males will spawn with several females on the same substrate, so that the nest may contain eggs in various stages of development. The number of eggs per nest may vary from as few as nine or 10 to as many as 12,000.

4.6 The ovaries of the females contain eggs in all stages of development, and they spawn repeatedly as the eggs mature. A female may deposit eggs in more than one nest. Although the average number of eggs per spawn is generally 100 to 150, large females may lay 400 to 500 eggs per spawn.

4.7 Gale and Buynak (1982), in a study using five captive pairs of fathead minnows in separate outdoor pools, observed that each pair produced 16 to 26 clutches of eggs between May and August. The time between spawns, which ranged from 2-l6 days, was affected by water temperature. As the temperature increased, the intervals between spawning sessions become shorter and more uniform. In their study, from nine to 1,136 (mean of 414) eggs were deposited per spawn. The average number of eggs deposited per spawn ranged from 371 to 480, and the total number of eggs spawned per female ranged from 6,803 to 10,164 (mean of 8,604). The length of the spawning period during a given season also varied greatly between females. The authors suggested that the fecundity of fathead minnows is much higher than has generally been recognized, but they noted that fecundity of fish in the natural environment, where conditions might be more or less favorable, might differ from that of captive fish.

4.8 The incubation time depends on temperature, and is 4.5 to 6 days at 25EC. The newly-hatched young (larvae) are about 5 mm long, white in color, and have large black eyes. The general appearance and typical pigmentation of the various larval stages are illustrated in Figures 3A-3M. In a warm, food-rich environment, growth is rapid. Markus (1934) stated that fish hatched in May in Iowa reached adult size and were spawning by late July. Hubbs and Cooper (1935) and others noted that such rapid growth is unlikely in more northerly waters, and that the young do not spawn the first year. In cooler water the adult size is probably not reached until the second year. The males generally grow faster than the females, a characteristic of minnow species.

4.9 The fathead minnow is short lived, and rarely survives to the third year. However, Scott and Crossman (1973) stated that longevity varies throughout the geographic range of the species. Post-spawning mortality was reported to be great by several authors, but was not observed by Gale and Buynak (1982). However, in defending their territory, male fish, may become weakened by a lack of food over a prolonged period and their resistance to disease may be lowered. Also, at spawning time, many waters are warm and somewhat stagnate, favoring the spread of fish parasites and disease.

5. **CULTURE METHODS**

5.1 OUTSIDE SOURCES OF FATHEAD MINNOWS

5.1.1 Fathead minnows are available from commercial biological supply houses. Fish obtained from outside sources for use as brood stock or in toxicity tests may not always be of suitable age and quality. Fish provided by supply houses should be guaranteed to be of (1) the correct species, (2) disease free, (3) in the requested age range,

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(4) and in good condition. The latter can be done by providing the record of the date on which the eggs were laid and hatched, and information on LC50 of contemporary fish using reference toxicants.

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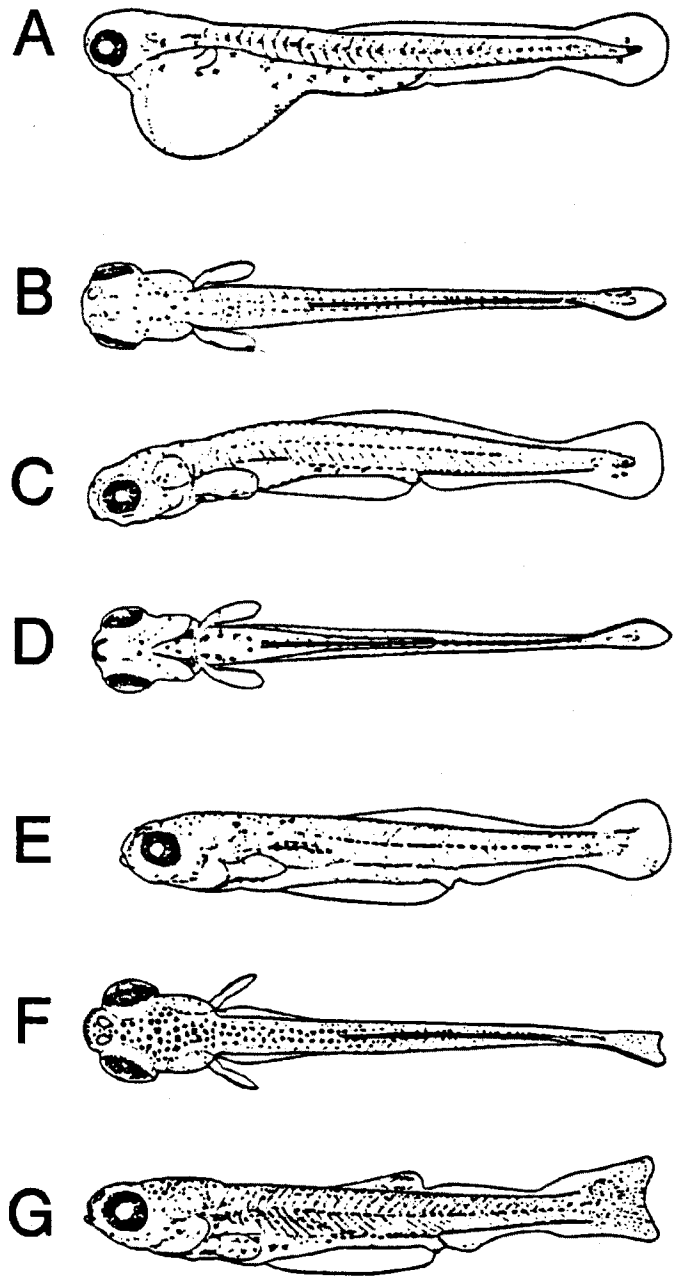

Figure 3. Fathead minnow (*Pimephales promelas*) larvae: A. protolarva, lateral view, 4.3 mm TL; B. protolarva, dorsal view, 5.6 mm TL; C. protolarva, lateral view, 5.6 mm TL; D. protolarva, ventral view, 5.6 mm TL; E. mesolarva, lateral view, 6.9 mm TL; F. mesolarva, dorsal view, 7.9 mm TL; G. mesolarva, lateral view, 7.9 mm TL; (From Snyder et al., 1977).

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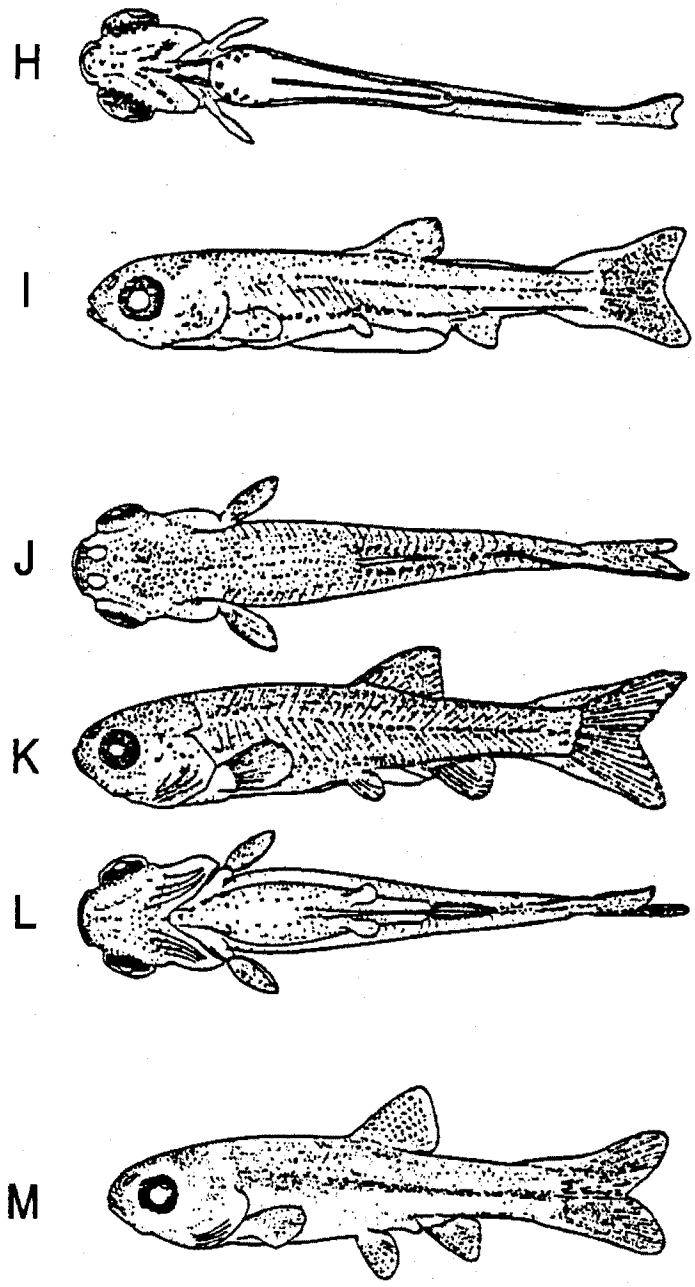

Figure 3. Fathead minnow (*Pimephales promelas*) larvae. H. mesolarva, ventral view, 7.9 mm TL; I. metalarva, lateral view, 9.3 mm TL; J. metalarva, dorsal view, 14.3 mm TL; K. metalarva, lateral view, 14.3 mm TL; L. metalarva, ventral view, 14.3 mm TL; M. late metalarva, lateral view, 19.6 mm TL (CONTINUED) (from Snyder et al., 1977).

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5.2 INHOUSE SOURCES OF FATHEAD MINNOWS

5.2.1 Problems in obtaining suitable fish from outside laboratories can be avoided by developing an inhouse laboratory culture facility. Fathead minnows can be easily cultured in static, recirculating, or flow-through systems.

5.2.2 Flow-through systems require large volumes of water and may not be feasible in some laboratories. The culture tanks should be shielded from extraneous disturbances using opaque curtains, and should be isolated from toxicity testing activities to prevent contamination.

5.2.3 To avoid the possibility of inbreeding of the inhouse brood stock, fish from an outside source should be introduced yearly into the culture unit.

5.2.4 The inhouse culture facility consists of the following components:

5.2.4.1 Water Supply

5.2.4.1.1 Water Quality

5.2.4.1.1.1 Reconstituted (synthetic) water or dechlorinated tap water can be used, but natural water may be preferred. To determine water quality, it is desirable to analyze the water for toxic metals and organics quarterly (see Section 4, Quality Assurance). Temperature, dissolved oxygen, pH, hardness, and alkalinity should also be measured periodically.

5.2.4.1.1.2 If a static or recirculating system is used, it is necessary to equip each tank with an outside activated carbon filter system, similar to those sold for tropical fish hobbyists (or one large activated carbon filter system for a series of tanks) to prevent the accumulation of toxic metabolic wastes (principally nitrite and ammonia) in the water.

5.2.4.1.2 Dissolved oxygen

5.2.4.1.2.1 The DO concentration in the culture tanks should be maintained near saturation, using gentle aeration with 15 cm air stones if necessary. Brungs (1971), in a carefully controlled long-term study, found that the growth of fathead minnows was reduced significantly at all DO concentrations below 7.9 mg/L. Soderberg (1982) presented an analytical approach to the re-aeration of flowing water for culture systems.

5.2.4.2 Maintenance

5.2.4.2.1 Adequate procedures for culture maintenance must be followed to avoid poor water quality in the culture system. The spawning and brood stock culture tanks should be kept free of debris (excess food, detritus, waste, etc.) by siphoning the accumulated materials (such as dead brine shrimp nauplii or cysts) from the bottom of the tanks daily with a glass siphon tube attached to a plastic hose leading to the floor drain. The tanks are more thoroughly cleaned as required. Algae, mostly diatoms and green algae, growing on the glass of the spawning tanks are left in place, except for the front of the tank, which is kept clean for observation. To avoid excessive build-up of algal growth, the walls of the tanks are periodically scraped. The larval culture tanks are cleaned once or twice a week to reduce the mass of fungus growing on the bottom of the tank.

5.2.4.2.2 Activated charcoal and floss in the tank filtration systems should be changed weekly, or more often if needed. Culture water may be maintained by preparation of reconstituted water or use of dechlorinated tap water. Distilled or deionized water is added as needed to compensate for evaporation.

5.2.4.2.3 Before new fish are placed in tanks, salt deposits are removed by scraping or with 5% acid solution, the tanks are washed with detergent, sterilized with a hypochlorite solution, and rinsed well with hot tap water and then with laboratory water.

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5.2.5 SPAWNING TANKS AND CULTURE CONDITIONS

5.2.5.1 For breeding tanks, it is convenient to use 60 L (l5 gal) or 76 L (20 gal) aquaria. The spawning unit is designed to simulate conditions in nature conductive to spawning, such as water temperature and photoperiod. Spawning tanks must be held at a temperature of 25 ±2EC. Each aquarium is equipped with a heater, if necessary, a continuous filtering unit, and spawning substrates. The photoperiod for the culture system should be maintained at 16 h light and 8 h darkness. For the spawning tanks, this photoperiod must be rigidly controlled. A convenient photoperiod is 5:00 AM to 9:00 PM. Fluorescent lights should be suspended about 60 cm above the surface of the water in the brood and larval tanks. Both DURATEST® and cool-white fluorescent lamps have been used, and product similar results. An illumination level of 10-20 $\mu E/m^2/s$ (50-100 ft-c) is adequate.

5.2.6 SPAWNING BEHAVIOR AND CONDITIONS

5.2.6.1 To simulate the natural spawning environment, it is necessary to provide substrates (nesting territories) upon which the eggs can be deposited and fertilized, and which are defended and cared for by the males. The recommended spawning substrates consist of inverted half-cylinders, such as 7.6 cm x 7.6 cm (3 in. X 3 in.) sections of schedule 40, PVC pipe. The substrates should be placed equi-distant from each other on the bottom of the tanks.

5.2.6.2 To establish a breeding unit, 15-20 pre-spawning adults six to eight months old are taken from a "holding" or culture tank and placed in a 76 L spawning tank. At this point, it is not possible to distinguish the sexes. However, after less than a week in the spawning tank, the breeding males will develop their distinct coloration and territorial behavior, and spawning will begin. As the breeding males are identified, all but two are removed, providing a final ratio of 5-6 females per male. The excess spawning substrates are used as shelter by the females.

5.2.6.3 Sexing of the fish to ensure a correct female/male ratio in each tank can be a problem. However, the task usually becomes easier as experience is gained (Flickinger, 1966). Sexually mature females usually have large bellies and a tapered snout. The sexually mature males are usually distinguished by their larger overall size, dark vertical color bands, and the spongy nuptial tubercles on the snout. Unless the males exhibit these secondary breeding characteristics, no reliable method has been found to distinguish them from females. However, using the coloration of the males and the presence of an enlarged urogenital structures and other characteristics of the females, the correct selection of the sexes can usually be achieved by trial and error.

5.2.6.4 Sexually immature males are usually recognized by their aggressive behavior and partial banding. These undeveloped males must be removed from the spawning tanks because they will eat the eggs and constantly harass the mature males, tiring them and reducing the fecundity of the breeding unit. Therefore, the fish in the spawning tanks must be carefully checked periodically for extra males.

5.2.6.5 A breeding unit will remain in their spawning tank about four months. Thus, each brood tank or unit is stocked with new spawners about three times a year. However, the restocking process is rotated so that at any one time the spawning tanks contain different age groups of brood fish.

5.2.7 EMBRYO COLLECTION

5.2.7.1 Fathead minnows spawn mostly in the early morning hours. They should not be disturbed except for a morning feeding (approximately 8:00 AM) and daily examination of substrates for eggs in late morning or early afternoon. In nature, the male protects, cleans, and aerates the eggs until they hatch. In the laboratory, however, it is necessary to remove the eggs from the tanks to prevent them from being eaten by the adults, and for ease of handling for purposes of recording embryo count and hatchability, and for the use of the newly hatched for young fish for toxicity tests.

5.2.7.2 Daily, beginning six to eight hours after the lights are turned on (i.e., 11:00 AM - 1:00 PM), the substrates in the spawning tanks are each lifted carefully and inspected for embryos. Substrates without embryos are

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immediately returned to the spawning tank. Those with embryos are immersed in clean water in a collecting tray, and replaced with a clean substrate. A daily record is maintained of each spawning site and estimated number of embryos on the substrate.

5.2.8 EMBRYO INCUBATION

5.2.8.1 Three different methods are described for embryo incubation.

5.2.8.1.1 Incubation of Embryos on the Substrates: Several (2-4) substrates are placed on end in a circular pattern (with the embryos on the inner side) in 10 cm of water in a tray. The tray is then placed in a constant temperature water bath, and the embryos are aerated with a 2.5 cm airstone placed in the center of the circle. The embryos are examined daily, and the dead and fungused embryos are counted, recorded, and removed with forceps. At an incubation temperature of 25EC, 75-100% hatch occurs in five days. At 22EC, embryos incubated on aerated tiles require seven days for 50% hatch.

5.2.8.1.2 Incubation of Embryos in a Separatory Funnel: The embryos are removed from the substrates with a rolling action of the index finger ("rolled off")(Gast and Brungs, 1973), their total volume is measured, and the number of embryos is calculated using a conversion factor of approximately 430 embryos/mL. The embryos are incubated in about 1.5 L of water in a 2 L separatory funnel maintained in a water bath. The embryos are stirred in the separatory funnel by bubbling air from the tip of a plastic micro-pipette placed at the bottom, inside the separatory funnel. During the first two days, the embryos are taken from the funnel daily, those that are dead and fungused are removed, and those that are alive are returned to the separatory funnel in clean water. The embryos hatch in four days at a temperature of 25EC. However, usually on day three the eyed embryos are removed from the separatory funnel and placed in water in a plastic tray and gently aerated with an air stone. Using this method, the embryos hatch in five days.

5.2.8.1.2.1 Hatching time is greatly influenced by the amount of agitation of the embryos and the incubation temperature. If on day three the embryos are transferred from the separatory funnel to a static, unaerated container, a 50% hatch will occur in six days (instead of five) and a 100% hatch will occur in 7 days.

5.2.8.1.3 Incubation in Embryo Incubation Cups: The embryos are "rolled off" the substrates, and the total number is estimated by determining the volume. The embryos are then placed in incubation cups attached to a rocker arm assembly (Mount, 1968). Both flow-through and static renewal incubation have been used. On day one, the embryos are removed from the cups and those that are dead and fungused are removed. After day one, only dead embryos are removed from the cups. Most of the embryos will hatch in five days if incubated at 25EC.

5.2.8.1.4 During the incubation period, the eggs are examined daily for viability and fungal growth, until they hatch. Unfertilized eggs, and eggs that have become infected by fungus, should be removed with forceps using a table top magnifier-illuminator. Non-viable eggs become milky and opaque, and are easily recognized. The non-viable eggs are very susceptible to fungal infection, which may then spread throughout the egg mass. Removal of fungused eggs should be done quickly, and the spawning substrates should be returned to the incubation tanks as quickly as possible so that the good eggs are not damaged by desiccation.

5.2.9 LARVAE REARING TANKS

5.2.9.1 Newly-hatched larvae are transferred daily from the egg incubation apparatus to small rearing tanks, using a large bore pipette, until the hatch is complete. New rearing tanks are set up on a daily basis to separate fish by age group. Approximately 1500 newly hatched larvae are placed in a 60 L (15 gal) or 76 L (20 gal) all-glass aquarium for 30 days. A density of 150 fry per liter is suitable for the first four weeks. The water temperature in the rearing tanks is allowed to follow ambient laboratory temperatures of 20-25EC, but sudden, extreme, variations in temperature must be avoided.

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5.2.10 HOLDING OR CULTURE TANKS FOR REPLACEMENT SPAWNERS

5.2.10.1 Replacement spawners (brood stock) are cultured from larvae produced in the spawning tanks. After 30 days in a larval rearing tank, a number of juveniles, equivalent to 2-4 days hatch are transferred to brood stock tanks for a 30-60 day growth period. The sub-adults then are transferred to 500-L brood stock tanks to provide about 500 sub-adult fish per month for the brood tank rotation. The surplus fish are transferred to 2000-L fiber glass, or equivalent, holding tanks.

5.2.10.2 Surplus young males removed from spawning tanks, and other surplus mature males, are placed in all-male holding tanks for future use as spawners. Similarly, young and surplus mature females are held in all-female holding tanks until needed as spawners. Tanks holding replacement spawners need not be temperature-controlled, but for ease of transfer to the spawning tanks, it is preferable to hold the water temperature close to that of the spawning tanks (25 ± 2EC).

5.2.11 FOOD AND FEEDING

5.2.11.1 Newly hatched brine shrimp nauplii or frozen adult brine shrimp and commercial fish starter are fed to the fish cultures in volumes based on age, size, and number of fish in the tanks. The amount of food and feeding schedule affects both growth and egg production.

5.2.11.2 Fish from hatch to 30 days old are fed starter food at the beginning and end of the work day, and newly hatched brine shrimp nauplii (from the brine shrimp culture unit) twice a day, usually mid-morning and mid-afternoon. Utilization of older (larger) brine shrimp nauplii may result in starvation of the young fish because they are unable to ingest the larger food organisms (see Appendix A.4 for instructions on the preparation of brine shrimp nauplii). Avoid introducing *Artemia* cysts and empty shells when the brine shrimp nauplii are fed to the fish larvae. Some of the mortality of the larval fish observed in cultures could be caused from the ingestion of these materials.

5.2.11.3 Fish older than four weeks are fed frozen brine shrimp and commercial fish starter (#1 and #2), which is ground fish meal enriched with vitamins. As the fish grow, larger pellet sizes are used, as appropriate.

5.2.11.4 The spawning fish and pre-spawners in holding tanks usually are fed all the adult frozen brine shrimp and tropical fish flake food or dry commercial fish food (No. 1 or No. 2 granules) that they can eat (*ad libitum*) at the beginning of the work day and in the late afternoon (i.e., 8:00 AM and 4:00 PM). The fish are feed twice a day, twice with dry food and once with adult shrimp, during the week, and once a day on weekends.

5.2.12 DISEASE CONTROL

5.2.12.1 Fish are observed daily for abnormal appearance or behavior. Bacterial or fungal infections are the most common diseases encountered. However, if normal precautions are taken, disease outbreaks will rarely, if ever, occur. Hoffman and Mitchell (1980) have put together a list of some chemicals that have been used commonly for fish diseases and pests.

5.2.12.2 Treatment of individual lots of infected fish should be carried out separate from the main culture. Use of treated fish should be avoided, if possible, and diseased cultures should be replaced.

5.2.12.3 In aquatic culture systems where filtration is utilized, the application of certain antibacterial agents should be used with caution. A treatment with a single dose of antibacterial drugs can interrupt nitrate reduction and stop nitrification for various periods of time, resulting in changes in pH, and in ammonia, nitrite and nitrate concentrations (Collins et al., 1976). These changes could cause the death of the culture organisms.

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5.2.12.4 To prevent possible rapid spread of disease, do not transfer equipment from one tank to another without first disinfecting tanks and nets. If an outbreak of disease occurs, any equipment, such as nets, airlines, tanks, etc., which has been exposed to diseased fish should be disinfected with sodium hypochlorite. Also to avoid the contamination of cultures or spread of disease, each time nets used to remove live or dead fish from tanks, they are first sterilized with sodium hypochlorite or formalin, and rinsed in hot tap water. Before a new lot of fish is transferred to culture tanks, the tanks are cleaned and sterilized as described above.

5.2.13 RECORD KEEPING

5.2.13.1 Records are kept in a bound notebook, include: (1) type of food and time of feeding for all fish tanks; (2) time of examination of the tiles for embryos, the estimated number of embryos on the tile, and the tile position number; (3) estimated number of dead embryos and embryos with fungus observed during the embryonic development stages; (4) source of all fish; and (5) daily observation of the condition and behavior of the fish.

5.2.14 REFERENCE TOXICANTS

5.2.14.1 It is recommended that static acute toxicity tests be performed monthly with a reference toxicant. Fathead minnow larvae one to 14 days old are used to monitor the acute toxicity of the reference toxicant to the test fish produced by the culture unit.

6. **TEST ORGANISMS**

6.1 Fish 1-14 days old are used in acute toxicity tests.

6.2 If the fish are kept in a holding tank or container, most of the water should be siphoned off to concentrate the fish. The fish are then transferred one at a time randomly to the test chambers until each chamber contain 10 fish. Alternately, fish may be placed one to two at a time into small beakers or plastic containers until they each contain five fish. Two of these beakers/plastic containers (total of 10 fish) are then assigned to each randomly-arranged control and exposure chamber.

6.3 The fish are transferred directly to the test vessels or intermediate chambers using a large-bore, fire-polished glass tube (6 mm to 9 mm I.D. X 30 cm long) equipped with a rubber bulb, or a large volumetric pipet with tip removed and fitted with a safety type bulb filler. The glass or plastic containers should only contain a small volume of dilution water.

6.4 It is important to note that larvae should not be handled with a dip net. Dipping small fish with a net may result in damage to the fish and cause mortality.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.6 RAINBOW TROUT, *ONCORHYNCHUS MYKISS* AND BROOK TROUT, *SALVELINIUS FONTINALIS*

1. RAINBOW TROUT

1.1 SYSTEMATICS AND TAXONOMY

1.1.1 Rainbow trout are native to the streams of the Pacific coast where several varieties or strains have developed. The seagoing form is known as the steelhead trout and is thought to be identical to the strictly freshwater rainbow form. Many other strains, for example, the inland lake form (Kamloops trout) are found in other watersheds. Because of the ease with which the eggs can be transported, different strains have been distributed all over the world.

1.1.2 Rainbow trout are a variable species that differ considerably over the whole of their range. Populations in different regions and watersheds of North America have been referred to over the years by different scientific names (e.g. species, distinct subspecies, or variants of a single species and different regional common names). In recent years the validity of the generic name, *Salmo*, for some western North American trout species has been questioned. Fish taxonomists agree that native "*Salmo*" trouts of the northern Pacific Ocean drainage are closely related with Pacific salmon *Oncorhynchus* spp. The American Society of Ichthyologists and Herpetologists and the American Fisheries Society have accepted *Oncorhynchus* as the appropriate generic name for all native Pacific drainage trouts that are presently called *Salmo*, based on new data and evidence by Smith and Stearly (1989). Furthermore, the Names of Fishes Committee of the American Fisheries Society has adopted the specific name, *Oncorhynchus mykiss*, for the rainbow trout and its anadromous form, steelhead trout. The new names for the other North American species affected are the following: Apache trout *(O. apache*), cutthroat trout (*O. clarki*), Gila trout (*O. gilae*), golden trout (*O. aguabonita)*, and Mexican golden trout (*O. chrysogaster)*.

1.2 DISTRIBUTION

1.2.1 The native range of the rainbow trout group (all varieties) in North America is west of the Rocky Mountains and along the eastern Pacific Ocean, but the species (*Oncorhynchus mykiss*) has now been introduced into many parts of the continent (Figure 1). Except for the northern and southern extremes of the rainbow trout range, anadromous populations occur in all coastal rivers. This species, under all its common names (rainbow trout, Kamloops trout, steelhead trout, steelhead, coast rainbow trout, and silver trout), has been so widely introduced in North America outside its natural range as to suggest it may occur throughout the United States in all suitable habitats. Rainbow trout are widely introduced and established in appropriate cold water habitats all over the world.

1.3 GENERAL LIFE HISTORY

1.3.1 In its natural environment of flowing streams of the western mountains, the rainbow trout (Figure 2) thrives best at temperatures ranging from 3EC in the winter to 21EC in the summer, but the optimum temperature is between 10-16EC. The rainbow trout can withstand higher and lower temperature if it is acclimated gradually. However, the rainbow trout's growth is impeded by extremes of temperature, for example, above 27EC which it can tolerate only for short periods of time.

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Figure 1. Map showing the distribution of the rainbow trout in North America. (Modified from Lee et al., 1980).

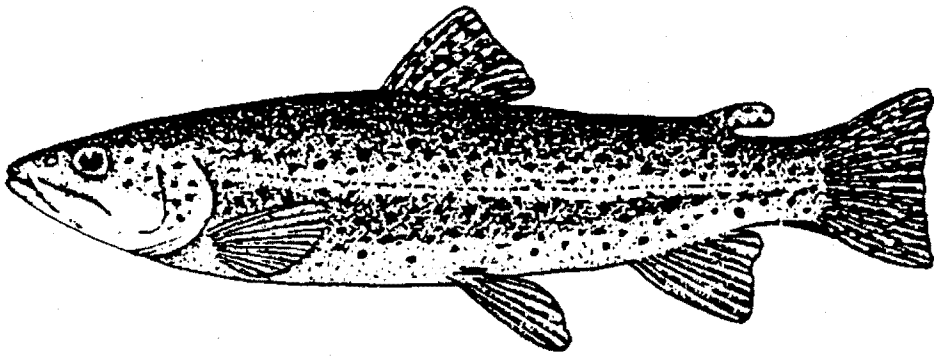

Figure 2. Rainbow trout (Modified from Eddy and Underhill, 1974).

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1.3.2 Rainbow trout are basically spring spawners, but they can spawn at the beginning of summer or early winter, depending on climate, elevation, and genetic strain. If the spawning occurs in late fall or in winter, the eggs do not hatch until spring. Prior to the spawning season adult males develop a kype (elongated hooked snout) on the lower jaw and their colors intensify. Males and females usually migrate upstream and select spawning sites in beds of fine, clean gravel in riffles or runs above pools in streams. Long journeys may be made by lake-dwelling rainbow (or Kamloops) and steelhead trouts or anadromous, ocean-run rainbow steelheads. If the rainbow trout are confined to land-locked lakes, they move into shallow shoals or reefs of gravel and sand for spawning. Females dig out pits or sweep out depressions (redds) in the gravel or sand and later spawn with males. Males are capable of displaying aggressive behavior on the spawning grounds and can drive other males away from a redd occupied by a female. In general, one or more males court the digging female by sliding along side and crossing over her body and rubbing their snout against her caudal peduncle with body pressing and body vibrations. The female deposits her eggs, which are 3-5 mm in diameter, demersal, and pink to orange in color. The eggs are immediately fertilized by one or more males, fall into spaces between the gravel, and are covered with loose gravel or sand to depths of 20 cm or more by the female. Females are capable of digging and spawning in several redds with the same male or different males. The number of eggs released can range from 400-3000, depending on the size of the female.

1.3.3 Eggs usually hatch in approximately four to seven weeks. The time of hatching, however, varies greatly with region and habitat. If the stream temperature averages 7EC, eggs will hatch in about 48 days. The newly hatched fish, called alevins, have a yolk sac, which is absorbed in three to seven days. After the yolk sac is absorbed, the young are called fry, and begin feeding in 10-15 days. In general, rainbow trout feed on a variety of invertebrates. Also, depending on their size and the habitat in which they live, other fishes and fish eggs, especially salmon, can be important food. The fry of lake-resident spawners move up or down the spawning river to the lake, or they may spend as much as one to three years in the streams. The stream-resident spawners remain in the streams, whereas the steelhead trout, which are stream-spawners, migrate to the sea, usually after 1-4 years in freshwater.

1.3.4 The growth of rainbow trout is highly variable with the area, habitat, type of life history, and quantity and type of food. Some males may be good breeders at two years of age, but few females produce eggs until their third year of life. Rainbow trout young attain fingerling size of about 76 mm by the end of their first summer. The length may range between 178-204 mm at the end of the second year, 279-382 mm after the third year, 356-406 mm after the fourth year, and 406 mm or more after the fifth year. Lake- and ocean-run rainbows may grow over twice as fast as this. However, the average length of rainbow trout (or Kamloops trout) is 305-458 mm and that of steelhead trout is 508-762 mm. Under favorable conditions of artificial propagation, yearlings average about 28 g, 2-year-olds about 255 g, 3-year-olds between 0.45-9 kg, and 4-year-olds between 1.4-1.8 kg. Returning sea-run individuals weigh up to 18 kg, or even more, but usually between 1.4-9 kg with the majority weighing less than 5.4 kg. Some western varieties weight up to 23 kg, but the midwest rainbows are much smaller. Those in streams are rarely over 1.4 kg, but in some large lakes (e.g., Lake Superior) and in some western lakes they may reach 7 kg or much larger. The life expectancy of rainbow trout can be as low as three or four years in many streams and Lake populations, but that of seagoing steelhead rainbow trout and Great lakes populations would appear to be 6 to 8 years (Scott and Crossman, 1973).

1.4 GENERAL DESCRIPTION

1.4.1 Adult rainbow trout are bluish or olive green above and silvery on the sides, with a broad pink lateral stripe that is enhanced during the spawning season. The back, the sides, and the dorsal and caudal fins are profusely dotted with small dark spots. Their color is variable with habitat, size, and sexual condition. Stream forms and spawners are generally darker with color more intense, lake forms lighter, brighter, and more silvery. Different color types are often called by different names, e.g., darker stream fish often called rainbows; larger, brighter, silvery fish in western lakes often called Kamloops trout, and large silvery specimens returning from the sea and in the Great Lakes or tributaries called steelhead trout. The scales are large, numbering 120 to 150 in the lateral line. The caudal fin is very slightly forked. The dorsal fin has 11 rays, and the anal fin has from 10 to 12 rays.

1.4.2 Young rainbow trout are typically blue to green on the dorsal surface, silver to white on the sides and white ventrally. There are 5-10 dark marks on the back between the head and dorsal fin. Also, there are 5-10 short, dark,

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oval parr marks widely spaced on the sides, straddling the lateral line with some small dark spots above but not below the lateral line. The dorsal fin has a white to orange tip and a dark leading edge, or a series of bars or spots. The adipose fin is edged with black, and the anal fin has an orange to white tip.

2. **BROOK TROUT**

2.1 SYSTEMATICS AND TAXONOMY

2.1.1 Brook trout can be found exhibiting some variation in growth rate and color throughout its range, but is considered a stable and well-defined species (American Fishery Society, 1980). Male brook trout may be crossed with female lake trout (*Salvelinus namaycush*) to produce fertile hybrids that are known as splake. Troutman (1981) and other papers cited in this section indicate that brook trout can naturally and artificially hybridize with brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*). For additional information and discussion on freshwater and anadromous brook trout stocks and systematic notes of brook trout, see Scott and Crossman (1973) and other papers cited in this section.

2.2 DISTRIBUTION

2.2.1 The native range of the brook trout (Figure 3) is eastern North America, extending throughout much of eastern Canada from Hudson Bay and Ungava Bay drainages and Labrador; southward through the New England States and in the Appalachian Mountains to the headwaters of the Savannah, Chattahoochee, and Tennessee Rivers in the Carolinas and Georgia. In the Great Lakes, brook trout are native to Lake Superior and tributaries to the northern tip of the Lower Peninsula, the interior of the Great Lakes basin. They are also native to a few far-northern headwaters of the upper Mississippi river system, and western Minnesota and northeastern Iowa.

2.2.2 The brook trout has been widely introduced to higher elevations in western North America. This species is also found in temperate regions of other continents. Inland forms are found in colder lakes and streams, and sea-run (anadromous) forms are found in the northeastern North American coastal water areas.

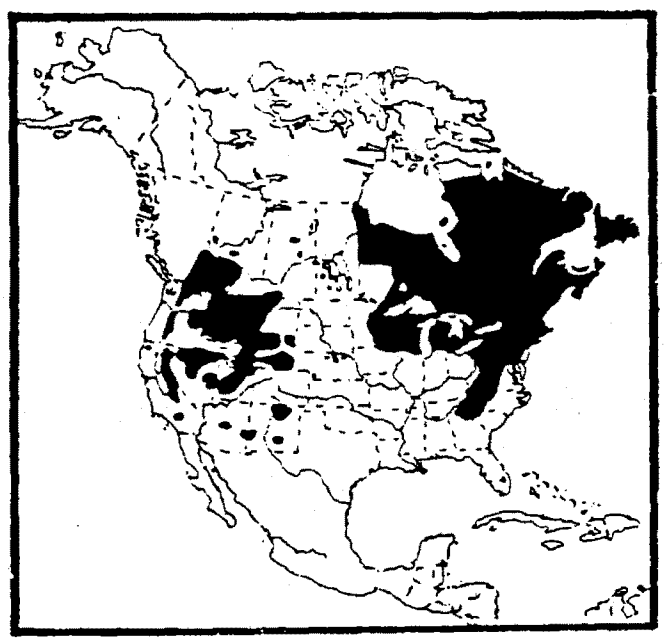

Figure 3. Map showing the distribution of the brook trout in North America. (Modified from Lee et al., 1980).

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2.3 GENERAL LIFE HISTORY

2.3.1 Brook trout (Figure 4) are generally found in clear brooks, streams, and rivers in which the mean temperature rarely exceeds 10EC. The optimum temperature is reported as ranging from 7 to 13EC, but they may be found living in waters with temperatures ranging from 1 to 22EC (Piper et al. 1982). The brook trout usually inhabits waters which flow less swiftly than those inhabited by the rainbow. Brook trout also thrive in the small cold-water lakes of the Great Lakes region, provided that suitable spawning conditions exist.

2.3.2 Brook trout spawn in late summer or autumn, the date varying with latitude and temperature, usually from late October to December when the water temperature is suitable although some may start spawning in September in certain streams flowing into large lakes. Some females are capable of spawning when they are a year old, while others do not mature until the second year. When the spawning season occurs, brook trout move upstream into small head waters or brooks where they select gravel and sand substrates usually in shallow riffle areas or the tail-ends of pools for the spawning beds. Spawning usually occurs during the day.

2.3.3 The female prepares a nest (redd), similar to those of the rainbow trout, by sweeping out a depression in the gravel and sand substrate. During preparation of the redd, the male starts courtship by quivering around the female and driving off all intruders. When the female is ready to spawn, she takes a position above and close to the redd. The male gets close to her side and arches his body over hers, discharging milt as the female deposits her eggs. Occasionally second male may join them in the spawning. After spawning, the male leaves.

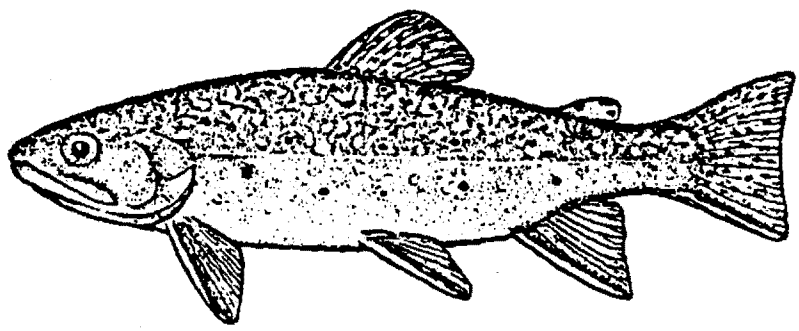

Figure 4. Brook trout. (Modified from Eddy and Underhill, 1974).

2.3.4 The eggs are 3.5 to 5.0 mm in diameter, are adhesive, and adhere to the gravel at the bottom of the redd. The female pushes loose gravel and sand to the center, covering the entire redd, and then desert the nest. A female may spawn several times, and the number of eggs can vary from 100 to 5000, depending on the size of the female.

2.3.5 The eggs remain in the redd until the water temperature rises during the following spring. If the level of DO is adequate, the eggs will hatch in approximately 75 days at an average water temperature of 6.1EC, and in approximately 50 days at an average temperature of 10EC. The upper lethal temperature limit for developing eggs is about 11.7EC (Scott and Crossman, 1973).

2.3.6 After the eggs hatch, the larvae (sac fry) remain in the gravel of the redd until the yolk is absorbed. Depending on the water temperature, it may take from one to three months for the yolk sac to be absorbed (Lagler,

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1956). While the yolk sac is absorbed, the fry work themselves free from the gravel and start feeding. They become free swimming at about 38 mm long. Under natural conditions, newly hatched brook trout establish small feeding territories in the stream and feed on small aquatic insects, insect larvae, and other organisms.

2.3.7 Growth of brook trout is extremely variable, depending on the suitability of the environment. The average length attained at various ages may approximate 8.9 cm the first year; 15.2 cm the second year, 22.9 cm the third year, 30.5 cm the fourth year, and 33 cm the fifth year. Brook trout generally do not exceed a length of 54 cm and a weight of 1.5 kg (Trautman, 1981). However, Scott and Crossman (1973) reported a brook trout as large as 6.6 kg. Rumors of larger brook trout have been circulated, but none have been verified. Brook trout may overpopulate small streams, resulting in large numbers of small trout less than 25.4 cm long. Wild brook trout seldom live longer than five years, and rarely live more than eight years.

2.4 GENERAL DESCRIPTION

2.4.1 The sides of large young and adult brook trout are dark olive, sprinkled with light spots and red spots outlined with purplish or blue hue. Some forms have red spots with light brown margins. The scales are cycloid, small, in about 215 to 250 rows at the lateral line. The top of the head and back is dark olive and heavily vermiculated. There are no black or brown spots on the head, back, adipose, or caudal fin. The anterior rays of the pectoral, pelvic, and anal fins are milk-white, bordered posteriorly with a dusky hue and the remainder of the fins yellowish or reddish.

2.4.2 The back of young or immature brook trout is olive, the sides are lighter and more silvery, and the belly is whitish. There are between 8-12 rectangular parr marks on the sides, also a few yellow and blue spots, but no black spots.

2.4.3 The dorsal fin has 10 rays, and the anal fin has 9 rays. The belly of breeding males is red, and some males may develop a hook (or kype) at the front of the lower jaw. The tail or caudal fin is slightly notched in the young but is generally square in older brook trout.

3. **HOLDING AND ACCLIMATION PROCEDURES FOR TROUT STOCKS**

3.1 SOURCES OF ORGANISMS

3.1.1 Trout fry are obtained from commercial hatcheries during March through July. However, if trout are needed for toxicity testing, it is advisable to contact the hatchery for its trout hatching and rearing schedule. If trout must be ordered from out-of-state, the State Fish and Game Agency should be contacted concerning regulations on fish importation. The recommended age for test organisms is approximately 15-30 days (after yolk sac absorption to 30 days) for rainbow trout and 30-60 days for brook trout. Trout are purchased 36 to 48 h prior to their use as testing organisms, but they must have time to stabilize over the acclimation period. Trout should appear disease-free and unstressed, with fewer than 5% of the animals dying during the 24-48 hours preceding use in a toxicity test.

3.1.2 Trout fry are usually transported in plastic bags of at least 4-mil plastic or thicker in shipping containers. The bags are partially filled with water saturated with oxygen. During warm weather the shipping containers are cooled with ice or cold packs to prevent temperature increases which will result in the loss of fish. Trout should be acclimated gradually from the temperature of the transportation unit to that of holding environment. Upon arrival at the destination the plastic bags should be allowed to float unopened in the holding tank for about 30 minutes to acclimate the fish.

3.2 HOLDING CONDITIONS

3.2.1 Trout are held in 200 L (50 gal) or larger tanks supplied with a flow-through water system, or with recirculated water and a biological filtration system. The holding water should be moderately hard and free of chlorine, have low concentrations of metals, and should have a pH between 6 and 9. Provide a daily photoperiod of

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16 hours light, 8 hours darkness with an illumination at 10-20 $\mu E/m^2/s$ (50-100 ft-c, or ambient laboratory levels). A 15 min dimmer timer should be used to gradually increase or decrease the illumination when lights are turned on or off. The gradual increase and decrease of illumination at the beginning and ending of the photoperiod is important because trout tend to jump when startled by a sudden change in light intensity. Holding water temperature is maintained at 12EC ±2EC and is aerated as close as possible to saturation. Measurements of temperature, DO, pH, conductivity, and ammonia are made on holding water daily.

3.3 FEEDING

3.3.1 Trout are fed fine texture trout chow. The fry in the holding tank are fed (*ad libitum*) up to 24 hours before the start of the acute toxicity test. Dead or moribund fish should be removed from the holding tanks every day. Excess food and feces are vacuum-siphoned off the bottom of the tank daily.

3.3.2 Daily records should be maintained for organism survival, health, and acclimation conditions.

4. TEST ORGANISMS

4.1 Rainbow trout fry 15-30 days old, and Brook trout 30-60 days old, are used in acute tests (see summary tables of test conditions in Section 9, Acute Toxicity Test Procedures). The fry in the holding tank are not fed for 24 hours prior to the start of the test. The fry are caught carefully with a fine mesh net and placed gently in the 5 L (4 L test solution volume) test chambers, until 10 fish are reached per test chamber. Larger test chambers or 5 fish/chambers may be necessary if DO or pH problems are encountered. Placement of the test chambers is random.

4.2 After the fish are introduced, the behavior should be noted and recorded throughout the test period. At the beginning and ending of the photoperiod, during the test, the light intensity should be raised and lowered gradually over a 15 min period using a dimmer switch or suitable device. Between observations the test vessels are covered to act as a dust barrier and to prevent fish from jumping out.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.7 SHEEPSHEAD MINNOW (*CYPRINODON VARIEGATUS*)

1. MORPHOLOGY AND TAXONOMY

1.1 The sheepshead minnow (*Cyprinodon variegatus*) belongs to the family Cyprinodonitidae (killifishes), which includes 45 genera and 300 species worldwide, occurring on all continents except Australia. Most species are freshwater, but some occur in brackish and coastal marine waters. There are thirteen species in the Genus *Cyprinodon* in the United States (American Fisheries Society, 1980). The sheepshead minnow is the only marine species, and is widely distributed in the coastal waters of the Atlantic and Gulf of Mexico.

1.2 Adult sheepshead minnows (see Hardy, 1978, for a complete description) can attain a total length of 93 mm, but the average standard length report for adults is 35-50 mm. The males are usually somewhat longer than females. The fish have the following morphological characteristics: lack a lateral line; have 24-29 lateral scale rows; have a large elongate humeral scale just above the pectoral base; the dorsal fin has nine to 13 rays; the anal fin has nine to 12 rays; the caudal fin has 14-16 principal rays and a total of 28-29 rays; the pectoral fin has 14-17 rays, and the ventral fin has five to seven rays.

1.3 The body of males is short, compressed, and deep. The depth increases with age. The upper profile is evenly elevated. The males are olivaceous above with a lustrous steel blue or bluish green area on the back from nape to dorsal or beyond, and have a series of poorly defined dark bars on the sides and a belly that is yellowish white to deep orange. The dorsal fin ocellus on posterior rays is lacking or developed as faint dusky spot.

1.4 The females are light olive, brown, brassy, or light orange above with 14 dark crossbars on the lower sides alternating with seven to eight crossbars on the back. The lower sides and belly are yellowish or white. The dorsal fin is olive or dusky and has one or two prominent ocelli on the posterior rays.

2. LIFE HISTORY

2.1 DISTRIBUTION AND GENERAL ECOLOGY

2.1.1 Sheepshead minnows occur in estuaries along the Atlantic and Gulf coasts (Figure 1). They are a schooling, euryhaline species that inhabit a variety of shallow water habitats, such as coves, bays, ponds, inlets, harbors, bayous, salt marshes, and along open beaches. In some cases, they may be very abundant where the bottom is partially sandy, emergent vegetation lacking, and little current or wave action are present. This species may establish populations in inland lakes containing relatively high concentrations of dissolved salts. They are tolerant of extreme changes in water temperatures, ranging from 0-40EC, and in salinities, ranging from 0.1-149‰ (Simpson and Grunter, 1956; Nordlie, 1987).

2.1.2 This omnivorous fish is an important component of the estuarine ecosystem serving as a link in transferring energy from lower trophic levels, detritus and benthic plants and animals, to carnivores in higher trophic levels (Hansen and Parrish, 1977). Sheepshead minnows serve as fo rage fish for commercially and recreationally valued fish species, such as the black drum (*Pogonias cromis*), red drum (*Sciaenops ocellata*), bluefish (*Pomatomus saltatrix*), spotted seatrout (*Cynoscion nebulosus*), striped bass (*Morone saxatilis*), and snook (*Centropomus undecimalis*) (Gunter, 1945; Darnell, 1958; Grant, 1962; Sekavec, 1974, and Carter et al., 1973).

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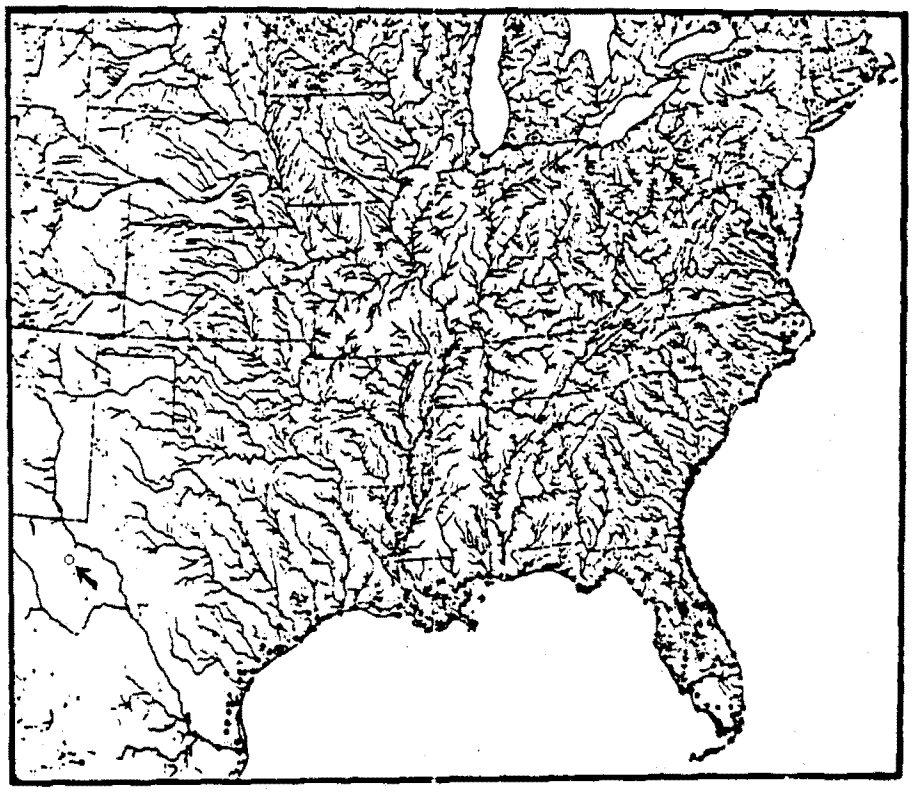

Figure 1. Map showing the distribution of the sheepshead minnow (*Cyprinodon variegatus*) in North America. Open circles represent transplanted populations (From Lee et al., 1980).

2.2 GENERAL SPAWNING BEHAVIOR

2.2.1 Sheepshead minnows (Figures 2, 3, 4) spawn at depths of 2.5-60 cm in shallow bays, tide pools, mangrove lagoons, and pools in shallow, gently flowing streams, and other similar habitats over bottoms of sand, black silt, or mud. Males occupy territories up to 0.3-0.6 m in diameter and may or may not construct nest pits. Spawning may take place out of both pit and territory. Besides temperature, Martin (1972) reported that sudden changes in salinity can initiates spawning activities. Eggs (Figure 2) are demersal, adhesive or semi-adhesive with very minute attachment filaments (threads) more or less evenly distributed over the chorion. They stick to a variety of substrates, such as plants, sand, rocks, logs, and to each other. Sometimes they stick to plants near the surface, and at other times become partially buried in the bottom. The yolk contains one very large and many minute oil globules. Adults spawn possibly throughout the year on the Gulf coast of the United States. Hansen and Parrish (1977) reported that in an estuary near Pensacola, Florida, spawning may occur during any month of the year. Ripe females are found April to October in North Carolina, throughout the summer in the Chesapeake Bay, May to August in Delaware Bay, May to September in New Jersey and New York, and June to mid-July in Massachusetts.

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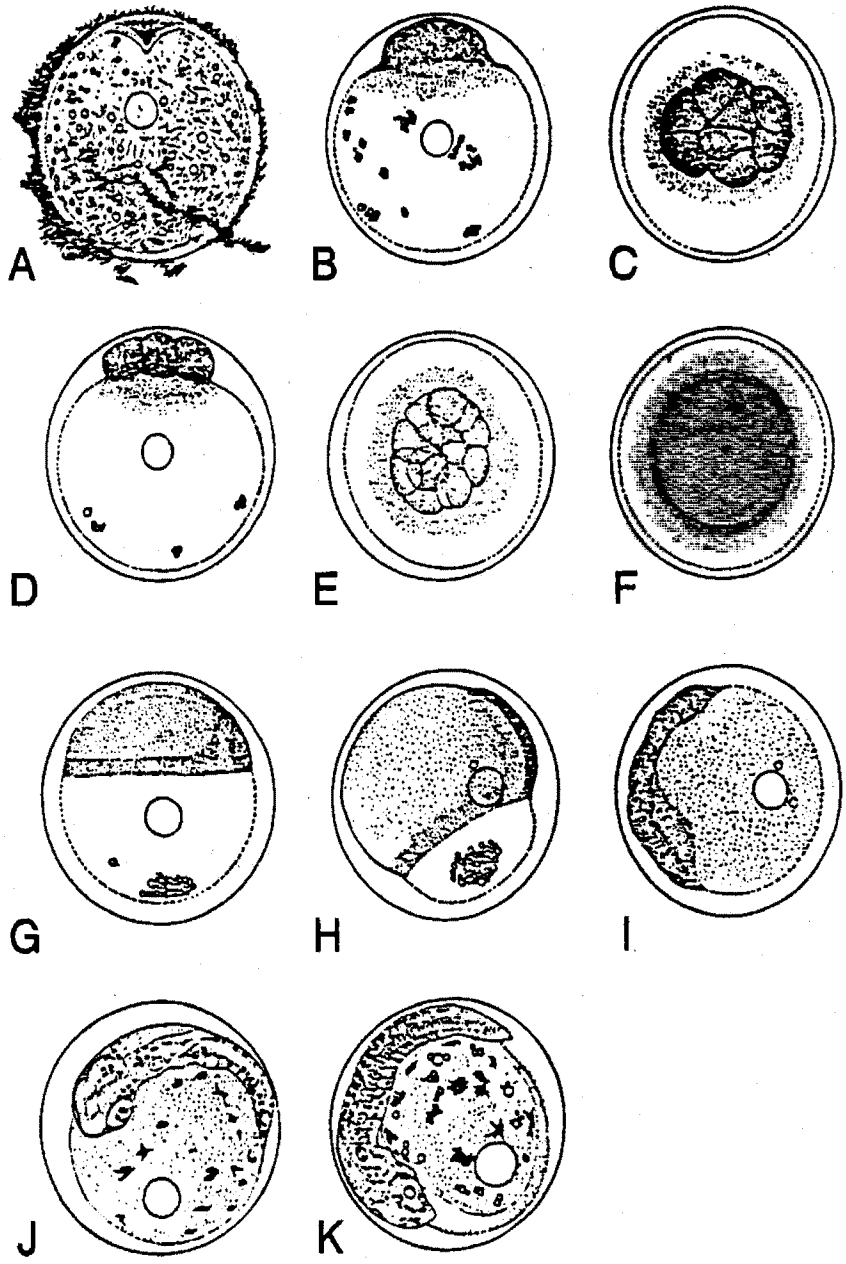

Figure 2. Sheepshead minnow (*Cyprinodon variegatus*). A. unfertilized egg; B. blastodisc stage; C-D. 8-cell stage; E. 16-cell stage; F. late cleavage; G. germ ring formed; H. blastoderm over 1/4 of yolk; I. early embryo; J. embryo 48-hours old; K. tail-free embryo. (From Kuntz, 1916).

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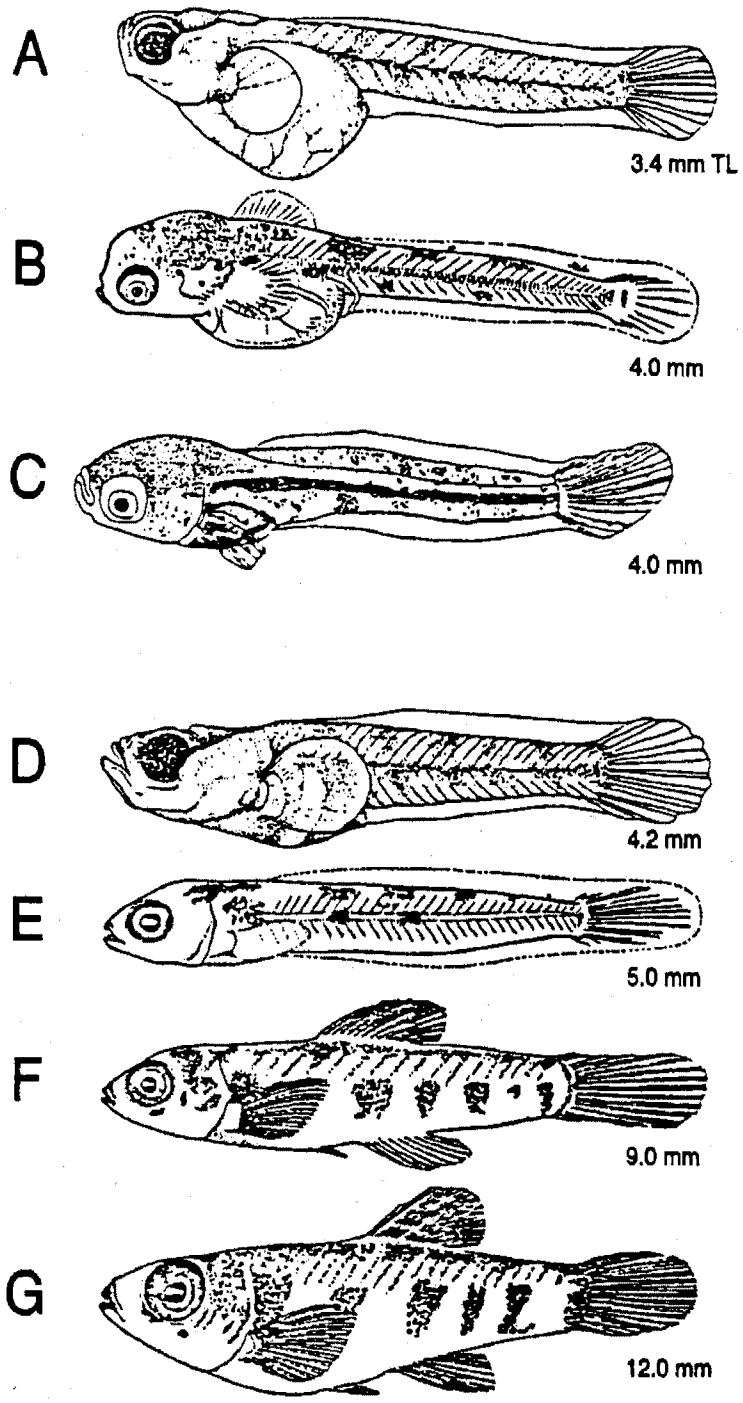

Figure 3. Sheepshead minnow (*Cyprinodon variegatus*). A-E. yolk-sac larvae; F. larvae; G. juvenile; (B-C, E-G, from Kuntz, 1916; A, D, from Foster, 1974).

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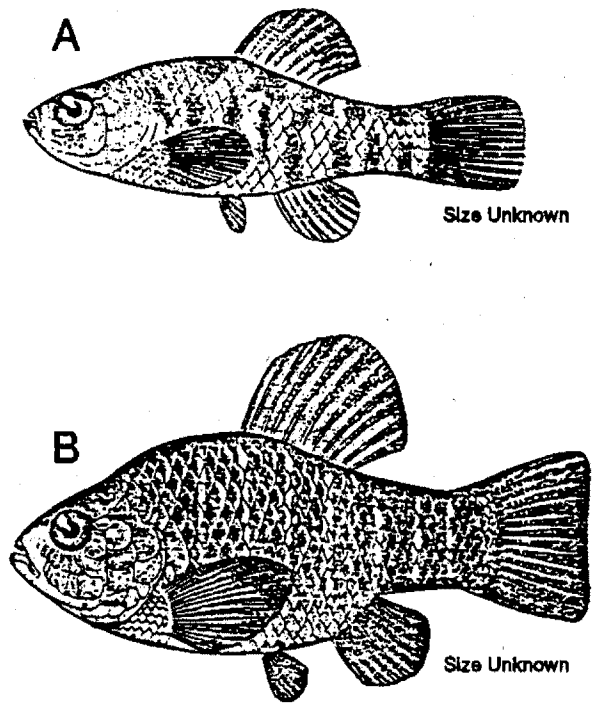

Figure 4. Sheepshead minnow (*Cyprinodon variegatus*). A. juvenile; B. adult (From Jordan and Evermann, 1896-1900).

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3. CULTURE METHODS AND FACILITIES

3.1 SOURCES OF ORGANISMS

3.1.1 Juvenile and adult sheepshead minnows (Figure 4) for use as brood stock spawners may be obtained from commercial biological supply houses or taken by seine in coastal estuaries of the Atlantic coast and Gulf of Mexico. They may also be obtained from young fish raised to maturity in the laboratory. Feral brood stock and first generation laboratory fish may be preferred, to minimize inbreeding. A continuous supply of wild stock, however, may be more cost effective. Neither fish nor eggs of feral stock should contain excessive contaminants nor exhibit excessive mortality, and the fish should demonstrate normal behavior. Before being used as a source of gametes, field-caught adults should be maintained and observed in the laboratory for at least one week to permit detection of disease and to allow time for acute mortality resulting from stress of capture. Injured or diseased fish should be discarded.

3.2 LABORATORY CULTURE FACILITIES

3.2.1 Sheepshead minnows can be cultured in a static, recirculated, or flow-through systems. Flow-through systems require large volumes of water and may not be feasible in some laboratories.

3.3 LABORATORY YEAR-ROUND SPAWNING

3.3.1 In the laboratory, adults may be kept in breeding condition year round. Females may spawn a number of times at intervals of one to seven days, and will generally produce an average of 10 to 30 eggs per spawning (USEPA, 1978a). To obtain large number of eggs at one particular time, adult fish of 27 mm standard length or greater should be used. If fish are taken in the field, they should be acclimated for at least one to two weeks in 20-30‰ salinity, a water temperature of 25-28EC, and a photoperiod of 16 h light and 8 h dark.

3.3.2 Sheepshead minnows can be continuously cultured in the laboratory from eggs to adults. The eggs (embryos), larvae, juveniles, and adults (Figures 2, 3, 4) should be kept in rearing and holding tanks of appropriate size and maintained at ambient laboratory temperature. The larvae should be fed sufficient newly-hatched *Artemia* nauplii daily to assure that live nauplii are always present. At the juvenile stage, they are fed frozen adult brine shrimp and a commercial flake food. Adult fish are fed flake food two or three times daily, supplemented with frozen adult brine shrimp.

3.3.3 Sheepshead minnows normally reach sexual maturity three to five months after hatching, and have an average standard length of approximately 27 mm for females and 34 mm for males, if held at a temperature of 25-30EC in rearing tanks of adequate size, and fed adequate ly. At this time, the males begin to exhibit sexual dimorphism and initiate territorial behavior. When the fish reach sexual maturity, and are to be used to obtain large number of embryos by natural spawning, the brood stock should be kept in a temperature controlled system at 18-20EC. To initiate spawning, the spawners are moved to spawning tanks with a temperature of 25EC. Adults can be maintained in natural or artificial seawater in a flow-through, static, or recirculating, aerated system consisting of an all-glass aquarium, or equivalent (see USEPA, 1985 and USEPA, 1987).

3.3.4 Static systems are equipped with an undergravel filter. Recirculating systems are equipped with an outside biological filter constructed in the laboratory using a reservoir system of crushed coral, crushed oyster shells, or dolomite and gravel, charcoal, floss, (see Spotte, 1973; 1979, Bower, 1983 for information on filters and conditioning the biological filter), or a commercially available cartridge filter or an equivalent system. The culture conditions should include seawater at 20-30‰, and a photoperiod of 16 h light and 8 h dark. Water temperature may be controlled or maintained at ambient laboratory levels.

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3.4 OBTAINING EGGS (EMBRYOS) FOR TOXICITY TESTS

3.4.1 Embryos can be shipped to the laboratory from an outside source or obtained from adults held in the laboratory. Ripe eggs can be obtained either by natural spawning or by intraperitoneal injection of the females with human chorionic gonadotrophin (HCG) hormone. If the culturing system for adults is temperature controlled, natural spawning can be induced to obtain large number of embryos by raising the temperature to 25 EC. Natural spawning is preferred because repeated spawning can be obtained from the same brood stock, whereas with hormone injection, the brood stock is sacrificed in obtaining gametes. It should be emphasized that the injection and hatching schedules given below are to be used only as guidelines. Response to the hormone varies with brood stock and temperature. Time-to-hatch and percent hatch also vary among stocks and among batches of embryos obtained from the same stock, and are dependent on temperature, DO, and salinity.

3.5 NATURAL SPAWNING

3.5.1 Adult fish should be maintained at 18-20EC in a temperature controlled system. The number of spawning chambers and fish to be spawned should be based on the requirements for providing sufficient numbers of viable embryos. As indicated above, an adult female in spawning condition will generally produce an average 10 to 30 eggs per spawn. To obtain embryos for a test, adult fish (generally, at least eight-to-ten females and three males) are transferred to a spawning chamber in a 57 L (15 gal) aquarium with the correct photoperiod and temperature (16 h light/8 h dark, and a temperature of 25EC), seven to eight days before the larval fish are needed. The spawning tank is fitted with a spawning chamber and an embryo collection tray. The spawning chamber consists of a basket of 3-5 mm NITEX® mesh, approximately 20 x 35 x 22 cm high (USEPA, 1978a), designed to fit into the aquarium. Spawning generally will begin within 24 h or less. The embryos will fall through the bottom of the spawning chamber and lightly adhere onto a collecting screen or tray placed on the bottom of the tank. The collecting tray should be checked for embryos the next morning. The number of eggs produced is highly variable. The number of spawning units required to provide the fish needed to perform a toxicity test (generally two to four) as determined by experience. If the collecting trays do not contain sufficient embryos after the first 24 h, discard the embryos, replace the tray, and collect the embryos for another 24 h. To help keep the embryos clean, the adults are fed while the screens are removed. Spawning fish should be shielded from excessive outside disturbance, e.g. an opaque curtain should surround the entire culture system. Care should also be taken so that outside light sources do not interfere with the photoperiod.

3.5.2 The embryos are collected in a tray placed on the bottom of the tank. The collecting trays are fabricated from plastic fluorescent light fixture diffusors (grids), with cells approximately 14 mm deep x 14 mm square. A screen consisting of 250-500 µm mesh is attached to one side (bottom) of the grid with silicone adhesive. The depth and small size of the grid protects the embryos from predation by the adult fish. The collecting trays with newly-spawned embryos are removed from the spawning tank, and the embryos are collected from the screens by washing them with a wash bottle or removing them gently with a fine brush. The embryos from several spawning units are generally pooled in a single container to provide a sufficient number to conduct the test(s). The embryos are transferred to a petri dish, or equivalent, filled with fresh culture water, and are examined using a dissecting microscope or other suitable magnifying device. Damaged and infertile eggs are discarded (see Figure 2). The embryos are then placed in incubation dishes (e.g. KIMAX® or PYREX® crystallizing dishes, Carolina culture dishes, or equivalent; see Subsection 3.8, Embryo Incubation and Hatching Facility). It is recommended that the embryos be obtained from fish cultured inhouse, rather than from outside sources, to eliminate the uncertainty of damage caused by shipping and handling that may not be observable, but which might affect the results of the test. After sufficient number embryos are collected for the test, the adult fish are returned to the (18-20EC) culture holding tanks.

3.6 SUSTAINED NATURAL EMBRYO PRODUCTION

3.6.1 Sustained (long-term), daily, embryo production can be achieved by maintaining mature fish (ratio of approximately 12-15 males to 50-60 females) in tanks, such as a 285-L LIVING STREAM® tank, or equivalent, at a temperature of 23-25EC. Embryos are collected seven or eight days prior to starting the acute or chronic toxicity

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tests for less than 24 hr or older larvae. Embryos are produced daily, and when needed, collecting trays are placed on the bottom of the tank. The next morning, the embryo collectors are removed, and the embryos are washed into a shallow glass culture dish using artificial seawater. Four collecting trays, each approximately 20 cm x 45 cm, will cover the bottom of a 285 L tank.

3.7 FORCED SPAWNING

3.7.1 Human chorionic gonadotrophin (HCG) is reconstituted with sterile saline or Ringer's solution immediately before use. The standard HCG vial contains 1,000 IU, which is reconstituted in 10 mL of saline. Freeze-dried HCG, which comes with premeasured and sterilized saline, is the easiest to use. The reconstituted HCG may be used for several weeks if kept in the refrigerator.

3.7.2 Each female is injected with HCG on two consecutive days. The HCG is injected into the peritoneal cavity, just below the skin, using the smallest needle possible. A 50 IU dose (0.5 mL of reconstituted hormone solution) is recommended for females approximately 27 mm in standard length. A larger or smaller dose may be used for fish which are significantly larger or smaller than 27 mm. It may be helpful if fish that are to be injected are maintained at 20EC before injection, and the temperature raised to 25EC on the day of the first injection. Injected females should be isolated from males.

3.7.3 With injections made on days one and two, females which are held at 25EC should be ready for stripping on Days 4, 5, or 6. Ripe females should show pronounced abdominal swelling, and release at least a few eggs in response to a gentle squeeze. Eggs are stripped from the ripe females and mixed with sperm derived from excised, macerated testes. At least ten females and five males are used per test to ensure that there is a sufficient number of viable embryos.

3.7.4 Prepare the testes immediately before stripping the eggs from the females. Remove the testes from three-to-five males. The testes are paired, dark-grey organs along the dorsal midline of the abdominal cavity. If the head of the male is cut off and pulled away from the rest of the fish, most of the internal organs can be pulled out of the body cavity, leaving the testes behind. The testes are placed in a few mL of seawater until the eggs are ready.

3.7.5 Strip the eggs from the females into a dish containing 50-100 mL of seawater, by firmly squeezing the abdomen. Sacrifice the females and remove the ovaries if all the ripe eggs do not flow out freely. Break up any clumps of ripe eggs and remove clumps of ovarian tissue and under-ripe eggs. Ripe eggs are spherical, approximately 1.0-1.7 mm in diameter, and almost clear. Place the testes in a fold of NITEX® screen (250-500 µm mesh), dampen with seawater, and macerate while holding over the dish containing the eggs. Rinse the testes with seawater to remove the sperm from the tissue, and wash the remaining sperm and testes into the dish with the eggs. Let the eggs and sperm stand together for 10-15 minutes, swirling occasionally.

3.7.6 Pour the contents of the dish into a crystallizing dish or equivalent and insert an airstone. Aerate gently, so that the water moves slowly over the eggs, and incubate at 25EC for 60-90 min. After this period of time, wash the fertilized eggs on a NITEX® screen, place them in clean seawater in an incubation chamber.

3.8 EMBRYO INCUBATION AND HATCHING FACILITY

3.8.1 Embryos are incubated in KIMAX® or PYREX® crystallizing dishes, Carolina culture dishes, or equivalent, at a temperature of 25EC and 14-h light/10-h dark photoperiod. An air stone is placed in each dish, and the contents are gently aerated for the duration of the incubation. The water in the incubation chambers is replaced daily. Approximately 24 h prior to hatching, the salinity of the seawater in the incubation chambers is changed to that of the test salinity, if different. The salinity must remain within the 20-30‰ range. The embryos should hatch in 6 to 7 days at 25EC, and in 4 to 5 days at 30EC.

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3.9 FEEDING AND STOCKING DENSITY

3.9.1 The sheepshead minnow cultures should be provided a sufficient amount of high quality nutrition without over-feeding. The adult and juvenile sheepshead minnows are fed, frozen adult brine shrimp and flake food, ad libitum, daily. The larvae are fed newly hatched *Artemia* nauplii and crushed flake food, ad libitum, daily. Methods for culturing brine shrimp are discussed in Appendix A.4. The stocking of adult fish in the holding tanks depends on the biological filter system (see Subsection 3.11, Biological Filters and Substrate Conditioning). A circular, 1.3 m (48 in.) diameter, 880 L (235 gal), fiberglass tank will hold approximately 30-50 adult fish with a varied sex ratio. A stocking density of about 300 larvae is suitable in a 76 L aquarium. Brood stock should be replaced with feral fish annually, or whenever the fecundity of the females diminishes, and they appear spent with age and from frequent breeding.

3.10 CULTURE TANKS

3.10.1 Larvae, juvenile, and adult fish should be kept in holding and rearing tanks of appropriate size. The tanks can be all-glass aquaria, fiberglass tanks, or equivalent. All tanks should have appropriate biological filtration systems, and the culture filtration system should be conditioned properly before adding the fish (see Spotte, 1973, 1979; Bower, 1983).

3.11 BIOLOGICAL FILTERS AND SUBSTRATE CONDITIONING

3.11.1 Holding and rearing aquaria and tanks can accommodate as many fish as its biological filter will permit. The substrate conditioning for the undergravel or outside filters is also important to the life and health of the fish. Substrate conditioning is the process to develop nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) that can convert ammonia and nitrite to nitrate. A conditioned filter bed is defined as one in which the capacity for ammonia and nitrite oxidation is sufficient to keep pace with the production of ammonia by the fish. Consult Spotte (1973; 1979) or Bower (1983) for a thorough understanding of the biological filter and conditioning process.

3.12 CULTURE WATER

3.12.1 Artificial seawater is prepared by dissolving FORTY-FATHOMS® or equivalent artificial sea salts in deionized water to a salinity of 20-30‰. Synthetic sea salts are packaged in plastic bags and mixed with deionized (MILLI-Q® or equivalent) water. The instructions on the package of sea salts should be followed carefully, and the salts should be mixed in a separate container, and not in the culture tank. The deionized water used in hydration should be in the temperature range of 21-26EC. Seawater made from artificial sea salts is conditioned (see Spotte, 1973, 1979; Bower, 1983) before it is used for culturing by aerating mildly for at least 24 h.

3.12.2 Adequate aeration will bring the pH and concentration of dissolved oxygen and other gases into equilibrium. The concentration of dissolved oxygen in the water supply should be 90-100% saturation before it is used. If a residue or precipitate is present, the solution should be filtered before use. The seawater should be monitored periodically to insure a constant salinity.

3.13 CULTURE CONDITIONS

3.13.1 Holding and rearing tanks and any area used for manipulating live sheepshead minnows should be located in a room or space separated from that in which toxicity test(s) are to be conducted. The salinity of the culture systems should be between 20 and 30‰. Water temperature for the brood stock should be maintained at 18-20EC. A photoperiod of 14 h illumination (10-20 $\mu E/m^2/s$, or 50-100 ft-c) and 10 h dark, should be provided. The holding and rearing tanks should be aerated so that the DO is not less than 1.0 ppm below saturation at any given temperature, with 5.0 ppm (60% saturation) being the absolute lowest limit.

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3.14 CULTURE MAINTENANCE

3.14.1 Replace approximately 10% of the culture water every two weeks, or 25% monthly. The culture water should be clear. If the water appears cloudy or discolored, replace at least 50% of it. Replacement water should be well oxygenated and at the same temperature and salinity as the culture water. Salinity is maintained at the proper level by adding deionized water to compensate for evaporation. A replenisher, made of the trace elements, iodine (KI) and bromine (KBr), is added (1 mL/400 L) to the culture water each week, or commercial trace elements replenisher should be used as directed by the artificial sea salt manufacturer.

3.14.2 To avoid excessive build up of algal growth, periodically scrape the walls of culture system. Some of the algae will serve as a supplement to the diet of the fish. A partial activated carbon "charcoal" change in the filtration systems should be done monthly or as needed. The detritus (dead brine shrimp nauplii and cysts, adult brine shrimp, other organic material accumulation) should be siphoned from the bottom of rearing and holding aquaria or tanks each week or as needed.

3.15 WATER QUALITY MONITORING

3.15.1 Checking the chemistry of the sea water is critical to the success of the marine culture system. The water quality will determine whether the life support processes in the filter bed work at reasonable and steady rates. The culture water is checked routinely for temperature, alkalinity, pH, DO, total ammonia, nitrite, and nitrate. More frequent monitoring of these parameters is recommended during periods of organism procurement and starting new culture systems with inside underground filters and outside-of-tank biological filtration. The DO should be maintained at greater than 60% saturation. The pH should not go below 7.5 with an acceptable range between 7.5 and 8.3. Low pH levels can result from overcrowding, overfeeding, or waste accumulation, especially in static or recirculating culture systems.

3.15.2 Acceptable pH levels can be re-established by siphoning off 50-75% of the water and replacing it with conditioned artificial seawater of the same temperature. Also, sodium bicarbonate or commercially available liquid buffers can be added to the tanks whenever the pH falls below 7.5. Un-ionized ammonia, total (NH_3 + NH_4), and nitrite ion (NO_2) levels should not exceed 0.1 ppm in the holding tanks. It is recommended that the ammonia and nitrite concentrations be determined prior to starting new culture systems. It is recommended that nitrate (NO_3) concentrations be determined prior to starting new culture systems, and the nitrate ion concentrations should not exceed 20 mg/L.

3.15.3 A specific schedule for water quality monitoring should be established for each culture system. All water quality measurements and data are recorded in the culture and environmental conditions log books.

3.16 DISEASE CONTROL AND TREATMENT

3.16.1 Discussions of identification and treatment of common parasites of marine fish culturing can be found in Spotte (1973), Sindermann (1970), and Bower (1983). Several commercial companies sell various kinds of medication to treat common parasites of marine fish.

3.16.2 A colorless medication, FORMALITE II®, has been used successfully for the treatment of the protozoan parasites, *Chilodonella*, *Costia*, *Trichoina*, *Scyphidia*, *Trichophrya*, and *Ichyophirius*.

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4. TEST ORGANISMS

4.1 Sheepshead minnows 1-14 days old are used in the acute toxicity test. If the larvae are used one or two days after hatching, they can be held in the crystallizing or culture dishes. If they are to be used later, they should be placed in larger holding aquarium or tanks. Prior to beginning the test, the larvae can be transferred to small beakers or plastic cups, using a large-bore, fire-polished glass tube (6 mm to 9 mm I.D. x 30 cm long) equipped with a rubber bulb.

4.2 If the larvae are to be moved to holding aquaria, a large-bore, fire-polished glass tube should also be used to move them. It is important to note that larvae and fry should not be handled with a dip net. Dipping larvae and fry with a net can result in very high mortality. Some of the water in the holding aquarium or tank containing the larvae should be siphoned off before they are transferred using the large-bore tube. This should make them easier to catch. The same large-bore, fire-polished glass tube discussed above should be used to gently transfer the fish from the holding vessels to the test vessels. As the fish are counted, they can be transferred to small plastic cups before they are added to the test vessels. It is more convenient to first transfer five fish to each of several small beakers or plastic containers with a few mL of 20-30‰ saline dilution water. The appropriate number of fish (multiples of five) can then be added to the test vessels.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.8 SILVERSIDES: INLAND SILVERSIDE *(MENIDIA BERYLLINA)* ATLANTIC SILVERSIDE (M. MENIDIA), AND TIDEWATER SILVERSIDE (M. PENINSULAE)

1. MORPHOLOGY AND TAXONOMY

1.1 Adult Atlantic silversides attain a total length of up to 117 mm (Figure 1A and 1B). Females in general are slightly larger than males. The first dorsal fin has three to seven, usually four or five spines. The second dorsal fin has one spine and eight or nine rays; the anal fin has one spine and 19 to 29, usually 21 to 26, rays; and the pectoral fin has 12 to 16, usually 14 or 15, rays (Robbins, 1969). Atlantic silverside embryos are easily distinguished from those of the closely related inland silverside, *Menidia beryllina*. The former have a bundle of elastic filaments attached to the chorion at one small area of insertion (Figure 1C and 1D). These filaments, typically longer than the diameter of the egg, are all the same diameter. In contrast, inland silverside eggs posses one or two thick, elongated filaments, up to 50 mm long and four to nine shorter, thinner filaments (Figures 1E and 1F).

2. GENERAL LIFE HISTORY

2.1 DISTRIBUTION

2.1.1 Silversides occur in estuaries along the Atlantic, Gulf, and Pacific coasts (Figures 2-4). The Atlantic silverside, *Menidia menidia*, is a resident of estuaries from Maine to northern Florida. It occurs at intermediate to high salinities, typically of 12 to 30 parts per thousand (ppt), and remains in Atlantic estuaries throughout most of the year (De Sylva et al., 1962; Dahlberg, 1972). Recent evidence indicates an offshore migration at northern latitudes in the fall and reappearance of adults in estuaries in late spring (Conover and Kynard, 1984). This species is an important component in estuarine ecosystems, serving as forage fish for commercially and recreationally valued species such as striped bass, bluefish and spotted seatrout (Merriman, 1941; Bayliff, 1950; Middaugh, 1981).

2.1.2 Although the culturing methods described in this section were written primarily for *Menidia menidia*, they are also suitable for the inland silverside, *M. beryllina*, and the tidewater silverside, *M. peninsulae* (USEPA, 1987). The staff of the Environmental Research Laboratory, Gulf Breeze, Florida, have developed procedures for spawning, culturing, and testing of other fishes, including the California grunion, *Leuresthes tenuis*, and the topsmelt, *Atherinops affinis*. The availability of these fishes as test organisms will permit the use of indigenous fish in toxicity tests of wastes discharged along the entire coast line of the contiguous United States and Alaska.

2.2 SPAWNING BEHAVIOR

2.2.1 The Atlantic silverside spawns during spring and summer. Spawning runs generally occur during April - June or July at northern latitudes, and March through July or August at southern latitudes (Bayliff, 1950; Hildebrand and Schroeder, 1928; Middaugh and Lempesis, 1976). Spawning occurs in the upper intertidal zone during daytime high tides (Middaugh, 1981). Eggs are deposited on a variety of substrates which provide protection from thermal stress and desiccation (Middaugh et al., 1981; Conover and Kynard, 1981). Females typically release 200 to 800 eggs, 1.0-1.2 mm diameter, as they spawn. Individuals may spawn up to five or six times, at two week intervals, during the reproductive season. The life span is generally 12-15 months, although year class-2 fish are occasionally found (Beck, 1979).

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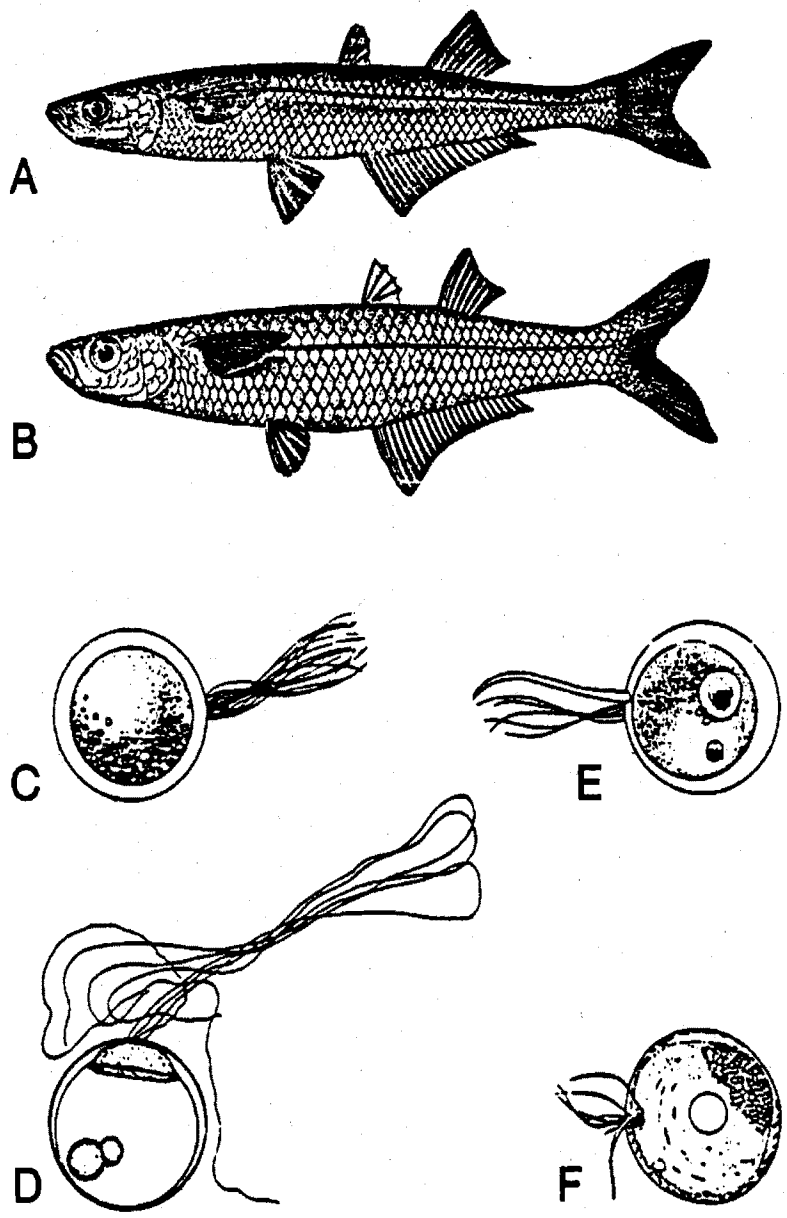

Figure 1. Silverside (*Menidia*): A-D, *M. menidia*, (Atlantic silverside); A, adult; ca. 95 mm SL (Massachusetts); B, adult, ca 102 mm SL (Florida); C, unfertilized egg (diagrammatic); D. developing embryo (note that filaments are all equal in diameter); E-F, *M. beryllina* (inland silverside); E, unfertilized egg (diagrammatic); F, developing embryo (note one thick filament and several thin filaments). (A, B from Kendall, 1902; C from Wang, 1974; D from Ryder, 1883; E from Wang, 1974; F from Hildebrand, 1922.)

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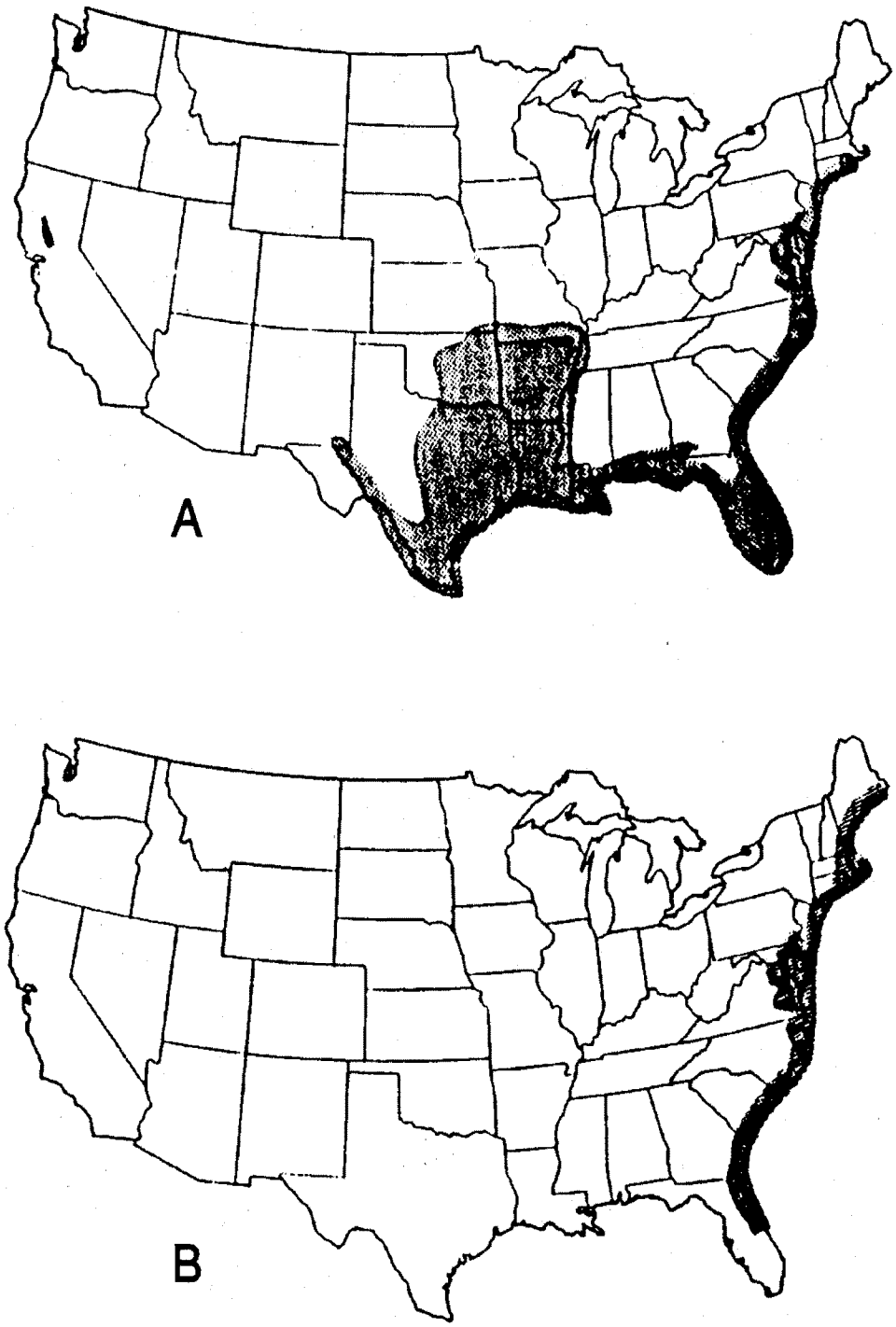

Figure 2. Biographical Distribution: A, inland silverside, *Menidia beryllina*; B, Atlantic silverside, *M. menidia*. (From USEPA, 1987).

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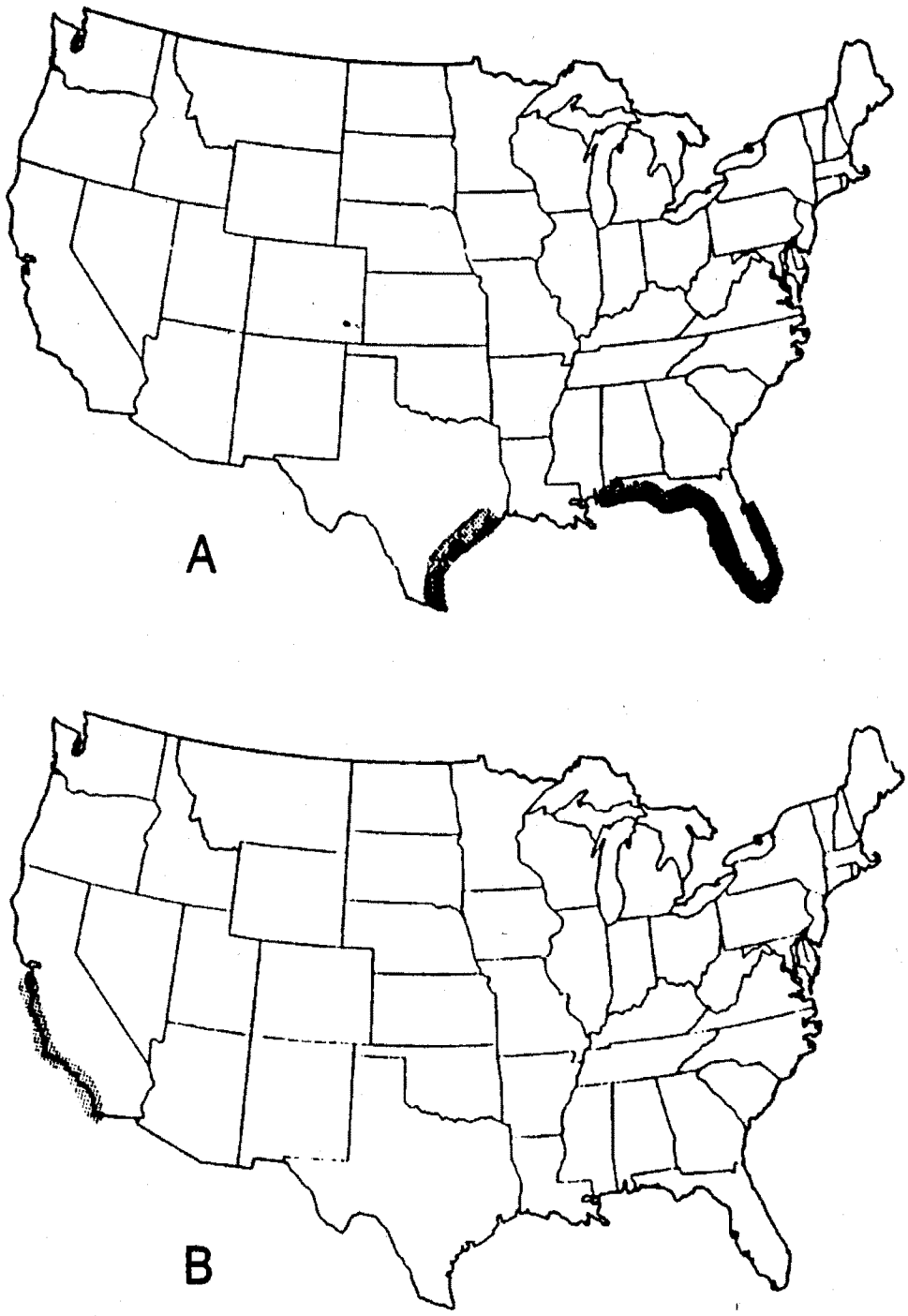

Figure 3. Biogeographical Distribution: A, tidewater silverside, *Menidia peninsulae*; B, California grunion, *Leuresthes tenuis*. (From USEPA, 1987).

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Figure 4. Biogeographical Distribution: Topsmelt, *Atherinops affinis*. (From USEPA, 1987).

3. CULTURING METHODS

3.1 SOURCES OF ORGANISMS

3.1.1 *Menidia* may be obtained from commercial biological supply houses or collected in the field.

3.1.1.1 The optimal time for collecting ripe *M. menidia* in the field is just prior to daytime high tides between 8:00 AM and Noon (usually one to four days after the occurrence of a new or full moon), when prespawning schools move into the upper intertidal zone (Middaugh, 1981; Middaugh et al., 1981). Since the Atlantic silverside prefers relatively high salinities, it is recommended that collections be made in areas with salinities of 20‰ or greater. Sandy beaches, bordering open but protected estuarine bays, are suitable for collecting adults. A 1 x 10 m bag seine with knotless 5 mm mesh is ideal for collecting. Since Atlantic silversides typically reside in shallow water, 1.5 m deep, they are easily captured by seining close to shore. It is important to avoid total beaching of the bag seine when collecting *M. menidia*. These fragile fish will quickly die if removed from water and, more importantly, ripe females often abort their eggs if stranded. Ideally, the bag portion of the seine, containing captured adults, should remain in water 5-15 cm deep (Middaugh and Lempesis, 1976).

3.1.1.2 It is possible to transport the spawn (fertilized eggs) or adults to the laboratory. The following procedure is recommended for stripping, fertilizing and transporting eggs from the field to the laboratory:

1. Immediately after seining (while still on the beach) three to five ripe females should be dipped into a bucket of seawater to remove sand and detritus.

2. Eggs are stripped into a glass culture dish containing seawater or onto a nylon screen (0.45-1.0 mm mesh) (Figure 5), which is then gently lowered into a culture dish of seawater with the eggs on the upper surface of the screen (Barkman and Beck, 1976). If excessive pressure is

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required to strip the eggs, the female should be discarded. Mature eggs, 1.0-1.2 mm in diameter, are clear, and have an amber hue.

3. Milt from several males can then be stripped into the culture dish and mixed with the eggs by gently tilting the dish from side to side. Upon contact with seawater, adhesive threads on mature eggs uncoil, making enumeration and separation difficult. If eggs are stripped directly into the culture dish, one end of a nylon string may be dipped into the dish and gently rolled so the embryos adhere (Middaugh and Lempesis, 1976). The Barkman and Beck (1976) technique for attaching the eggs to nylon screening minimizes the natural clumping tendency due to entanglement of the filaments on M. menidia eggs.

4. Strings of embryos or embryos on screens may be transported to the laboratory by placing them in an insulated glass container filled with seawater at the approximate temperature and salinity of fertilization. If gravid fish are transported to the laboratory for subsequent spawning, care must be taken to avoid overcrowding of fish in transport containers. Continuous, vigorous aeration is required and any increase in container water temperature should be minimized (Beck, 1979). A mass culture system for incubating the screen-adhered eggs and collecting the hatched larvae in a flowing seawater system (Figure 5) was described in detail by Beck (1979). A similar procedure utilizing a recirculating system was described by Middaugh and Lempesis (1976).

3.2 Laboratory Year-round Spawning

3.2.1 Atlantic, inland, and tidewater silversides may be spawned in the laboratory on a year-round basis. Procedures described by Middaugh and Takita (1983), and Middaugh and Hemmer (1984), provide for maintenance of a brood stock of 30 to 50 fish, sex ratio 1:1, in 1.3 m diameter, circular holding tanks which are part of a recirculating seawater system (Figure 6). The photoperiod should be adjusted to 14 L:10 D (lights on at 5:00 AM and off at 7:00 PM, intensity 10-20 $\mu E/m^2/s$, or 50-100 ft c), with the water temperature maintained at 18-20EC for fish from northern latitudes, and 20-25EC for southern latitudes. Suitable salinities for the culture units would be 25-30 ppt for the Atlantic and tidewater silversides, and 7‰ for the inland silverside. Fish are fed 8 g Tetramin® each morning and afternoon, and concentrated *Artemia* nauplii (hatch obtained from approximately 15 mL of eggs after 48 h of incubation at 25EC) in mid-afternoon (see section on *Artemia* culture). Excess food should be siphoned from the holding tanks weekly. Filter media (activated charcoal) located in a reservoir tray should be changed weekly, immediately after cleaning the holding tanks. To induce spawning by the Atlantic silverside, the circulation current velocity in the holding tanks should be reduced to zero (from 8 to 0 cm/sec) twice daily by turning off the seawater circulation pump from midnight to 1:00 AM, and from Noon to 1:00 PM. Atlantic silversides will spawn in response to interrupted current velocities during daytime (Noon to 1:00 PM). Spawning of the tidewater silverside also is enhanced by reducing the current velocity twice daily, but spawns primarily during nighttime. No interruption in current is necessary to enhance spawning by the inland silverside.

3.2.2 A suitable spawning substrate can be made by cutting enough 25 cm lengths of No. 18 nylon string to form a small bundle, and tying a string around the middle of the bundle to form a "mop." The mop is suspended just below the surface of the water, in contact with the side of the holding tanks. Spawning fish will deposit eggs on this substrate. The mops are removed from the holding tanks daily and suspended in incubation vessels. Typical egg production ranges from 300 to 1200 per spawn. Fish generally can be expected to spawn three to four days each week.

3.2.3 It is essential that light-tight curtains surround the holding tanks. These curtains should remain closed except during periodic feedings, tanks cleaning, and during removal and replacement of spawning substrates.

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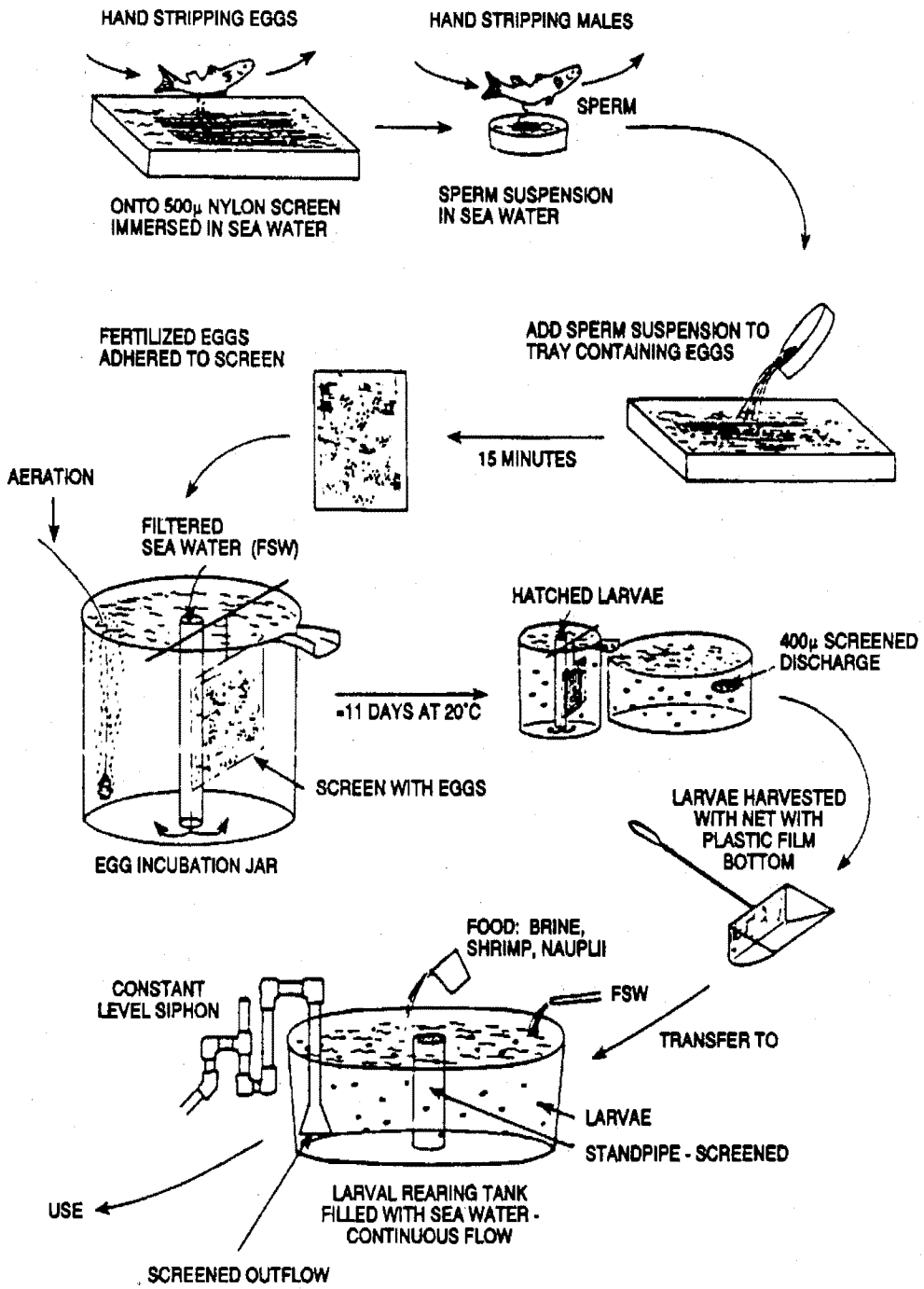

Figure 5. Techniques for collection of silverside eggs in the field, and production of larvae in the laboratory (From Beck, 1979).

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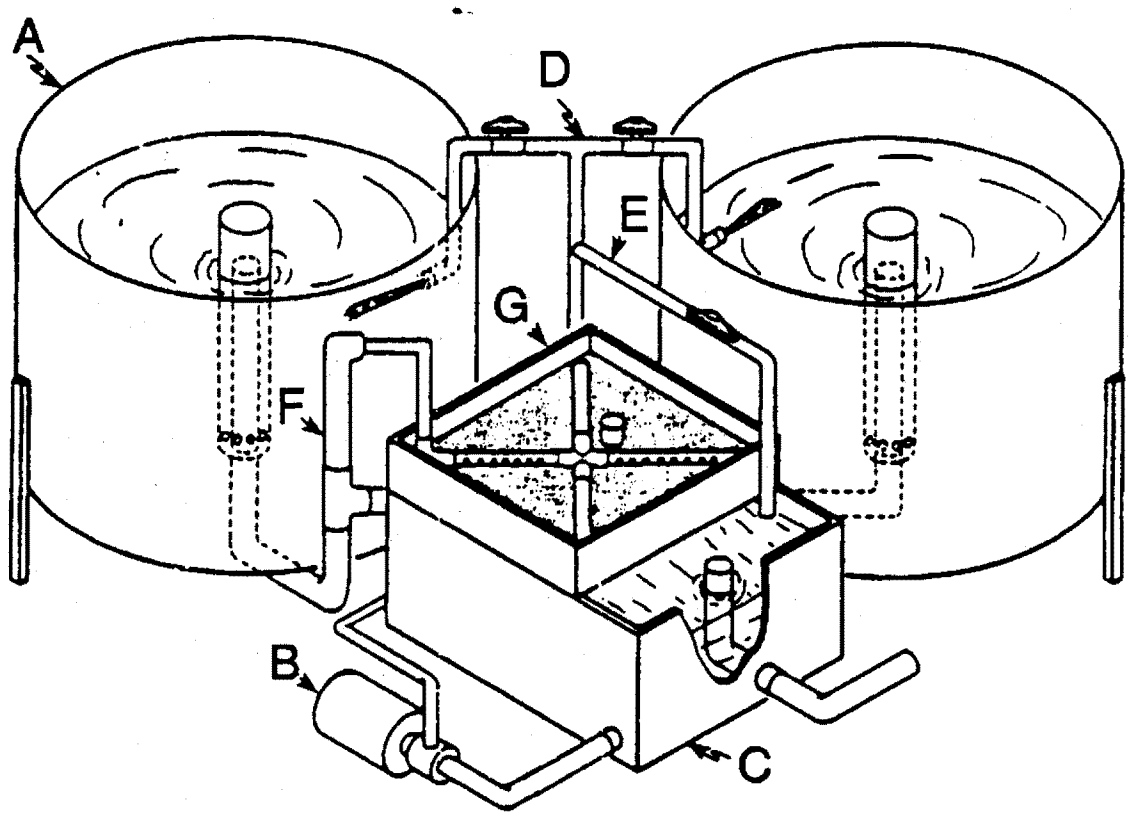

Figure 6. Holding and spawning system utilized in the culture of silversides (*Menidia*). A, 1.3 m diameter tanks; B, circulation pump; C, reservoir; D, seawater distribution system; E, by-pass line; F, seawater return line; and G, reservoir filter system. (From Middaugh and Hemmer, 1984).

3.2.4 Embryos attached to nylon screening or nylon string may be suspended in a culture system such as shown in Figure 6. The culture chambers for embryos should be constructed of glass. Upon hatching, larvae may be transferred from the collection container to a 90 cm diameter glass or fiberglass tank with a volume of 350 L. Tanks receive a continuous flow of seawater at 2 L/min. Water is introduced at the tank periphery causing a gentle current sufficient to induce orientation to water movement and normal schooling behavior. Water is discharged from the tank by two automatic siphons. Siphon openings are protected by a 400 μm nylon screen to prevent escape of larvae. An inverted funnel is used at the siphon to decrease the velocity of discharge water, thus preventing impingement of larvae.

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3.2.5 Embryos can also be incubated in small (4-10 L) glass aquaria, by placing the nylon screening or strings just below the surface of the water. Gentle aeration should be provided by an airstone positioned near the bottom of the holding aquaria.

3.3 CULTURE MEDIA

3.3.1 Use natural seawater if it is available and unpolluted. Otherwise use synthetic seawater prepared by adding artificial marine salts, such as FORTY FATHOMS®, to deionized water. If synthetic seawater is used, it should be aged for a least one week before being utilized in culture aquaria.

3.4 CULTURE CONDITIONS

3.4.1 The salinity maintained during incubation should be similar to that of the water from which the adults were taken, if collected in the field, or at which the adults are being maintained in the laboratory, if the embryos originate from laboratory brood stock. Water temperature should be maintained at 20 to 25EC depending upon the latitude where fish are collected. Provide a photoperiod of 12-14 h of illumination daily at 10-20 $\mu E/m^2/s$, or 50-100 ft-c (12 h minimum light/24 h). Embryos will hatch in seven to 14 days, depending upon the incubation temperature and salinity (Middaugh and Lempesis, 1976).

3.5 FEEDING AND STOCKING DENSITY

3.5.1 Upon hatching, *Menidia* larvae should be fed immediately. Newly hatched brine shrimp (*Artemia*) nauplii (less than eight hours old) are fed to the larvae twice daily. It is essential to feed *M. menidia* and *M. peninsulae* larvae newly-hatched brine shrimp nauplii (USEPA, 1987). Utilization of older, larger, brine shrimp nauplii will result in starvation of the larvae since they are unable to ingest the larger food organisms. Three to four days after hatching, the fish are able to consume older (larger) brine shrimp nauplii. Because of their small size *M. beryllina* larvae must be fed a mixohaline rotifer, *Branchionus plicatilus* from day of hatch through day five. Thereafter, they are able to consume newly-hatched and older *Artemia* nauplii (USEPA, 1987; USEPA 1988). Methods for culturing brine shrimp are discussed in the Appendix. A stocking density of about 300 larvae is suitable in an 76 L aquarium.

3.6 CULTURE MAINTENANCE

3.6.1 To avoid excessive build up of algal growths, periodically scrape the walls of aquaria. Activated charcoal in the aquarium filtration systems should be changed weekly and detritus (dead brine shrimp nauplii or cysts) siphoned from the bottom of holding aquaria each week. Salinity may be maintained at the proper level by addition of distilled or deionized water to compensate for evaporation.

4. **TEST ORGANISMS**

4.1 Fish one to 14 days old are used in acute toxicity tests. Most of the water in the holding aquarium should be siphoned off before removal of larvae. Larvae can then be siphoned from the holding tanks into a holding vessel. It is essential that larvae not be handled with a dip net, because it will result in very high mortality. A large-bore, fire-polished glass tube, 6 mm I.D. x 500 mm long (1/4 in. ID x 18 in. long), equipped with a rubber squeeze bulb should be used to transfer the larvae from the holding vessel to the test vessels. It is more convenient to first transfer five fish to each of several small beakers containing 20 mL of saline dilution water. The appropriate number of fish (multiples of five) can then be added to test vessels.

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APPENDIX B

SUPPLEMENTAL LIST OF ACUTE TOXICITY TEST SPECIES

TEST ORGANISM		TEST TEMP (EC)	LIFE STAGE
FRESHWATER SPECIES: VERTEBRATES - WARMWATER			
Cyprinella leedsi[1]	Bannerfin shiner	25	1-14 days
Lepomis macrochirus	Bluegill sunfish	20,25	" "
Ictalurus punctatus	Channel catfish	"	" "
FRESHWATER SPECIES: INVERTEBRATES - COLDWATER			
Pteronarcys spp.	Stoneflies*	12	larvae
Pacifastacus leniusculus	Crayfish*	"	juveniles
Baetis spp.	Mayflies*	"	nymphs
Ephemerella spp.	"	"	"
FRESHWATER SPECIES: INVERTEBRATES - WARMWATER			
Hyalella spp.	Amphipods	20,25	juveniles
Gammarus lacustris	"	"	"
G. fasciatus	"	"	"
G. pseudolimnaeus	"	"	"
Hexagenia limbata	Mayflies	"	nymphs
H. bilineata	"	"	"
Chironomus spp.	Midges	"	larvae

*Stoneflies, crayfish, and mayflies may have to be field collected and acclimated for a period of time to ensure the health of the organisms and that stress from collection is past. Species identification must be verified.

[1] Test conditions for *Cyprinella leedsi* and *Holmesimysis costata* are found in Table 14 and Table 19, respectively, in Section 9.

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SUPPLEMENTAL LIST OF ACUTE TOXICITY TEST SPECIES (CONTINUED)

TEST ORGANISM		TEST TEMP (EC)	SALINITY (‰)	LIFE STAGE
MARINE AND ESTUARINE SPECIES: VERTEBRATES - COLDWATER				
Parophrys vetulus	English sole	12	32-34	1-90 days
Citharichys sitigmaeus	Sanddab	"	"	" "
Pseudopleuronectes americanus	Winter flounder	"	"	post metamorphosis
MARINE AND ESTUARINE SPECIES: VERTEBRATES - WARMWATER				
Paralichthys dentatus	Flounder	20,25	32-34	1-90 days
P. lethostigma	"	"	"	" "
Fundulus simillis	Killifish	"	20-32	1-30 days
Fundulus heteroclitus	Mummichog	"	25-32	" "
Lagodon rhomboides	Pinfish	"	20-32	1-90 days
Orthipristis chrysoptera	Pigfish	"	15-30	" "
Leostomus xanthurus	Spot	"	10-30	" "
Gasterosteus aculeatus	Threespine stickleback	"	20-32	1-30 days
Atherinops affinis	Topsmelt	21	10-30	7-15 days
MARINE AND ESTUARINE SPECIES: INVERTEBRATES - COLDWATER				
Pandalus jordani	Oceanic shrimp	12	25-32	juvenile
Strongylocentrotus droebachiensis	Green sea urchin	"	32-34	gametes/embryo
Strongylocentrotus purpuratus	Purple sea urchin	"	"	" "
Dendraster excentricus	Sand dollar	"	"	" "
Cancer magister	Dungeness crab	"	"	juvenile
Holmesimysis costata[2]	Mysid	"	"	1-5 days
MARINE AND ESTUARINE SPECIES: INVERTEBRATES - WARMWATER				
Callinectes sapidus	Blue crab	20,25	10-30	juvenile
Palaemonetes pugio	Grass shrimp	"	10-32	1-10 days
P. vulgaris	" "	"	"	" "
P. intermedius	" "	"	"	" "
Penaeus setiferus	White shrimp	"	20-32	post-larval
Penaeus duorarum	Pink shrimp	"	"	" "
Penaeus aztecus	Brown shrimp	"	"	" "
Crangon septemspinosa	Sand shrimp	"	25-32	" "
Mysidopsis almyra	Mysid	"	10-32	1-5 days
Neomysis americana	"	"	"	" "
Metamysidopsis elongata	"	"	"	" "
Crassostrea virginica	American oyster	"	20-32	embryo
Crassostrea gigas	Pacific oyster	"	25-32	"
Arbacia punctulata	Purple sea urchin	"	32-34	gametes/embryo

[2] Test conditions for *Holmesimysis costata* are found in Table 19.

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APPENDIX C

DILUTOR SYSTEMS

Two proportional dilutor systems are illustrated: the solenoid valve system, and the vacuum siphon system.

1. Solenoid and Vacuum Siphon Dilutor Systems

The designs of the solenoid and vacuum siphon dilutor systems incorporate features from devices developed by many other Federal and state programs, and have been shown to be very versatile for on-site bioassays in mobile laboratories, as well as in fixed (central) laboratories. The Solenoid Valve system is fully controlled by solenoids (Figures 1, 2, and 3), and is preferred over the vacuum siphon system. The Vacuum Siphon system (Figures 1, 4, and 5), however, is acceptable. The dilution water, effluent, and pre-mixing chambers for both systems are illustrated in Figures 6, 7, and 8. Both systems employ the same control panel (Figure 9).

If in the range-finding test, the LC50 of the effluent falls in the concentration range, 6.25-100%, pre-mixing is not required. The pre-mixing chamber is bypassed by running a TYGON® tube directly from the effluent in-flow pipe to chamber E-2 (see Figures 3 and 5), and Chambers E-l and D-1 and the pre-mixing chamber are deactivated.

The dilutor systems described here can also be used to conduct tests of the toxicity of pure compounds by equipping the control panel with an auxiliary power receptacle to operate a metering pump to deliver an aliquot of the stock solution of the pure compound directly to the mixing chamber during each cycle. In this case, chamber E-l is de-activated and chamber D-1 is calibrated to deliver a volume of 2000 mL, which is used to dilute the aliquot to the highest concentration used in the toxicity test.

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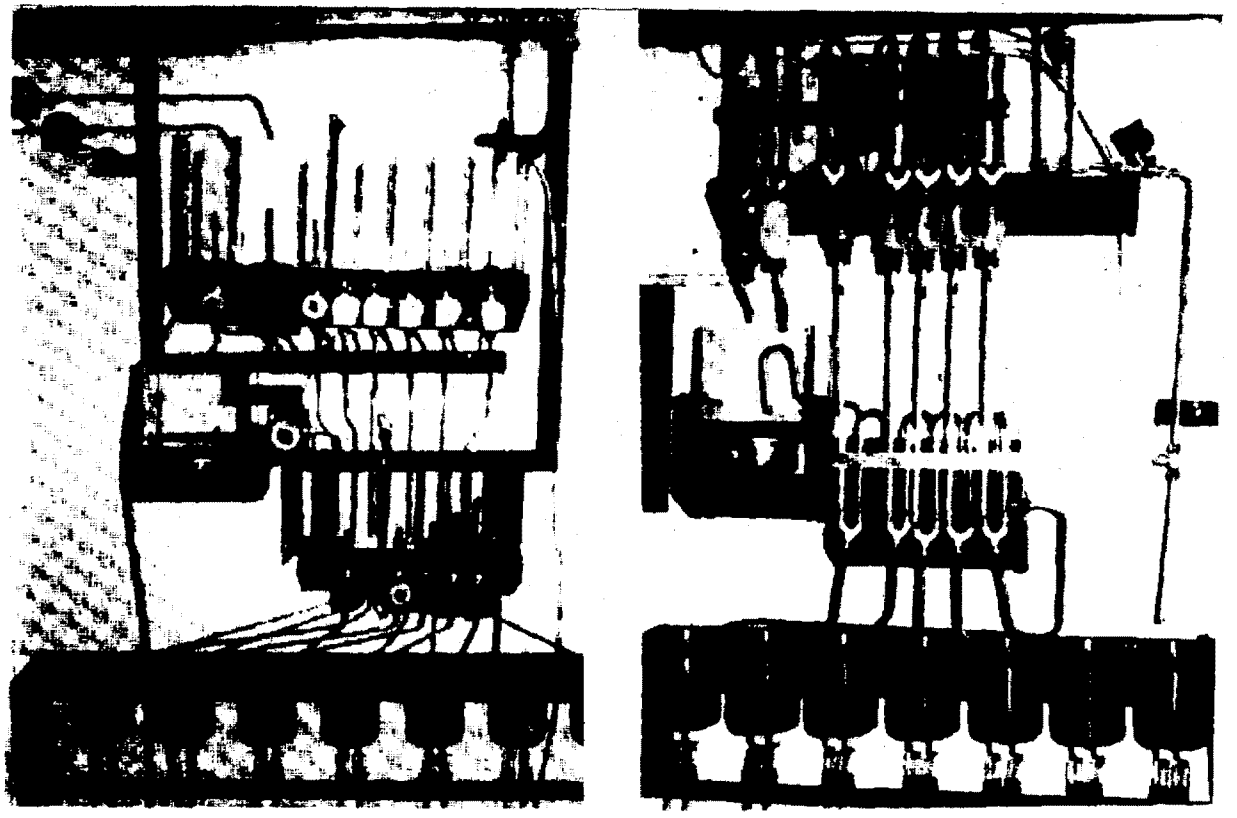

Figure 1. Photographs of the solenoid valve system (left), and the vacuum siphon system (right).

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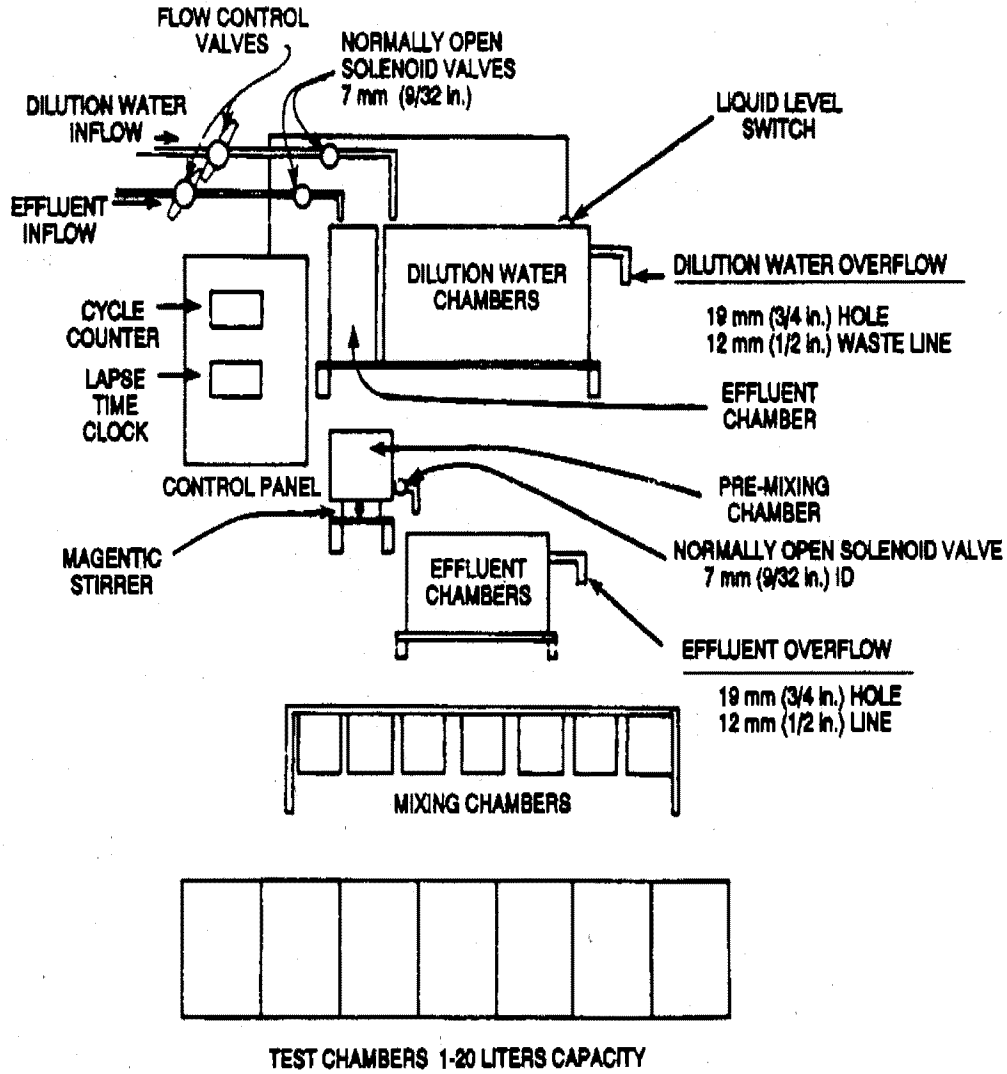

Figure 2. Solenoid valve dilutor system, general diagram (not to scale).

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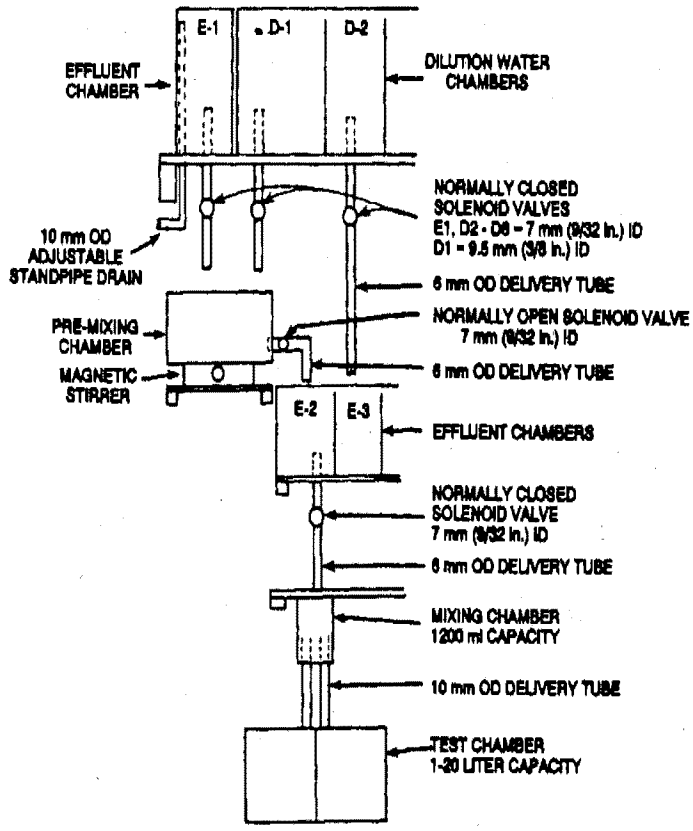

NOTE: WHEN 100% EFFLUENT IS USED AS THE HIGHEST EFFLUENT CONCENTRATION, E-1, D-1, AND THE PRE-MIXING CHAMBER ARE BYPASSED BY CONNECTING A TYGON TUBE TO THE EFFLUENT INFLOW, AND RUNNING IT DIRECTLY TO E-2. IN THIS CASE, SOLENOIDS FOR E-1 AND D-1, AND THE PRE-MIXING CHAMBER ARE DISCONNECTED. D-2 + E-3 = 50% EFFLUENT; D-3 + E-4 = 25% EFFLUENT, ETC.

Figure 3. Solenoid valve dilutor system, detailed diagram (not to scale).

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SOLENOID SYSTEM EQUIPMENT LIST

1. Dilator Glass.
2. Stainless Steel Solenoid Valves
   a. 3, normally open, two-way, 55 psi, water, 1/4" pipe size, 9/32" orifice size, ASCO 8262152, for incoming effluent and dilution water pipes and mixing chamber pipe.
   b. 1, normally closed, two-way, 15 psi, water, 3/8" pipe size, 3/8" orifice size, ASCO 8030865, for D-l chamber evacuation pipe.
   c. 12, normally closed, two-way, 36 psi, water, 1/4" pipe size, 9/32" orifice size. ASCO 8262C38, for remaining dilution chambers (D2-D6) and effluent chamber (E1-E6) evacuation pipes.
3. Stainless steel tubing, seamless, austenitic, 304 grade for freshwater and 316 grade for saline water.
   a. 10 ft of 3/8" OD, 0.035" wall thickness, for dilution water and effluent pipes.
   b. 60 ft of 1/4" OD, 0.035" wall thickness, for dilution water and effluent pipes.
   c. 1 ft of 3/4" OD, 0.035" wall thickness, for standpipe in D1 chamber.
4. Swagelok tube connectors, stainless steel.
   a. 4, male tube connectors, male pipe size 1/4", tube OD 3/8".
   b. 2, male tube connectors, male pipe size 1/2", tube OD 3/8".
   c. 26, male tube connectors, male pipe size 1/4", tube OD 1/4".
   d. 2, male tube connectors, male pipe size 3/8", tube OD 3/8".
   e. 2, male adaptor, tube to pipe, male size 1/2", tube OD 3/8".
5. 7, 1200 mL stainless steel beakers.
6. Several Ibs each of Neoprene stoppers, sizes 00, 0, and 1; 1 lb of size 5.
7. 14 - aquarium (1-20 liters).
8. Magnetic stirrer.
9. 2 - PVC ball valves, 1/2" pipe size.
10. Dilutor control panel - see Fig. 9 and equipment list.
11. Plywood sheeting, exterior grade: one - 4' x 8' x 3/4", one - 4' x 8' x 1/2".
12. Pineor redwood board, 1" x 8", 20 ft.
13. Epoxy paint, 1 gal.
14. Assorted wood screws, nails, etc.
15. 25 ft - 14" ID, TEFLON® tubing, to connect the mixing chambers to the test chambers.

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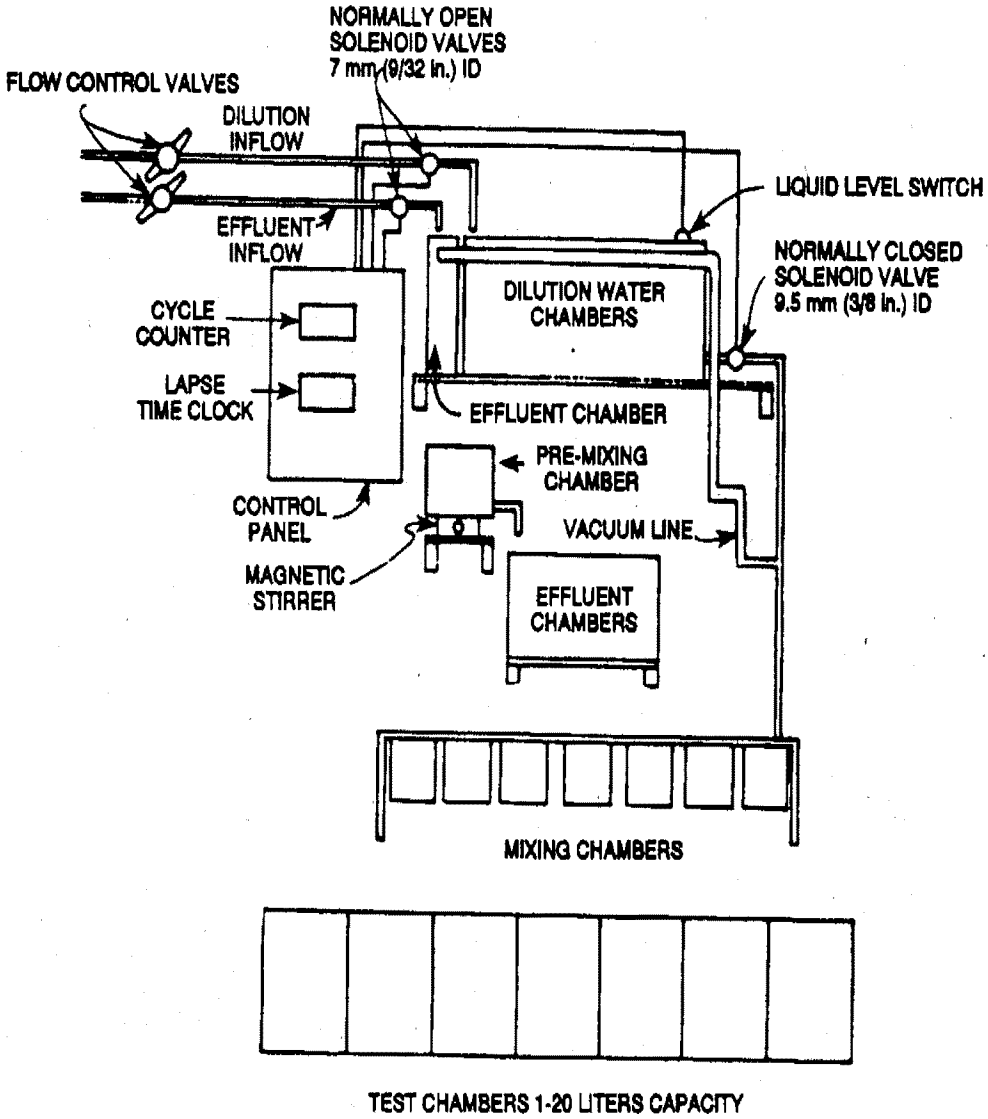

Figure 4. Vacuum siphon dilutor system, general diagram (not to scale).

VACUUM SIPHON SYSTEM EQUIPMENT LIST

1. Dilutor Glass.
2. Stainless steel solenoid valves.
   a. 2, normally open, two-way, 55 psi, water, 1/4" pipe size, 9/32" orifice size, ASCO 8262152, for incoming effluent and dilution water pipes.
   b. 2, normally closed, two-way, 15 psi, water, 3/8" pipe size, 3/8" orifice size, ASCO 8030865, for dilution water chamber D-6 and effluent chamber E-2.
3. Stainless steel tubing, seamless, austenitic, 304 grade for freshwater and 316 grade for saline water.
   a. 60 ft of 3/8" OD, 0.035" wall thickness, for dilution water and effluent pipes.
   b. 20 ft of 5/16" OD, 0.035" wall thickness, for standpipes in mixing chambers.
   c. 1 ft of 3/4" OD, 0.035" wall thickness, for standpipe in D1 chamber.
4. Swagelok tube connectors, stainless steel.
   a. 4, male tube connectors, male pipe size l/4", tube OD 3/8".
   b. 2, male tube connectors, male pipe size 3/8", tube OD 3/8".
   c. 2, male adaptor, tube to pipe, male pipe size 1/2", tube OD 3/8".
   d. 2, male tube connectors, male pipe size 1/2", tube OD 3/8".
5. 7, 1,200 mL stainless steel beakers.
6. Several Ibs each of Neoprene stoppers, sizes 00, 0, and 1; 1 lb of size 5.
7. 14 - aquarium (1-20 liters).
8. Magnetic stirrer.
9. 2, PVC Ball valves, 1/2" pipe size.
10. Dilutor control panel equipment - see Fig. 9 and equipment list.
11. 7, 120 mL NALGENE® bottles.
12. 3 ft, 1-in-2 aluminum bar, for siphon support brackets.
13. Stainless steel set screws, box of 50, for securing SS tubing in siphon support brackets.
14. Stainless steel hose clamps, box of 10, size #4 or 5, (need 3 boxes).
15. 6, NALGENE® T's, 5/16" OD.
16. 12, TYGON® Y connectors, 3/8" I.D.
17. TYGON® tubing, 3/8" OD, 10 ft.
18. Plywood sheeting, exterior grade: one - 4' x 8' x 3/4", one - 4' x 8' x 1/2".
19. Pine or redwood board, 1" x 8", 20 ft.
20. Epoxy paint, 1 gal.
21. Assorted wood screws, nails, etc.
22. 25 ft of 5/16" ID, TEFLON® tubing, to connect the mix5ng chambers to the test chambers.

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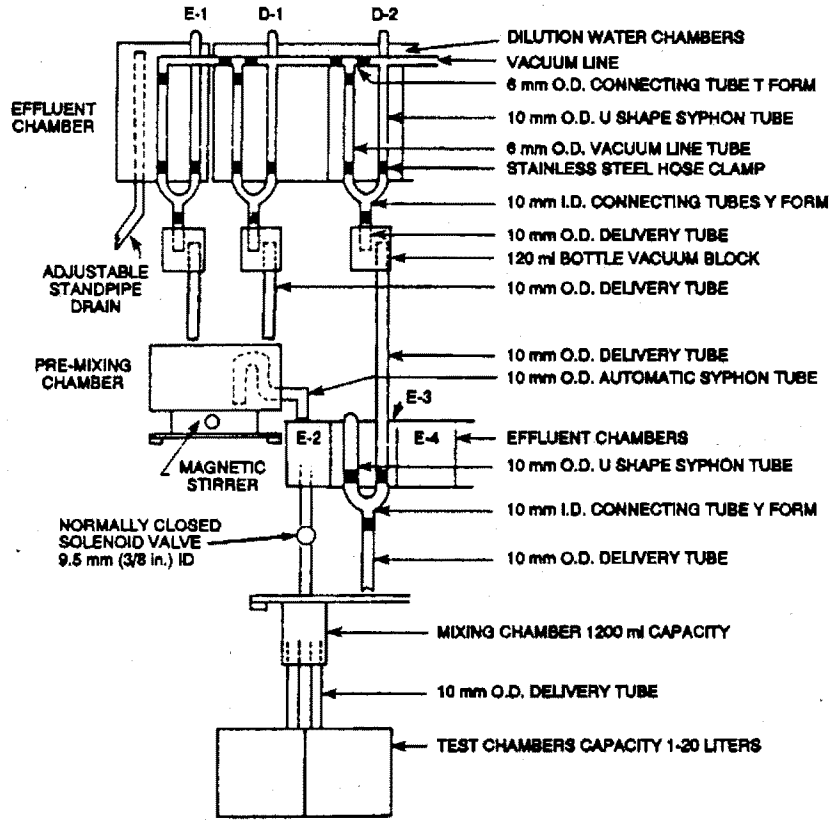

Figure 5. Vacuum siphon dilutor system, detailed diagram (not to scale).

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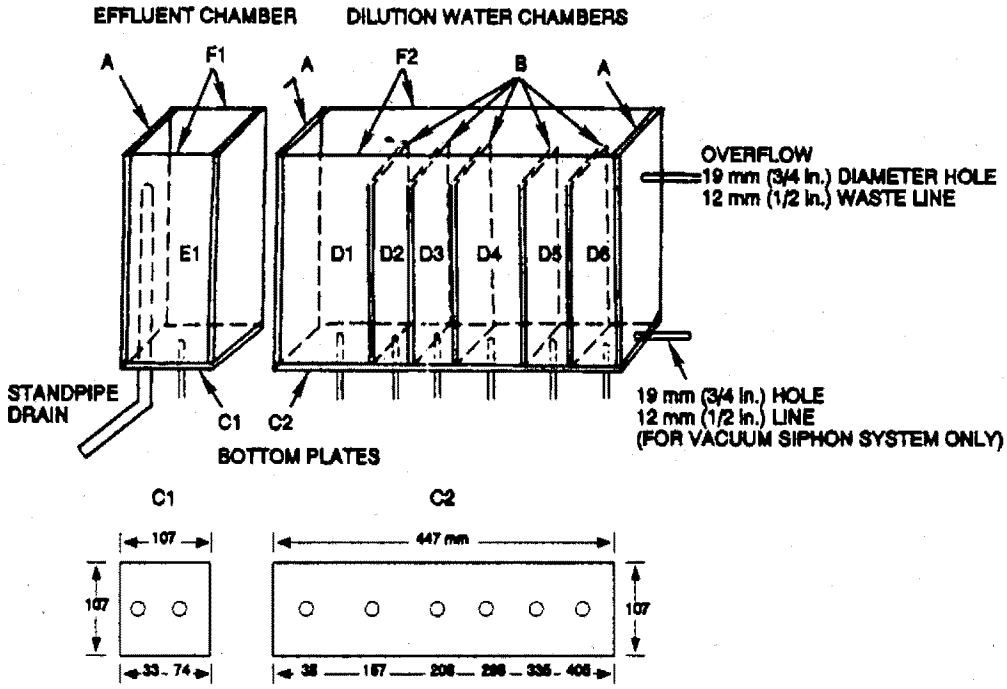

DRAIN HOLES IN BOTTOM PLATE (C1 AND C2) SHOWN FOR SOLENOID VALVE DILUTOR SYSTEM. FOR VACUUM SIPHON DILUTOR SYSTEM, DRAIN HOLE IS REQUIRED ONLY FOR CHAMBER E1.

INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING 6 mm (1/4 in.) PLATE GLASS. NOTE: 1.6 mm (1/16 in.) No. 304 GRADE (FOR FRESH WATER) OR No. 316 GRADE (FOR SALINE WATER) STAINLESS STEEL MAY BE SUBSTITUTED FOR GLASS.

	LENGTH	WIDTH	NO. PIECES (18)
A:	331 mm	x 95 mm	= 4 (END PLATES)
B:	200 mm	x 95 mm	= 6 (PARTITIONS)
C1:	107 mm	x 107 mm	= 1 (BOTTOM PLATE FOR E1)
C2:	447 mm	x 107 mm	= 1 (BOTTOM PLATE FOR D1-D6)
F1:	107 mm	x 331 mm	= 2 (FRONT AND BACK PANELS FOR E1)
F2:	447 mm	x 331 mm	= 2 (FRONT AND BACK PANELS FOR D1-D6)

INSIDE CELL MEASUREMENTS AND APPROXIMATE VOLUMES

	WIDTH	LENGTH	HEIGHT	VOLUME
E1:	95 mm	x 95 mm	x 331 mm	= 3045 mL
D1:	185 mm	x 95 mm	x 200 mm	= 3375 mL
D2:	40 mm	x 95 mm	x 200 mm	= 760 mL
D3:	50 mm	x 95 mm	x 200 mm	= 950 mL
D4:	60 mm	x 95 mm	x 200 mm	= 1140 mL
D5:	60 mm	x 95 mm	x 200 mm	= 1140 mL
D6:	70 mm	x 95 mm	x 200 mm	= 1330 mL

Figure 6. Effluent and dilution water chambers (not to scale).

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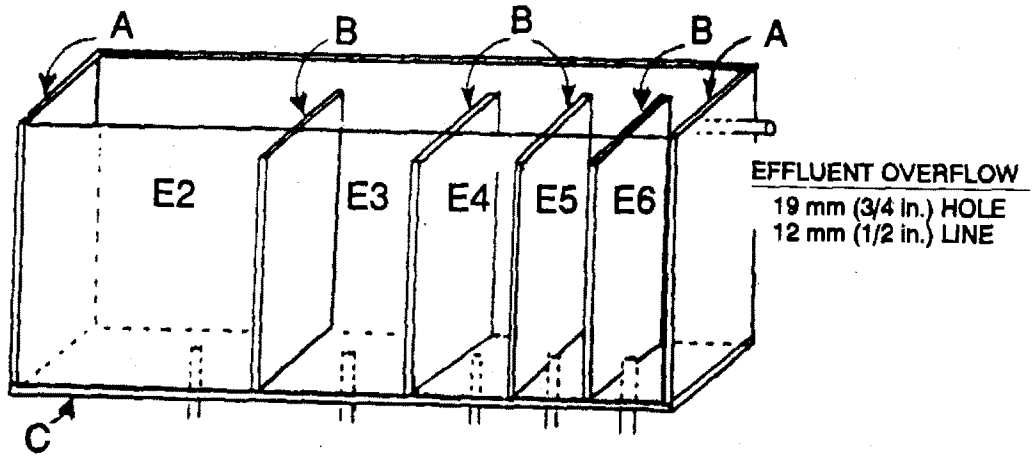
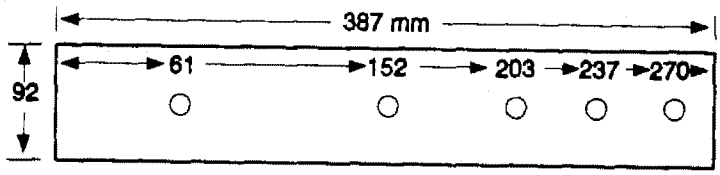


BOTTOM PLATE (C)



DRAIN HOLES IN BOTTOM PLATE (C) SHOWN FOR SOLENOID VALVE DILUTOR SYSTEM ONLY. FOR VACUUM SIPHON DILUTOR SYSTEM, A DRAIN HOLE IS REQUIRED ONLY FOR CHAMBER E2.

INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING A 6 mm (1/4 in.) PLATE GLASS ARE SHOWN BELOW. NOTE: 1/16 in. No. 304 (FOR FRESH WATER) OR No. 316 STAINLESS STEEL (FOR SALINE WATER) MAY BE SUBSTITUTED FOR GLASS.

	LENGTH	WIDTH	NO. PIECES (9)
A	180 mm x	80 mm	= 2 (END PLATES)
B	155 mm x	80 mm	= 4 (PARTITIONS)
C	296 mm x	92 mm	= 1 (BOTTOM PLATE)
D	296 mm x	180 mm	= 2 (FRONT AND BACK PLATES)

INSIDE CHAMBER MEASURMENTS AND APPROXIMATE VOLUMES.

	WIDTH	LENGTH	HEIGHT	VOLUME
E2:	110 mm x	80 mm	x 155 mm	= 1364 mL
E3:	60 mm x	80 mm	x 155 mm	= 744 mL
E4:	30 mm x	80 mm	x 155 mm	= 372 mL
E5:	30 mm x	80 mm	x 155 mm	= 372 mL
E6:	30 mm x	80 mm	x 155 mm	= 372 mL

Figure 7. Effluent chambers (not to scale).

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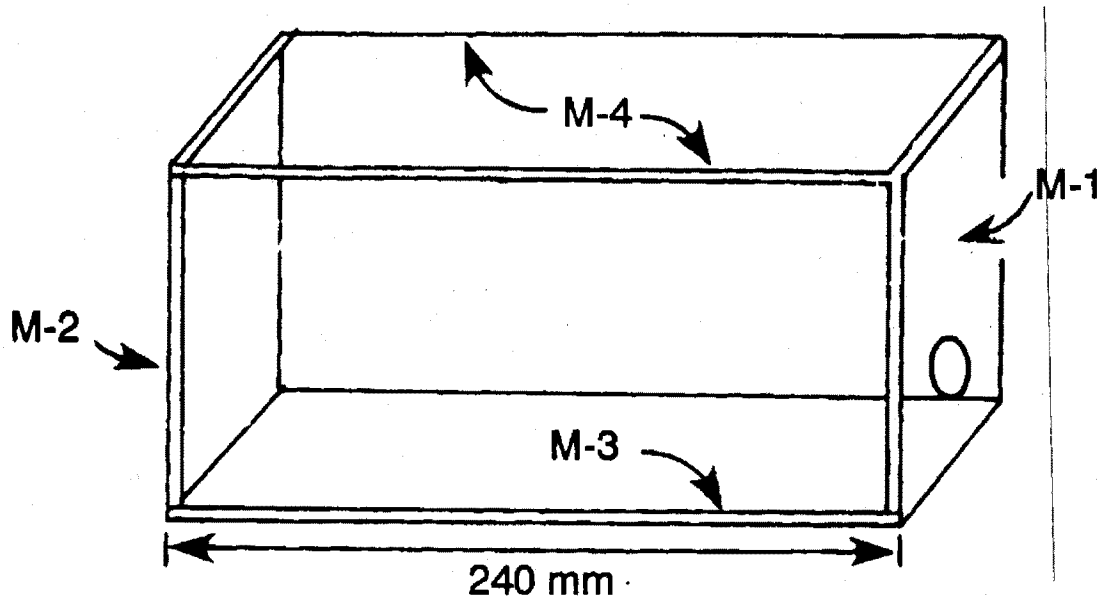
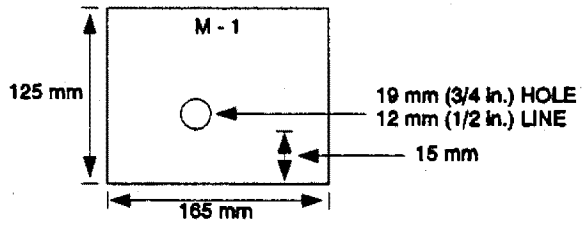


SIDE VIEW

END VIEW



INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING 6 mm (1/4 in.) PLATE GLASS. APPROXIMATE CAPACITY 4360 mL

M-1	125	mm	x	153	mm	-	1	(END PLATE, WITH HOLE)
M-2	125	mm	x	153	mm	-	1	(END PLATE)
M-3	240	mm	x	165	mm	-	1	(BOTTOM PLATE)
M-4	240	mm	x	125	mm	-	2	(SIDE PLATES)

Figure 8. Pre-mixing chamber (not to scale).

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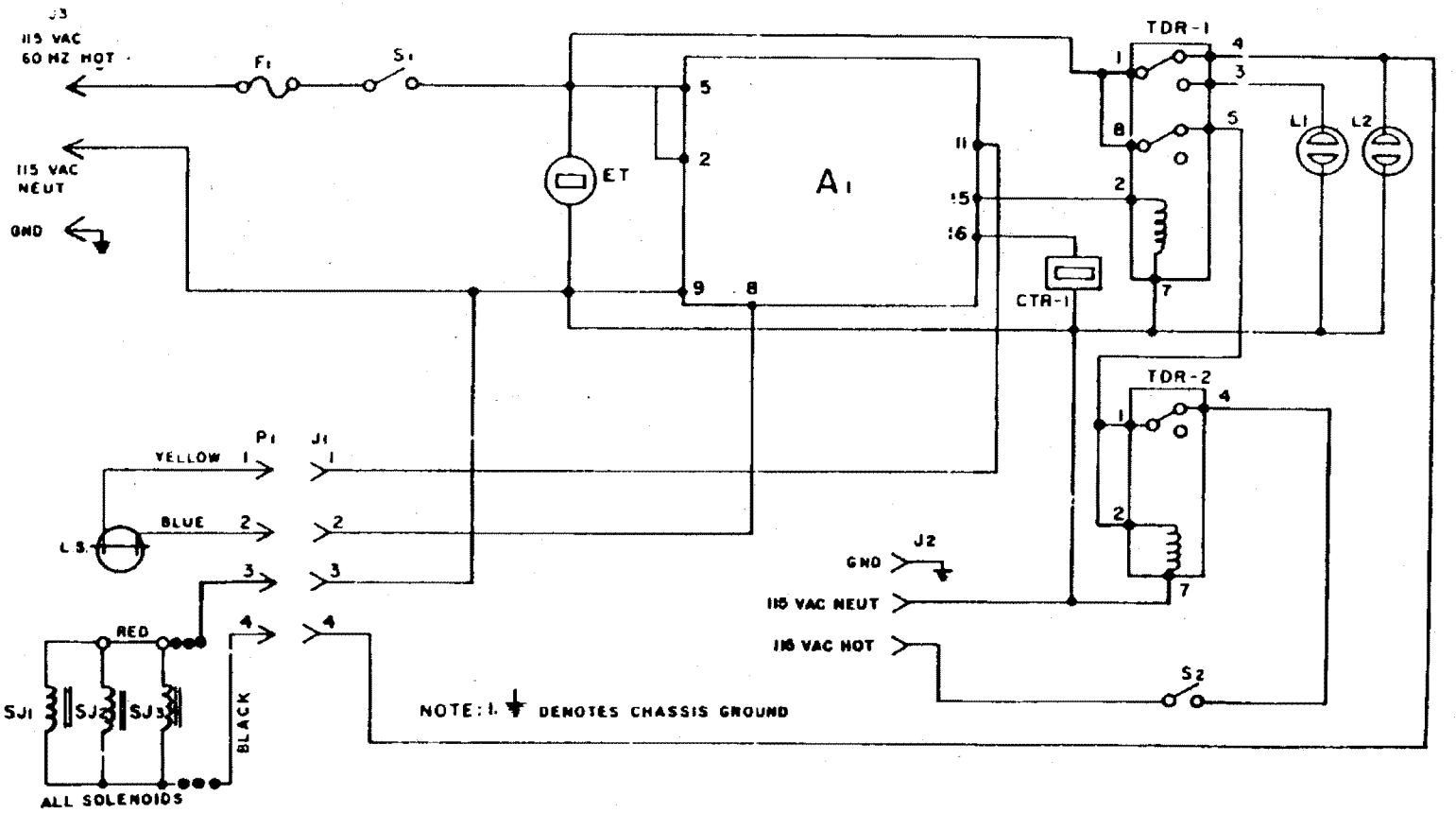

Figure 9. Dilutor control panel wiring diagram.

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DILUTOR CONTROL PANEL EPUIPMENT LIST*

Designation	CKT Description	Manufacturer
A_1	Encapsulated amplifier	Cutler Hammer 13535H98C
CTR-1	Cycle counter	Redington #P2-1006
ET	Elapsed time indicator	Conrac #636W-AA H&T
F_1	Input power fuse	Little fuse 342038
J_1	Receptacle	Amphenol 91PC4F
J_2	Aux A.C. output jack	Stand. 3-prong AC Rcpt.
J_3	Main input power cord	Stand. 3-prong AC male plug
L_1	Fill indicator light	Dialco 95-0408-09-141
L_2	Emptying indicator light	Dialco 95-0408-09-141
L.S.	Liquid level sensor (Dual Sensing Probe)	Cutler Hammer 13653H2
P_1	Plug	Amphenol 91MC4M
S_1	On-off main power switch (spst)	Cutler Hammer 7580 K7
S_2	On-off aux power switch (spst)	Cutler Hammer 7580 K7
SJ_1	Solenoid	(See Solenoid and Vacuum System equipment lists)
SJ_2	"	" " "
SJ_3	"	" " "
SJ_4-SJ_6	Additional Solenoids for Solenoid Valve System	" " "
TDR-1	Time delay relay	Dayton 5x829
TDR-2	Aux time delay relay	Dayton 5x829

*Consult local electric supply house.

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APPENDIX D

PLANS FOR MOBILE TOXICITY TEST LABORATORY

D.1. TANDEM-AXLE TRAILER

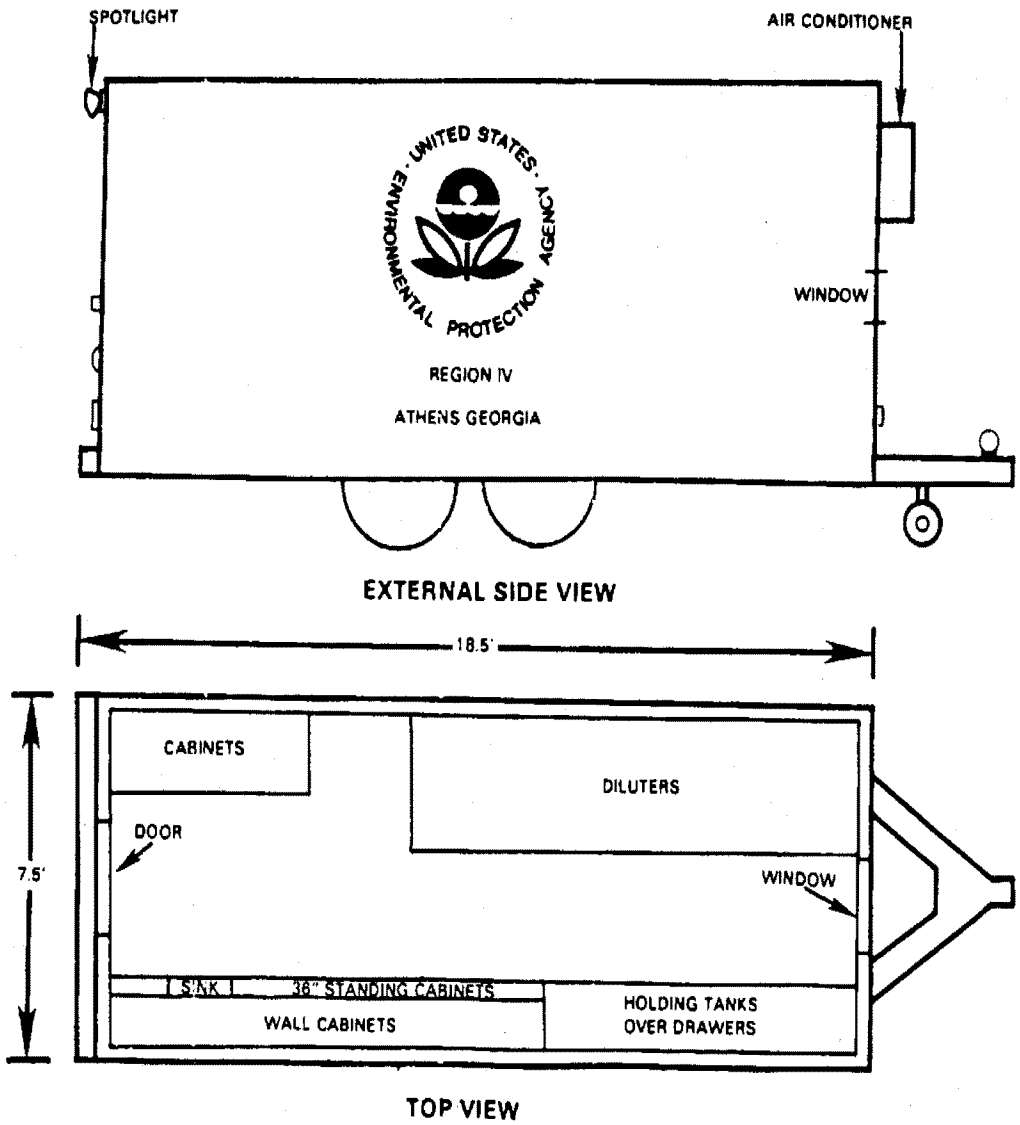

Figure 1. Mobile bioassay laboratory, tandem axle trailer. Above - external side view; below - internal view from above.

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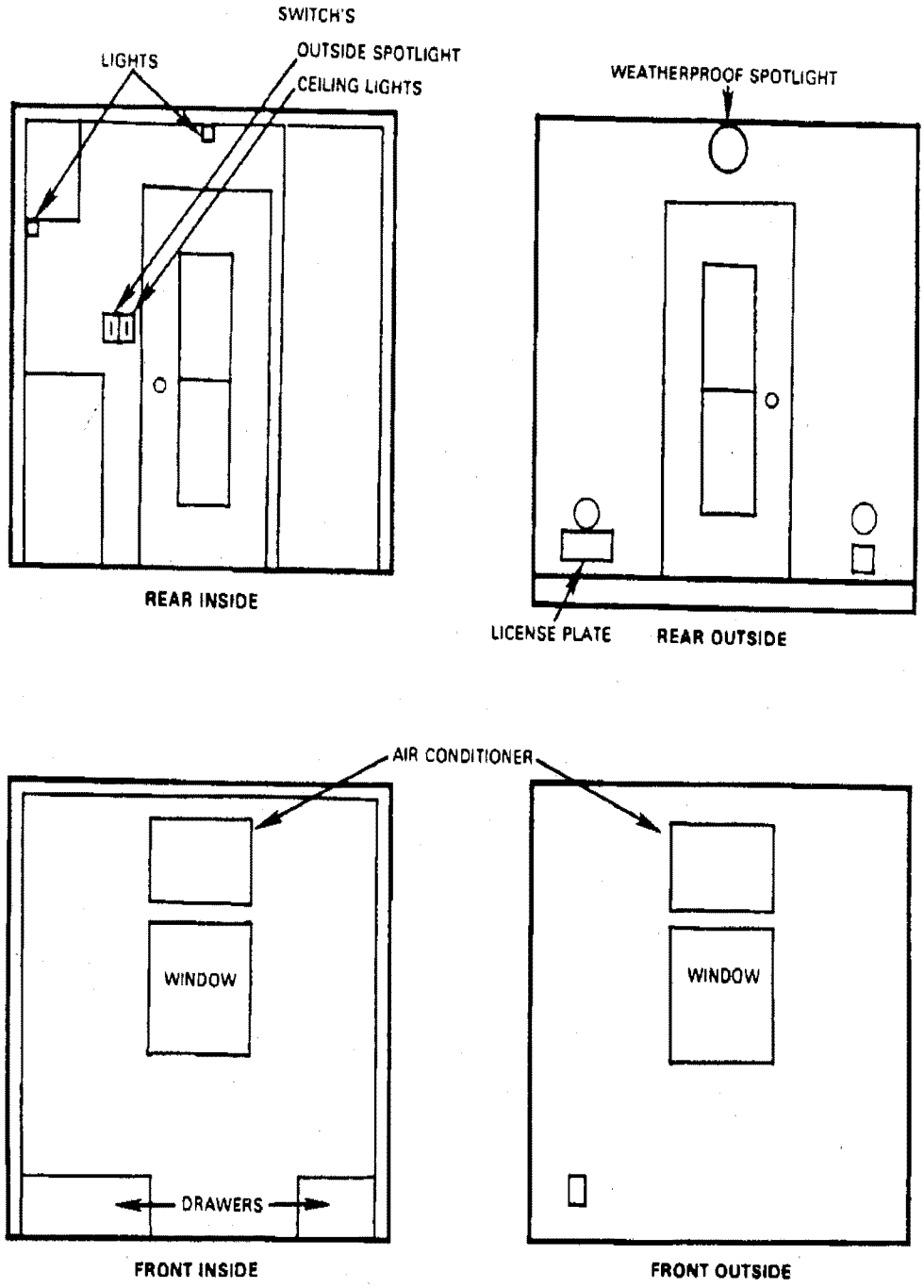

Figure 2. Mobile bioassay laboratory, tandem-axle trailer, external and internal end views.

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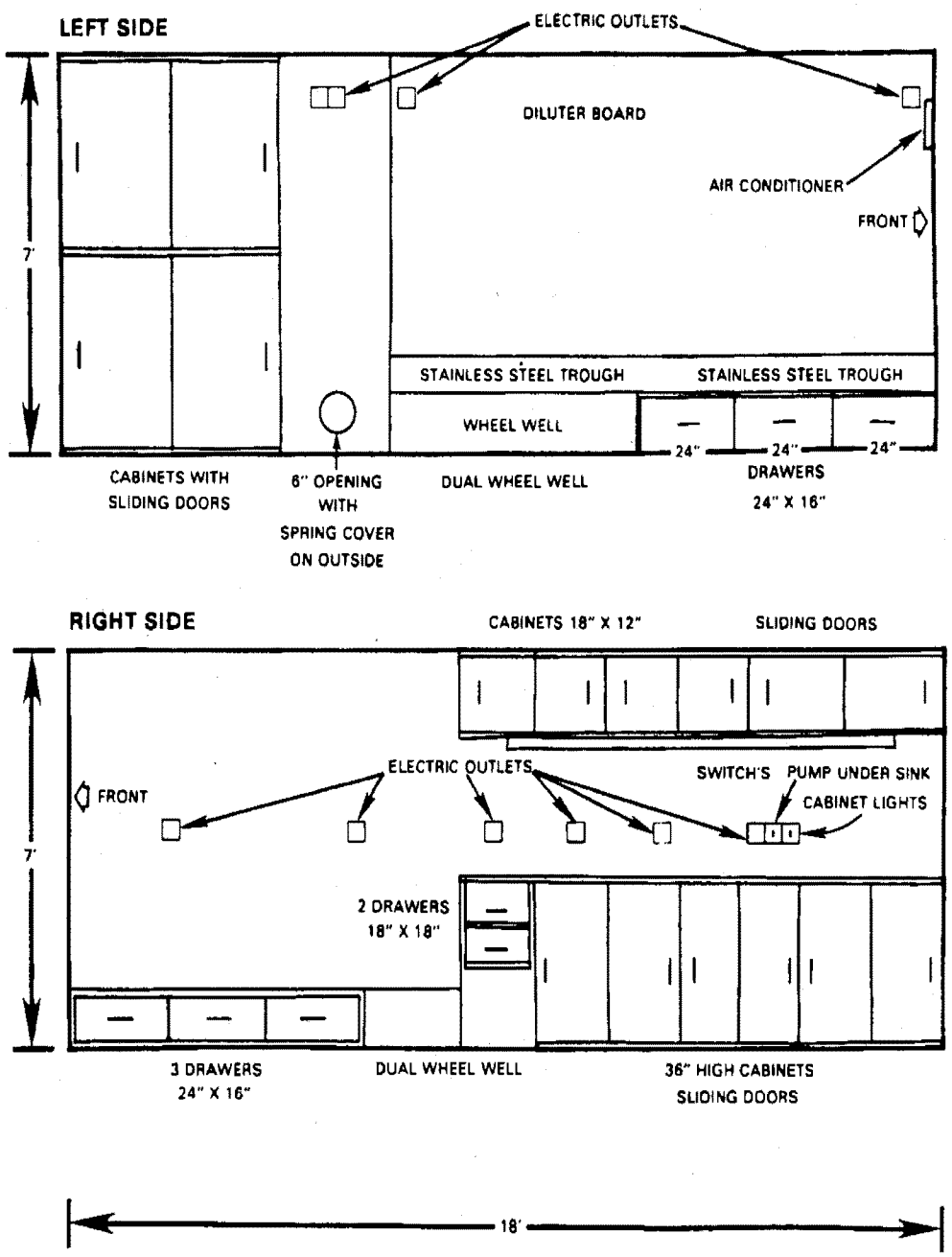

Figure 3. Mobile bioassay laboratory, tandem-axle trailer, internal views of side walls.

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APPENDIX D

PLANS FOR MOBILE TOXICITY TEST LABORATORY

D.2. FIFTH WHEEL TRAILER

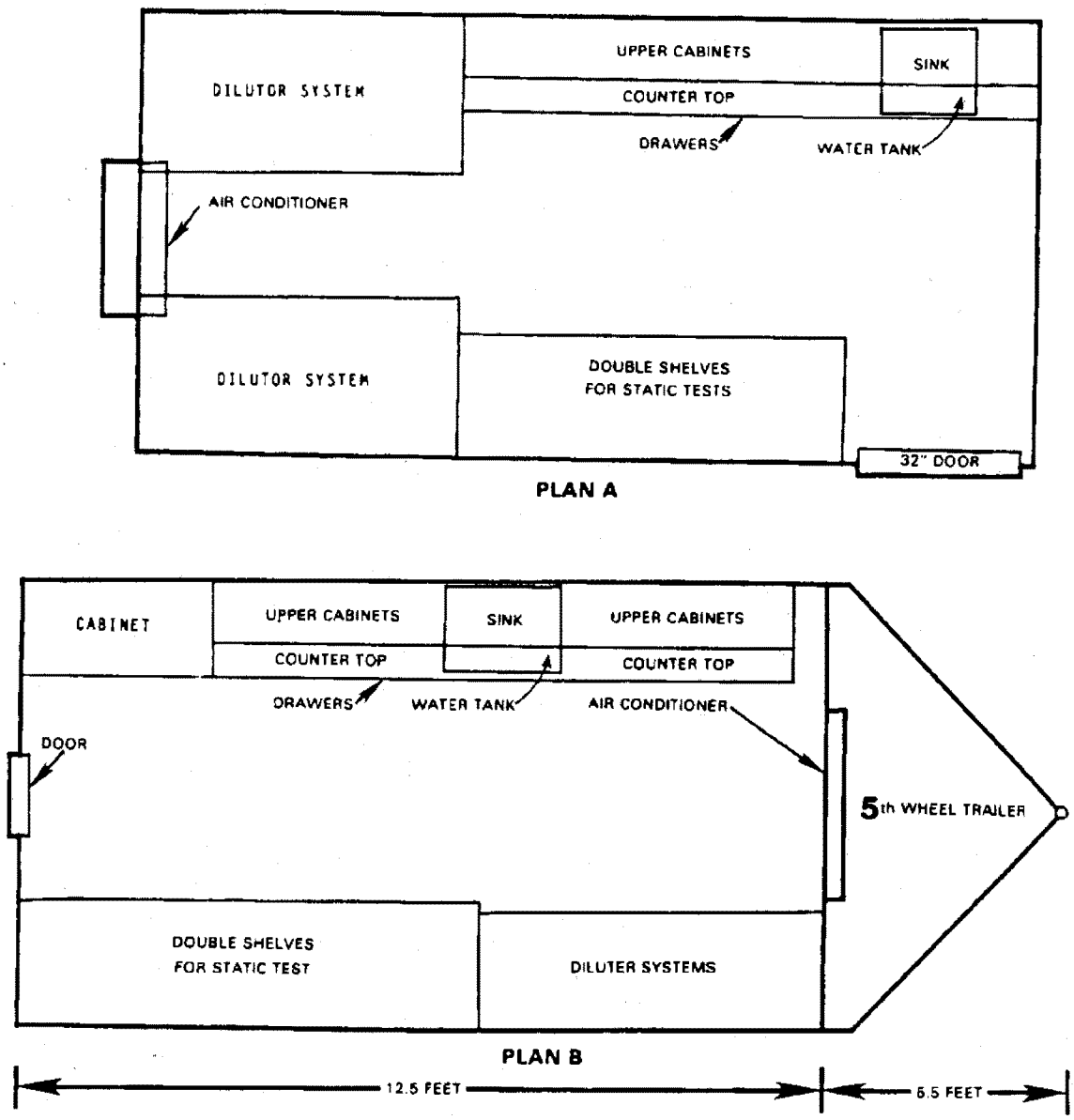

Figure 4. Mobile bioassay trailer, fifth-wheel trailer, internal view from above.

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APPENDIX E

CHECK LISTS AND INFORMATION SHEETS

E.1. TOXICITY TEST FIELD EQUIPMENT LIST

Truck

____Boards
____Cinder blocks
____Drums: ____500 gal nalgene
____ 55 gal metal - diesel fuel
____ 22 gal
____Gas can ____ 5 gal
____Jacks
____Jumper cables
____Oil
____Pumps: ____ (2) Homelite
____ ____ Hoses & couplings
____Shovels
____Spare tires (trailer, generator)

Trailer

____Acetone
____Aerators (battery operated)
____Air line: _____Clamps
_____Aerators (battery operated)
_____Air line: ____Clamps
____Stones
____Tubing
____Valves
____Alcohol
____Aluminum foil
____Alkalinity analysis (0.02 N H_2SO_4)
____Boots: ____safety
____wading
____Batteries ____D cell
____Beakers: ____150 mL nalgene
____200 mL glass (3 boxes)
____Bottles: ____D.O.
____wash
____Sample
____VOA vials
____500 mL plastic
____Glass organic
____Qt. w/teflon liner

____Brine shrimp eggs
____Broom
____Brushes (wash)
____Buckets
____Camera
____Chlorine kit (w/chem)
____Cleanser
____Clip board (lg, sm)
____Cork borer set
____Culture dishes (200 mL, Daphnia)
____Daphnia food
____Data sheets: ____Bioassay (static)
____Bioassay (flow-thru)
____Dilutor volume delivery
____Calibrator delivery sheet
____Daily events log
____Dish pan
____Dish rack
____Dissolved oxygen:
____KCL membrane solution
____Membranes
____Meter (YSI)
____Probes
____Reagent: ____$MnSo_4$
____Alkaline azide
____H_2SO_4
____0.0375 Na thiosulfate
____Starch
____Distilled H_2O
____Emergency road kit
____Enamel pans (lg, sm)
____Erlenmeyer flasks: ____ 500 mL (2)
____1000 mL
____2000 mL
____Extension cords
____Fire extinguisher
____First aid kit
____Fish nets, (lg, sm)
____Flash light
____Generator: ____Oil
____Filter - fuel
____Funnel
____Grease gun (wheels)
____Credit card
____Lock/key
____Siphon hose

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E.1. TOXICITY TEST FIELD EQUIPMENT LIST (CONT.)

____Glass cutter
____Gloves (plastic)
____Graduated cylinders:
25 mL, 50 mL, 100 mL
250 mL, 500 mL, 1000 mL, 2000 mL
____Ground wire & rod
____Hand soap
____Hard hats
____Hardness analysis: ____Buffer
____EDTA
____indicator
____HCl (20%)
____Heaters: ____Aquarium
____Space
____Hose: ____Clamps
____Connectors
____Ice chests

____Jars: ____750 mL (4 boxes)
____3 gal (glass) (1)
____5 gal (glass) (1)
____Sample jugs (2)
____Kimwipes (lg, sm)
____Lab coats (2)
____Level
____Light 110 V
____Log book
____Magnetic stirrers: ____Lighted
____Other

____Mop

____Paper towels
____Parachute cord
____Parafilm
____Pencils, pens
____pH: ____Meters, Orion
____ ____Meters, corning
____Buffers, 4,7,10
____Probes (extras)
____Pipets: ____Bulbs
____Eyedroppers
____Volumetric (1 mL, 5 mL, 10 mL)
____Plastic bags
____Quality assurance - SPCP
____Rain gear
____Reconstituted hard water
____Refractometer
____Respirators (cartridges)

____Rubber bands
____Ruler
____Safety glasses
____Safety manual
____Sample labels
____Scissors
____Screen bioassay cups
____Sea salts
____Separatory funnels & racks
____Silent gients
____Silicon sealant
____Solenoids (spare)
____Stainless steel tubing pieces
____Standard Methods Hand Book
____Stirring bars
____Stoppers (assorted)
____Submersible pumps: ____lg, sm.
____screens

____Super ice
____Tablets (paper)
____Tape: ____Cellophane
____Color coded
____Electrician
____Masking
____Nylon
____Thermometers: ____Dial
____Glass
____Tools (lock/key)
____Tygon tubing, 1/8", 1/4", 3/8"
____Volumetric flasks (1000 mL, 2000 mL)
____WD40
____Weigh boats
____Wire tags

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APPENDIX E

CHECK LISTS AND INFORMATION SHEETS

E.2. INFORMATION CHECK LIST FOR ON-SITE INDUSTRIAL OR MUNICIPAL WASTE TOXICITY TEST

1. PRE-TRIP INFORMATION

Facility Name: _____

Address: _____

Phone number: _____

Plant Representative(s): _____

Names, Titles, Addresses of Company Personnel:

A. To Receive Correspondence: _____

_____

_____

B. To Receive Carbons: _____

_____

_____

Date of Notification Letter: _____

State Making Notification and Arrangements: _____

Special Plant Safety/Security Requirements for EPA Personnel to Observe:

_____

Local Accommodation Recommendations: _____

Directions to Plant: _____

_____

_____

Availability of Power Hookups (three 20-amp, 110-V Circuits): _____

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Distance from Power Source to Trailer: _____ (Feet)

Trailer Location: _____

Possible Source of Dilution Water: _____

_____

_____

Major Products: _____

Raw Materials: _____

_____

Name of Receiving Water: _____

Schedule of Plant Operation (continuous, weekdays only, etc.): _____

_____

Treatment Steps: _____

_____

_____

_____

Treatment Level (BPT, BAT, etc.): _____

Wastewater Retention Time by Lagoon or Treatment Step:

Lagoon Designation	Retention Time Hours	Days
_____	____	____
_____	____	____
_____	____	____
_____	____	____
_____	____	____

Total Wastewater Retention Time: _____ Hours; _____ Days

Retention Time Determination: _____ Calculated; _____ Actual

Calculation method: _____

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Description of Wastewater Tap Point: _____

_____

_____

Description of Outfall (surface, submerged diffuser, etc.): _____

_____

_____

Description of Other Waste Disposal Alternatives in Use (spray irrigation, deepwell, municipal discharge, etc.): _____

_____

_____

2. ON-SITE INFORMATION

Wastewater General Characteristics:

Color: _____

Odor: _____

Solids: _____

Other: _____

Serial Number(s) of Discharge(s) to be Tested: _____

Description of Receiving Water: ____ Uniflow; ____ Tidal;
____ Approximate amplitude, feet

Color: _____

Odor: _____

Solids: _____

Salinity: High tide _____; Low tide _____

Other: _____

_____

7Q10: _____; Ave. flow _____

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Description of Receiving Water Zone of Dilution: _____

_____

_____

Location and Description of Water Sampling Point(s): _____

_____

Fresh: _____

Salt: _____

Dilution Waste General Characteristics:

Color: _____

Odor: _____

Solids: _____

Other: _____

_____

Description of Toxicity Test Anomalies (plant production changes, power failure, rain events, etc.):

Duration		
Time & Date	Time & Date	Anomaly

Description of Plant maintenance: _____

_____

_____

_____

Attach: DIAGRAM OF WASTEWATER TREATMENT FACILITIES.

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3. FOLLOW-UP INFORMATION

Date of follow-up letter: _____

Wastewater Flow (data supplied by discharger):

Week Prior to Testing		Week of Testing	
Date	Discharge (MGD)	Date	Discharge (MGD)

Average Discharge (MGD): _____

Organisms Tested On-site or In-Lab:

Species	Flow-thru test duration (h)	Static test duration (h)	Test Location	Dates	Results

Possible Recommended Action as a Result of These Findings:

_____

_____

_____

_____

_____

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APPENDIX E

CHECK LISTS AND INFORMATION SHEETS

E.3. DAILY EVENTS LOG

Date: _____ Page ____ of ____ Pages

Site: _____ Day #____ of Study

Initials: _____ Day #____ of Flow-through Test

Time: _____ Notes: _____

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APPENDIX E

CHECK LISTS AND INFORMATION SHEETS

E.4. DILUTOR CALIBRATION FORM

Calibration Site: _____ Calibrator: _____
Dilutor Number: _____ Date: _____

Effluent Concentration (%)	100.0	50.0	25.0	12.5	6.25	3.12	1.56
Dilution Water (mL)	0	500	750	876	938	969	984
Trial 1							
2							
3							
Average							
Effluent (mL)	1000	500	250	125	62	31	16
Trial 1							
2							
3							
Average							

Mixing Chamber (%): _____
Wastewater (mL): _____
Dilution Water (mL): _____

	Dilution Water	Effluent
Vol (mL)		
Trial 1		
2		
3		
Average		

Remarks:

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APPENDIX E

CHECK LISTS AND INFORMATION SHEETS

E.5. DAILY DILUTOR CALIBRATION CHECK

DILUTOR VOLUME DELIVERY INFORMATION

Industry: _____
Date: _____
Test Start Date: _____ Time: _____

Observation Number	Date	Time	Volume Delivery to Metering Boxes Each 10 Seconds (mL)		Measured Cycle Time (min.)	Control Box		Comments
			Dilution Water	Wastewater		Hour	Cycles	
Final								Ave 4-day turnover = Min/turnover

USEPA
Athens, GA

STANDARD DILUTOR FLOW RATES

Cycle Time (min)	Flow rate (mL/10 seconds) Dilution Water	Wastewater	Turnover Time (min)
2.5	532	246	43
5.0	199	92	85
7.5	123	57	128
10.0	89	41	170
12.5	69	32	213
15.0*	67	22	300

* This turnover rate provides approximately five 90% turnovers per day.

*U.S. GOVERNMENT PRINTING OFFICE:1993-750-002/80302

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United States
Environmental Protection
Agency

Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms

Fourth Edition

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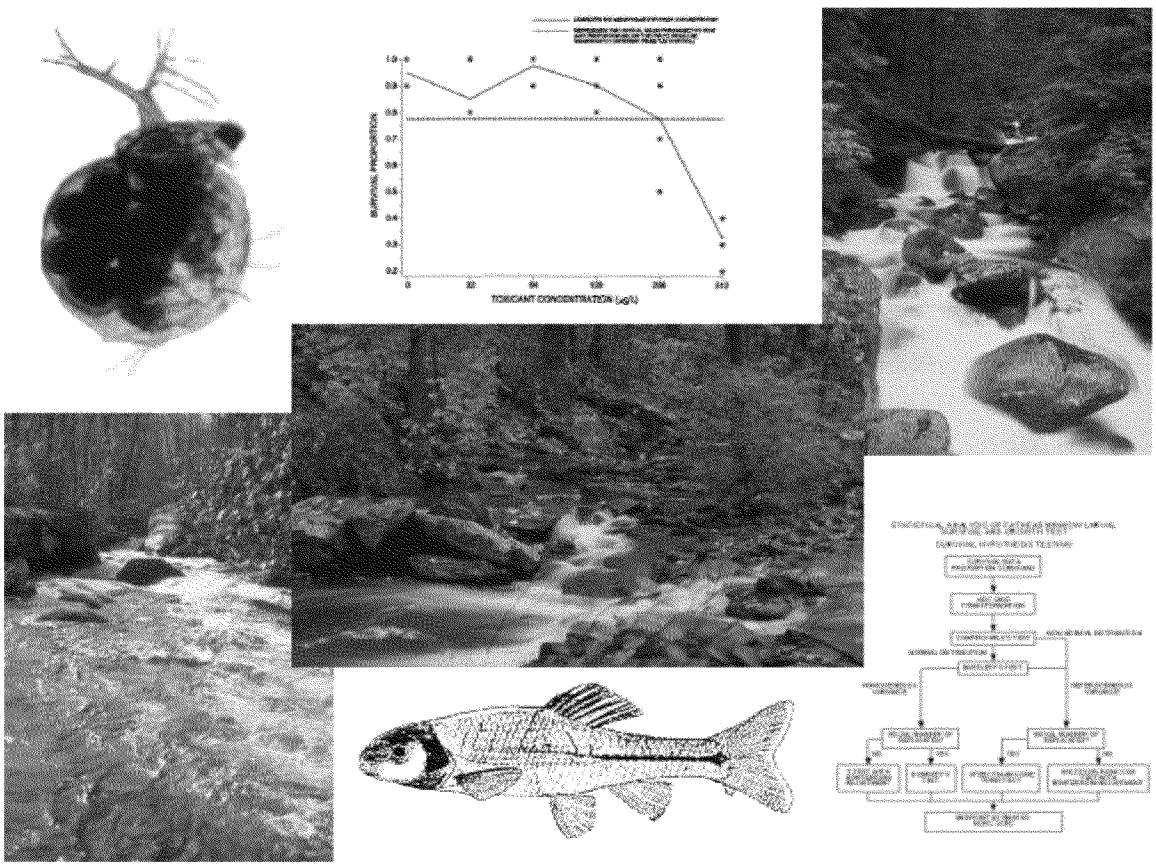

U.S. Environmental Protection Agency
Office of Water (4303T)
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SECTION 1

INTRODUCTION

1.1 This manual describes chronic toxicity tests for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in chronically toxic concentrations. The methods included in this manual are referenced in Table IA, 40 CFR Part 136 regulations and, therefore, constitute approved methods for chronic toxicity tests. They are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a central laboratory or on-site, by the regulatory agency or the permittee.

1.2 The data are used for NPDES permits development and to determine compliance with permit toxicity limits. Data can also be used to predict potential acute and chronic toxicity in the receiving water, based on the LC50, NOEC, IC50 or IC25 (see Section 9, Chronic Toxicity Endpoints and Data Analysis) and appropriate dilution, application, and persistence factors. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations. Data from chronic toxicity tests performed as part of permit requirements are evaluated during compliance evaluation inspections and performance audit inspections.

1.3 Modifications of these tests are also used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, and to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA, 1988c; USEPA, 1989b; USEPA 1989c; USEPA, 1989d; USEPA, 1989e; USEPA, 1991a; USEPA, 1991b; and USEPA, 1992).

1.4 This methods manual serves as a companion to the acute toxicity test methods for freshwater and marine organisms (USEPA, 2002a), the short-term chronic toxicity test methods for marine and estuarine organisms (USEPA, 2002b), and the manual for evaluation of laboratories performing aquatic toxicity tests (USEPA, 1991c). In 2002, EPA revised previous editions of each of the three methods manuals (USEPA, 1993a; USEPA, 1994a; USEPA, 1994b).

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991a).

1.6 These freshwater short-term toxicity tests are similar to those developed for marine and estuarine organisms to evaluate the toxicity of effluents discharged to marine and estuarine waters under the NPDES permit program. Methods are presented in this manual for three species of freshwater organisms from three phylogenetic groups. The methods are all static renewal type seven-day tests except the green alga, *Selenastrum capricornutum*, test which lasts four days.

1.7 The three species for which test methods are provided are the fathead minnow, *Pimephales promelas*; the daphnid, *Ceriodaphnia dubia*; and the green alga, *Selenastrum capricornutum*.

1.7.1 Two of the methods incorporate the chronic endpoint of growth in addition to lethality and one incorporates reproduction. The fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test incorporates teratogenic effects in addition to lethality. The green alga, *Selenastrum capricornutum*, growth test has the advantage of a relatively short exposure period (96 h).

1.8 The validity of the freshwater chronic methods in predicting adverse ecological impacts of toxic discharges was demonstrated in field studies (USEPA, 1984; USEPA, 1985b; USEPA, 1985c; USEPA, 1985d; USEPA, 1986a; USEPA, 1986b; USEPA, 1986c; USEPA, 1986d; Birge et al., 1989; and Eagleson et al., 1990).

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1.9 The use of any test species or test conditions other than those described in the methods summary tables in this manual shall be subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.

1.10 These methods are restricted to use by, or under the supervision of, analysts experienced in the use or conduct of aquatic toxicity tests and the interpretation of data from aquatic toxicity testing. Each analyst must demonstrate the ability to generate acceptable test results with these methods using the procedures described in this methods manual.

1.11 This manual was prepared in the established EMSL-Cincinnati format (USEPA, 1983).

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SECTION 2

SHORT-TERM METHODS FOR ESTIMATING CHRONIC TOXICITY

2.1 INTRODUCTION

2.1.1 The objective of aquatic toxicity tests with effluents or pure compounds is to estimate the "safe" or "no effect" concentration of these substances, which is defined as the concentration which will permit normal propagation of fish and other aquatic life in the receiving waters. The endpoints that have been considered in tests to determine the adverse effects of toxicants include death and survival, decreased reproduction and growth, locomotor activity, gill ventilation rate, heart rate, blood chemistry, histopathology, enzyme activity, olfactory function, and terata. Since it is not feasible to detect and/or measure all of these (and other possible) effects of toxic substances on a routine basis, observations in toxicity tests generally have been limited to only a few effects, such as mortality, growth, and reproduction.

2.1.2 Acute lethality is an obvious and easily observed effect which accounts for its wide use in the early period of evaluation of the toxicity of pure compounds and complex effluents. The results of these tests were usually expressed as the concentration lethal to 50% of the test organisms (LC50) over relatively short exposure periods (one-to-four days).

2.1.3 As exposure periods of acute tests were lengthened, the LC50 and lethal threshold concentration were observed to decline for many compounds. By lengthening the tests to include one or more complete life cycles and observing the more subtle effects of the toxicants, such as a reduction in growth and reproduction, more accurate, direct, estimates of the threshold or safe concentration of the toxicant could be obtained. However, laboratory life-cycle tests may not accurately estimate the "safe" concentration of toxicants because they are conducted with a limited number of species under highly controlled, steady-state conditions, and the results do not include the effects of the stresses to which the organisms would ordinarily be exposed in the natural environment.

2.1.4 An early published account of a full life-cycle, fish toxicity test was that of Mount and Stephan (1967). In this study, fathead minnows, *Pimephales promelas*, were exposed to a graded series of pesticide concentrations throughout their life cycle, and the effects of the toxicant on survival, growth, and reproduction were measured and evaluated. This work was soon followed by full life-cycle tests using other toxicants and fish species.

2.1.5 McKim (1977) evaluated the data from 56 full life-cycle tests, 32 of which used the fathead minnow, *Pimephales promelas*, and concluded that the embryo-larval and early juvenile life-stages were the most sensitive stages. He proposed the use of partial life-cycle toxicity tests with the early life-stages (ELS) of fish to establish water quality criteria.

2.1.6 Macek and Sleight (1977) found that exposure of critical life-stages of fish to toxicants provides estimates of chronically safe concentrations remarkably similar to those derived from full life-cycle toxicity tests. They reported that "for a great majority of toxicants, the concentration which will not be acutely toxic to the most sensitive life stages is the chronically safe concentration for fish, and that the most sensitive life stages are the embryos and fry". Critical life-stage exposure was considered to be exposure of the embryos during most, preferably all, of the embryogenic (incubation) period, and exposure of the fry for 30 days post-hatch for warm water fish with embryogenic periods ranging from one-to-fourteen days, and for 60 days post-hatch for fish with longer embryogenic periods. They concluded that in the majority of cases, the maximum acceptable toxicant concentration (MATC) could be estimated from the results of exposure of the embryos during incubation, and the larvae for 30 days post-hatch.

2.1.7 Because of the high cost of full life-cycle fish toxicity tests and the emerging consensus that the ELS test data usually would be adequate for estimating chronically safe concentrations, there was a rapid shift by aquatic toxicologists to 30 - 90-day ELS toxicity tests for estimating chronically safe concentrations in the late 1970s. In

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1980, USEPA adopted the policy that ELS test data could be used in establishing water quality criteria if data from full life-cycle tests were not available (USEPA, 1980a).

2.1.8 Published reports of the results of ELS tests indicate that the relative sensitivity of growth and survival as endpoints may be species dependent, toxicant dependent, or both. Ward and Parrish (1980) examined the literature on ELS tests that used embryos and juveniles of the sheepshead minnow, *Cyprinodon variegatus*, and found that growth was not a statistically sensitive indicator of toxicity in 16 of 18 tests. They suggested that the ELS tests be shortened to 14 days posthatch and that growth be eliminated as an indicator of toxic effects.

2.1.9 In a review of the literature on 173 fish full life-cycle and ELS tests performed to determine the chronically safe concentrations of a wide variety of toxicants, such as metals, pesticides, organics, inorganics, detergents, and complex effluents, Woltering (1984) found that at the lowest effect concentration, significant reductions were observed in fry survival in 57%, fry growth in 36%, and egg hatchability in 19% of the tests. He also found that fry survival and growth were very often equally sensitive, and concluded that the growth response could be deleted from routine application of the ELS tests. The net result would be a significant reduction in the duration and cost of screening tests with no appreciable impact on estimating MATCs for chemical hazard assessments. Benoit et al. (1982), however, found larval growth to be the most significant measure of effect, and survival to be equally or less sensitive than growth in early life-stage tests with four organic chemicals.

2.1.10 Efforts to further reduce the length of partial life-cycle toxicity tests for fish without compromising their predictive value have resulted in the development of an eight-day, embryo-larval survival and teratogenicity test for fish and other aquatic vertebrates (USEPA, 1981; Birge et al., 1985), and a seven-day larval survival and growth test (Norberg and Mount, 1985).

2.1.11 The similarity of estimates of chronically safe concentrations of toxicants derived from short-term, embryo-larval survival and teratogenicity tests to those derived from full life-cycle tests has been demonstrated by Birge et al. (1981), Birge and Cassidy (1983), and Birge et al. (1985).

2.1.12 Use of a seven-day, fathead minnow, *Pimephales promelas*, larval survival and growth test was first proposed by Norberg and Mount at the 1983 annual meeting of the Society for Environmental Toxicology and Chemistry (Norberg and Mount, 1983). This test was subsequently used by Mount and associates in field demonstrations at Lima, OH (USEPA, 1984), and at many other locations. Growth was frequently found to be more sensitive than survival in determining the effects of complex effluents.

2.1.13 Norberg and Mount (1985) performed three single toxicant fathead minnow larval growth tests with zinc, copper, and DURSBAN®, using dilution water from Lake Superior. The results were comparable to, and had confidence intervals that overlapped with, chronic values reported in the literature for both ELS and full life-cycle tests.

2.1.14 Mount and Norberg (1984) developed a seven-day cladoceran partial life-cycle test and experimented with a number of diets for use in culturing and testing the daphnid, *Ceriodaphnia reticulata* (Norberg and Mount, 1985). As different laboratories began to use this cladoceran test, it was discovered that apparently more than one species was involved in the tests conducted by the same laboratory. Berner (1986) studied the problem and determined that perhaps as many as three variant forms were involved and it was decided to recommend the use of the more common *Ceriodaphnia dubia* rather than the originally reported *Ceriodaphnia reticulata*. The method was adopted for use in the first edition of the freshwater short-term chronic methods (USEPA, 1985e).

2.1.15 The green alga, *Selenastrum capricornutum*, bottle test was developed, after extensive design, evaluation, and application, for the National Eutrophication Research Program (USEPA, 1971). The test was later modified for use in the assessment of receiving waters and the effects of wastes originating from industrial, municipal, and agricultural point and non-point sources (USEPA, 1978a).

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2.1.16 The use of short-term toxicity tests including subchronic and chronic tests in the NPDES Program is especially attractive because they provide a more direct estimate of the safe concentrations of effluents in receiving waters than was provided by acute toxicity tests, at an only slightly increased level of effort, compared to the fish full life-cycle chronic and 28-day ELS tests and the 21-day daphnid, *Daphnia magna*, life-cycle test.

2.2 TYPES OF TESTS

2.2.1 The selection of the test type will depend on the NPDES permit requirements, the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity.

2.2.2 Effluent chronic toxicity is generally measured using a multi-concentration, or definitive test, consisting of a control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that affects the hatchability, gross morphological abnormalities, survival, growth, and/or reproduction within the prescribed period of time (four to seven days). The results of the tests are expressed in terms of the highest concentration that has no statistically significant observed effect on those responses when compared to the controls or the estimated concentration that causes a specified percent reduction in responses versus the controls.

2.2.3 Use of pass/fail tests consisting of a single effluent concentration (e.g., the receiving water concentration or RWC) and a control **is not recommended**. If the NPDES permit has a whole effluent toxicity limit for acute toxicity at the RWC, it is prudent to use that permit limit as the midpoint of a series of five effluent concentrations. This will ensure that there is sufficient information on the dose-response relationship. For example, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) (RWC + 100)/2, (3) RWC, (4) RWC/2, and (5) RWC/4. More specifically, if the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

2.2.4 Receiving (ambient) water toxicity tests commonly employ two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

2.2.5 A negative result from a chronic toxicity test does not preclude the presence of toxicity. Also, because of the potential temporal variability in the toxicity of effluents, a negative test result with a particular sample does not preclude the possibility that samples collected at some other time might exhibit chronic toxicity.

2.2.6 The frequency with which chronic toxicity tests are conducted under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

2.2.7 Tests recommended for use in this methods manual may be static non-renewal or static renewal. Individual methods specify which static type of test is to be conducted.

2.3 STATIC TESTS

2.3.1 Static non-renewal tests - The test organisms are exposed to the same test solution for the duration of the test.

2.3.2 Static-renewal tests - The test organisms are exposed to a fresh solution of the same concentration of sample every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

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2.4 ADVANTAGES AND DISADVANTAGES OF TOXICITY TEST TYPES

2.4.1 STATIC NON-RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Simple and inexpensive.
2. Very cost effective in determining compliance with permit conditions.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform many more tests in the same amount of time.
4. Smaller volume of effluent required than for static renewal or flow-through tests.

Disadvantages:

1. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
2. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Generally less sensitive than static renewal, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.4.2 STATIC RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

1. Reduced possibility of DO depletion from high COD and/or BOD, or ill effects from metabolic wastes from organisms in the test solutions.
2. Reduced possibility of loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Test organisms that rapidly deplete energy reserves are fed when the test solutions are renewed, and are maintained in a healthier state.

Disadvantages:

1. Require greater volume of effluent than non-renewal tests.
2. Generally less chance of temporal variations in waste properties.

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SECTION 3

HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

3.1.1 Each laboratory should develop and maintain an effective health and safety program, requiring an ongoing commitment by the laboratory management. This program should include (1) a safety officer with the responsibility and authority to develop and maintain a safety program, (2) the preparation of a formal, written, health and safety plan, which is provided to each of the laboratory staff, (3) an ongoing training program on laboratory safety, and (4) regularly scheduled, documented, safety inspections.

3.1.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

3.1.3 Prior to sample collection and laboratory work, personnel will determine that all necessary safety equipment and materials have been obtained and are in good condition.

3.1.4 Guidelines for the handling and disposal of hazardous materials must be strictly followed.

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

3.2.1.1 Personnel should use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes. Plastic netting on glass beakers, flasks, and other glassware minimizes breakage and subsequent shattering of the glass.

3.2.2 LABORATORY SAFETY EQUIPMENT

3.2.2.1 Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, chemical spill clean up kits, and eye fountains.

3.2.2.2 Mobile laboratories should be equipped with a telephone or other means to enable personnel to summon help in case of emergency.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1 Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see safety manuals listed in Section 3, Health and Safety, Subsection 3.5). It is recommended that personnel collecting samples and performing toxicity tests not work alone.

3.3.2 Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the toxicity test areas must be used whenever possible.

3.3.3 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.4 All containers are to be adequately labeled to indicate their contents.

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3.3.5 Staff should be familiar with safety guidelines on Material Safety Data Sheets for reagents and other chemicals purchased from suppliers. Incompatible materials should not be stored together. Good housekeeping contributes to safety and reliable results.

3.3.6 Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or under an exhaust canopy over the work area.

3.3.7 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.8 Mobile laboratories should be properly grounded to protect against electrical shock.

3.4 DISEASE PREVENTION

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, polio, and hepatitis B.

3.5 SAFETY MANUALS

3.5.1 For further guidance on safe practices when collec ting effluent samples and conducting toxicity tests, check with the permittee and consult general safety manuals, including USEPA (1986e) and Walters and Jameson (1984).

3.6 WASTE DISPOSAL

3.6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state, and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in performing the toxicity testing activities. Local fire officials should be notified of any potentially hazardous conditions.

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SECTION 4

QUALITY ASSURANCE

4.1 INTRODUCTION

4.1.1 Development and maintenance of a toxicity test laboratory quality assurance (QA) program (USEPA, 1991a) requires an ongoing commitment by laboratory management. Each toxicity test laboratory should (1) appoint a quality assurance officer with the responsibility and authority to develop and maintain a QA program; (2) prepare a quality assurance plan with stated data quality objectives (DQOs); (3) prepare a written description of laboratory standard operating procedures (SOPs) for culturing, toxicity testing, instrument calibration, sample chain-of-custody procedures, laboratory sample tracking system, glassware cleaning, etc.; and (4) provide an adequate, qualified technical staff for culturing and testing the organisms, and suitable space and equipment to assure reliable data.

4.1.2 QA practices for toxicity testing laboratories must address all activities that affect the quality of the final effluent toxicity test data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

4.1.3 Quality control practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance and general guidance on good laboratory practices and laboratory ev aluation related to toxicity testing, see FDA, (1978); USEPA, (1979d), USEPA (1980b), USEPA (1980c), and USEPA (1991c); DeWoskin (1984); and Taylor (1987).

4.1.4 Guidance for the evaluation of laboratories performing toxicity tests and laboratory evaluation criteria may be found in USEPA (1991c).

4.2 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.2.1 Separate test organism culturing and toxicity testing areas should be provided to avoid possible loss of cultures due to cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into organism culturing or testing areas, and from testing and sample preparation areas into culture rooms.

4.2.2 Laboratory and toxicity test temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities, Equipment and Supplies; and specific toxicity test method).

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are the fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia*, and the green alga, *Selenastrum capricornutum*. The fish and invertebrates should appear healthy, behave normally, feed well, and have low mortality in the cultures, during holding, and in test controls. Test organisms should be positively identified to species (see Section 6, Test Organisms).

4.4 LABORATORY WATER USED FOR CULTURING AND TEST DILUTION WATER

4.4.1 The quality of water used for test organism culturing and for dilution water used in toxicity tests is extremely important. Water for these two uses should come from the same source. The dilution water used in effluent toxicity tests will depend in part on the objectives of the study and logistical constraints, as discussed in detail in Section 7, Dilution Water. For tests performed to meet NPDES objectives, synthetic, moderately hard water should be used.

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The dilution water used for internal quality assurance tests with organisms, food, and reference toxicants should be the water routinely used with success in the laboratory. Types of water are discussed in Section 5, Facilities, Equipment and Supplies. Water used for culturing and test dilution should be analyzed for toxic metals and organics at least annually or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. The concentration of the metals Al, As, Cr, Co, Cu, Fe, Pb, Ni, and Zn, expressed as total metal, should not exceed 1 mg/L each, and Cd, Hg, and Ag, expressed as total metal, should not exceed 100 ng/L each. Total organochlorine pesticides plus PCBs should be less than 50 ng/L (APHA, 1992). Pesticide concentrations should not exceed USEPA's Ambient Water Quality chronic criteria values where available.

4.5 EFFLUENT AND RECEIVING WATER SAMPLING AND HANDLING

4.5.1 Sample holding times and temperatures of effluent samples collected for on-site and off-site testing must conform to conditions described in Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

4.6 TEST CONDITIONS

4.6.1 Water temperature should be maintained within the limits specified for each test. The temperature of test solutions must be measured by placing the thermometer or probe directly into the test solutions, or by placing the thermometer in equivalent volumes of water in surrogate vessels positioned at appropriate locations among the test vessels. Temperature should be recorded continuously in at least one test vessel for the duration of each test. Test solution temperatures should be maintained within the limits specified for each test. DO concentration and pH should be checked at the beginning of each test and daily throughout the test period.

4.7 QUALITY OF TEST ORGANISMS

4.7.1 The health of test organisms is primarily assessed by the performance (survival, growth, and/or reproduction) of organisms in control treatments of individual tests. The health and sensitivity of test organisms is also assessed by reference toxicant testing. In addition to documenting the sensitivity and health of test organisms, reference toxicant testing is used to initially demonstrate acceptable laboratory performance (Subsection 4.15) and to document ongoing laboratory performance (Subsection 4.16).

4.7.2 Regardless of the source of test organisms (in-house cultures or purchased from external suppliers), the testing laboratory must perform at least one acceptable reference toxicant test per month for each toxicity test method conducted in that month (Subsection 4.16). If a test method is conducted only monthly, or less frequently, a reference toxicant test must be performed concurrently with each effluent toxicity test.

4.7.3 When acute or short-term chronic toxicity tests are performed with effluents or receiving waters using test organisms obtained from outside the test laboratory, concurrent toxicity tests of the same type must be performed with a reference toxicant, unless the test organism supplier provides control chart data from at least the last five monthly short-term chronic toxicity tests using the same reference toxicant and control conditions (see Section 6, Test Organisms).

4.7.4 The supplier should certify the species identification of the test organisms, and provide the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted.

4.7.5 If routine reference toxicant tests fail to meet test acceptability criteria, then the reference toxicant test must be immediately repeated.

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4.8 FOOD QUALITY

4.8.1 The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. This is especially true for the unsaturated fatty acid content of brine shrimp nauplii, *Artemia*. Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. *Artemia* cysts, and other foods must be obtained as described in Section 5, Facilities, Equipment, and Supplies.

4.8.2 Problems with the nutritional suitability of food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. If a batch of food is suspected to be defective, the performance of organisms fed with the new food can be compared with the performance of organisms fed with a food of known quality in side-by-side tests. If the food is used for culturing, its suitability should be determined using a short-term chronic test which will determine the affect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source. Where applicable, foods used only in chronic toxicity tests can be compared with a food of known quality in side-by-side, multi-concentration chronic tests, using the reference toxicant regularly employed in the laboratory QA program.

4.8.3 New batches of food used in culturing and testing should be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. If the concentration of total organochlorine pesticides exceeds 0.15 mg/g wet weight, or the concentration of total organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight, or toxic metals (Al, As, Cr, Cd, Cu, Pb, Ni, Zn, expressed as total metal) exceed 20 µg/g wet weight, the food should not be used (for analytical methods see AOAC, 1990 and USDA, 1989). For foods (e.g., such as YCT) which are used to culture and test organisms, the quality of the food should meet the requirements for the laboratory water used for culturing and test dilution water as described in Section 4.4 above.

4.9 ACCEPTABILITY OF SHORT-TERM CHRONIC TOXICITY TESTS

4.9.1 For the tests to be acceptable, control survival in fathead minnow, *Pimephales promelas*, and the daphnid, *Ceriodaphnia dubia*, tests must be 80% or greater. At the end of the test, the average dry weight of surviving seven-day-old fathead minnows in control chambers must equal or exceed 0.25 mg. In *Ceriodaphnia dubia* controls, 60% or more of the surviving females must have produced their third brood in 7 ± 1 days, and the number of young per surviving female must be 15 or greater. In algal toxicity tests, the mean cell density in the controls after 96 h must equal or exceed 1×10^6 cells/mL and not vary more than 20% among replicates. If these criteria are not met, the test must be repeated.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of the test would depend on the experience and professional judgment of the laboratory investigator and the reviewing staff of the regulatory authority. Any deviation from test specifications must be noted when reporting data from the test.

4.10 ANALYTICAL METHODS

4.10.1 Routine chemical and physical analyses for culture and dilution water, food, and test solutions must include established quality assurance practices outlined in USEPA methods manuals (USEPA, 1979a and USEPA, 1979b).

4.10.2 Reagent containers should be dated and catalogued when received from the supplier, and the shelf life should not be exceeded. Also, working solutions should be dated when prepared, and the recommended shelf life should be observed.

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4.11 CALIBRATION AND STANDARDIZATION

4.11.1 Instruments used for routine measurements of chemical and physical parameters such as pH, DO, temperature, and conductivity, must be calibrated and standardized according to instrument manufacturer's procedures as indicated in the general section on quality assurance (see USEPA Methods 150.1, 360.1, 170.1, and 120.1 in USEPA, 1979b). Calibration data are recorded in a permanent log book.

4.11.2 Wet chemical methods used to measure hardness, alkalinity and total residual chlorine must be standardized prior to use each day according to the procedures for those specific USEPA methods (see USEPA Methods 130.2 and 310.1 in USEPA, 1979b).

4.12 REPLICATION AND TEST SENSITIVITY

4.12.1 The sensitivity of the tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data.

4.13 VARIABILITY IN TOXICITY TEST RESULTS

4.13.1 Factors which can affect test success and precision include (1) the experience and skill of the laboratory analyst; (2) test organism age, condition, and sensitivity; (3) dilution water quality; (4) temperature control; and (5) the quality and quantity of food provided. The results will depend upon the species used and the strain or source of the test organisms, and test conditions, such as temperature, DO, food, and water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. Jensen (1972) discussed the relationship between sample size (number of fish) and the standard error of the test, and considered 20 fish per concentration as optimum for Probit Analysis.

4.14 TEST PRECISION

4.14.1 The ability of the laboratory personnel to obtain consistent, precise results must be demonstrated with reference toxicants before they attempt to measure effluent toxicity. The single-laboratory precision of each type of test to be used in a laboratory should be determined by performing at least five tests with a reference toxicant.

4.14.2 Test precision can be estimated by using the same strain of organisms under the same test conditions and employing a known toxicant, such as a reference toxicant.

4.14.3 Interlaboratory precision data from a 1991 study of chronic toxicity tests with two species using the reference toxicants potassium chloride and copper sulfate are shown in Table 1. Table 2 shows interlaboratory precision data from a study of three chronic toxicity test methods using effluent, receiving water, and reference toxicant sample types (USEPA, 2001a; USEPA, 2001b). The effluent sample was a municipal wastewater spiked with KCl, the receiving waster sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Additional precision data for each of the tests described in this manual are presented in the sections describing the individual test methods.

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TABLE 1. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 1991: SUMMARY OF RESPONSES USING A REFERENCE TOXICANT[1]

Organism	Endpoint	No. Labs	% Effluent[2]	SD	CV(%)
Pimephales promelas	Survival, NOEC	146	NA	NA	NA
	Growth, IC25	124	4.67	1.87	40.0
	Growth, IC50	117	6.36	2.04	32.1
	Growth, NOEC	142	NA	NA	NA
Ceriodaphnia dubia	Survival, NOEC	162	NA	NA	NA
	Reproduction, IC25	155	2.69	1.96	72.9
	Reproduction, IC50	150	3.99	2.35	58.9
	Reproduction, NOEC	156	NA	NA	NA

[1] From a national study of interlaboratory precision of toxicity test data performed in 1991 by the Environmental Monitoring Systems Laboratory- Cincinnati, U.S. Environmental Protection Agency, Cincinnati, OH 45268. Participants included Federal, state, and private laboratories engaged in NPDES permit compliance monitoring.

[2] Expressed as % effluent; in reality it was a reference toxicant (KCl) but was not known by the persons conducting the tests.

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TABLE 2. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 2000: PRECISION OF RESPONSES USING EFFLUENT, RECEIVING WATER, AND REFERENCE TOXICANT SAMPLE TYPES[1].

Organism	Endpoint	Number of Tests[2]	CV (%)[3]
Pimephales promelas	Growth, IC25	73	20.9
Ceriodaphnia dubia	Reproduction, IC25	34	35.0
Selenastrum capricornutum (with EDTA)	Growth, IC25	21	34.3
	Growth, IC50	22	32.2
Selenastrum capricornutum (without EDTA)	Growth, IC25	21	58.5
	Growth, IC50	22	58.5

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 Represents the number of valid tests (i.e., those that met test acceptability criteria) that were used in the analysis of precision. Invalid tests were not used.

3 CVs based on total interlaboratory variability (including both within-laboratory and between-laboratory components of variability) and averaged across sample types. IC25s or IC50s were pooled for all laboratories to calculate the CV for each sample type. The resulting CVs were then averaged across sample types.

4.14.4 Additional information on toxicity test precision is provided in the Technical Support Document for Water Quality-based Control (see pp. 2-4, and 11-15 in USEPA, 1991a).

4.14.5 In cases where the test data are used in Probit Analysis or other point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis), precision can be described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated endpoints from the replicated tests. In cases where the test data are used in the Linear Interpolation Method, precision can be estimated by empirical confidence intervals derived by using the ICPIN Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). However, in cases where the results are reported in terms of the No-Observed-Effect Concentration (NOEC) and Lowest-Observed-Effect Concentration (LOEC) (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis) precision can only be described by listing the NOEC-LOEC interval for each test. It is not possible to express precision in terms of a commonly used statistic. However, when all tests of the same toxicant yield the same NOEC-LOEC interval, maximum precision has been attained. The "true" no effect concentration could fall anywhere within the interval, NOEC ± (NOEC minus LOEC).

4.14.6 It should be noted here that the dilution factor selected for a test determines the width of the NOEC-LOEC interval and the inherent maximum precision of the test. As the absolute value of the dilution factor decreases, the width of the NOEC-LOEC interval increases, and the inherent maximum precision of the test decreases. When a dilution factor of 0.3 is used, the NOEC could be considered to have a relative variability as high as ± 300%. With a dilution factor of 0.5, the NOEC could be considered to have a relative variability of ± 100%. As a result of the variability of different dilution factors, **USEPA recommends the use of the dilution factor of 0.5 or greater**. Other factors which can affect test precision include: test organism age, condition, and sensitivity; temperature

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control; and feeding.

4.15 DEMONSTRATING ACCEPTABLE LABORATORY PERFORMANCE

4.15.1 It is a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes. To meet this requirement, the intralaboratory precision, expressed as percent coefficient of variation (CV%), of each type of test to be used in the laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (i.e., the same test duration, type of dilution water, age of test organisms, feeding, etc.), and the same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations.

4.16 DOCUMENTING ONGOING LABORATORY PERFORMANCE

4.16.1 Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method conducted in the laboratory during that month. For a given test method, successive tests must be performed with the same reference toxicant, at the same concentrations, in the same dilution water, using the same data analysis methods. Precision may vary with the test species, reference toxicant, and type of test. Each laboratory's reference toxicity data will reflect conditions unique to that facility, including dilution water, culturing, and other variables; however, each laboratory's reference toxicity results should reflect good repeatability.

4.16.2 A control chart should be prepared for each combination of reference toxicant, test species, test conditions, and endpoints. Toxicity endpoints from five or six tests are adequate for establishing the control charts. Successive toxicity endpoints (NOECs, IC25s, LC50s, etc.) should be plotted and examined to determine if the results (X_1) are within prescribed limits (Figure 1). The chart should plot logarithm of concentration on the vertical axis against the date of the test or test number on the horizontal axis. The types of control charts illustrated (see USEPA, 1979a) are used to evaluate the cumulative trend of results from a series of samples, thus reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. For endpoints that are point estimates (LC50s and IC25s), the cumulative mean ($\bar{X}$) and upper and lower control limits ($\pm$ 2S) are re-calculated with each successive test result. Endpoints from hypothesis tests (NOEC, NOAEC) from each test are plotted directly on the control chart. The control limits would consist of one concentration interval above and below the concentration representing the central tendency. After two years of data collection, or a minimum of 20 data points, the control chart should be maintained using only the 20 most recent data points.

4.16.3 Laboratories should compare the calculated CV (i.e., standard deviation / mean) of the IC25 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-2 in USEPA, 2000b). If the calculated CV exceeds the 75th percentile of CVs reported nationally, the laboratory should use the 75th and 90th percentiles to calculate warning and control limits, respectively, and the laboratory should investigate options for reducing variability. Note: Because NOECs can only be a fixed number of discrete values, the mean, standard deviation, and CV cannot be interpreted and applied in the same way that these descriptive statistics are interpreted and applied for continuous variables such as the IC25 or LC50.

4.16.4 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified. In the case of endpoints that are point estimates (LC50s and IC25s), at the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone. If more than one out of 20 reference toxicant tests fall outside the control limits, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Control limits for the NOECs will also be exceeded occasionally, regardless of how well a laboratory performs. In those instances when the laboratory can document the cause for the outlier (e.g., operator error, culture health or test system failure), the outlier should be excluded from the future calculations of the control limits. If two or more consecutive tests do not fall within the control limits, the results

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must be explained and the reference toxicant test must be immediately repeated. Actions taken to correct the problem must be reported.

4.16.5 If the toxicity value from a given test with a reference toxicant falls well outside the expected range for the other test organisms when using the standard dilution water and other test conditions, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Performance should improve with experience, and the control limits for endpoints that are point estimates should gradually narrow. However, control limits of ± 2S will be exceeded 5% of the time by chance alone, regardless of how well a laboratory performs. Highly proficient laboratories which develop very narrow control limits may be unfairly penalized if a test result which falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits should be considered in determining whether or not a reference toxicant test result falls "well" outside the expected range. The width of the control limits may be evaluated by comparing the calculated CV (i.e., standard deviation / mean) of the IC25 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-2 in USEPA, 2000b). In determining whether or not a reference toxicant test result falls "well" outside the expected range, the result also may be compared with upper and lower bounds for ±3S, as any result outside these control limits would be expected to occur by chance only 1 out of 100 tests (Environment Canada, 1990). When a result from a reference toxicant test is outside the 99% confidence intervals, the laboratory must conduct an immediate investigation to assess the possible causes for the outlier.

4.16.6 Reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. Reference toxicant testing is used for evaluating the health and sensitivity of organisms over time and for documenting initial and ongoing laboratory performance. While reference toxicant test results should not be used as a *de facto* criterion for test rejection, effluent and receiving water test results should be reviewed and interpreted in the light of reference toxicant test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increased test organism sensitivity or toward decreased test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test.

4.17 REFERENCE TOXICANTS

4.17.1 Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests. EMSL-Cincinnati hopes to release USEPA-certified solutions of cadmium and copper for use as reference toxicants through cooperative research and development agreements with commercial suppliers, and will continue to develop additional reference toxicants for future release. Standard reference materials can be obtained from commercial supply houses, or can be prepared inhouse using reagent grade chemicals. The regulatory agency should be consulted before reference toxicant(s) are selected and used.

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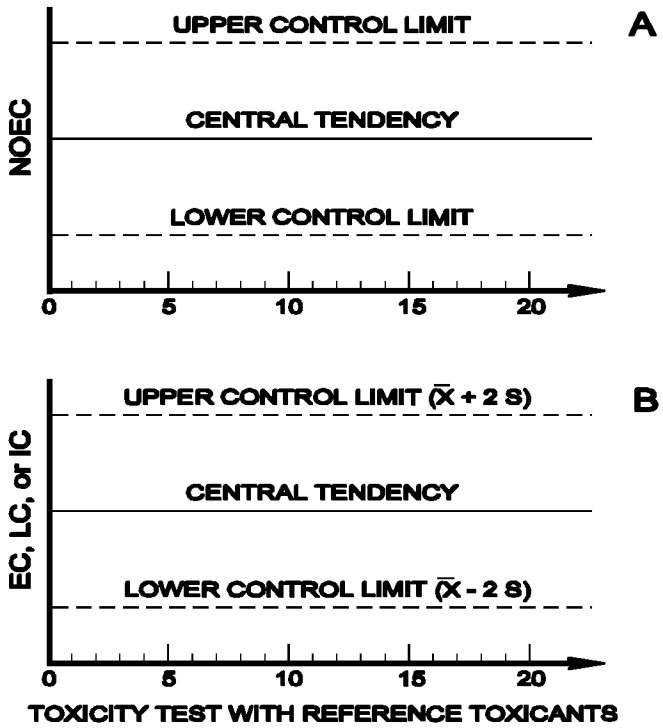

Figure 1. Control charts. (A) hypothesis testing results; (B) point estimates (LC, EC, or IC).

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

$$S = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - \frac{\left(\sum_{i=1}^{n} X_i\right)^2}{n}}{n-1}}$$

Where: X_i = Successive toxicity values from toxicity tests.

n = Number of tests.

$\bar{X}$ = Mean toxicity value.

S = Standard deviation.

4.18 RECORD KEEPING

4.18.1 Proper record keeping is important. A complete file should be maintained for each individual toxicity test or group of tests on closely related samples. This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions employed; and results of reference toxicant tests. Laboratory data should be recorded on a real-time basis to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed and dated by the laboratory personnel performing the tests.

4.18.2 The regulatory authority should retain records pertaining to discharge permits. Permittees are required to retain records pertaining to permit applications and compliance for a minimum of 3 years [40 CFR 122.41(j)(2)].

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SECTION 5

FACILITIES, EQUIPMENT, AND SUPPLIES

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities must include equipment for rearing and/or holding organisms. Culturing facilities for test organisms may be desirable in fixed laboratories which perform large numbers of tests. Temperature control can be achieved using circulating water baths, heat exchangers, or environmental chambers. Water used for rearing, holding, acclimating, and testing organisms may be ground water, receiving water, dechlorinated tap water, or reconstituted synthetic water. Dechlorination can be accomplished by carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg (anhydrous) sodium thiosulfate/L will reduce 1.0 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable. Air used for aeration must be free of oil and toxic vapors. Oil-free air pumps should be used where possible. Particulates can be removed from the air using BALSTON® Grade BX or equivalent filters, and oil and other organic vapors can be removed using activated carbon filters (BALSTON®, C-1 filter, or equivalent).

5.1.2 The facilities must be well ventilated and free from fumes. Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories and/or sample holding areas is not circulated to test organism culture rooms or toxicity test rooms, or that air from toxicity test rooms does not contaminate culture areas. Sample preparation, culturing, and toxicity test areas should be separated to avoid cross contamination of cultures or toxicity test solutions with toxic fumes. Air pressure differentials between such rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely- fitting doors. Organisms should be shielded from external disturbances.

5.1.3 Materials used for exposure chambers, tubing, etc., that come in contact with the effluent and dilution water should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON ®) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Containers made of plastics, such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used as test chambers or to ship, store and transfer effluents and receiving waters, but they should not be reused unless absolutely necessary, because they could carry over adsorbed toxicants from one test to another, if reused. However, these containers may be repeatedly reused for storing uncontaminated waters, such as deionized or laboratory-prepared dilution waters and receiving waters. Glass or disposable polystyrene containers can be used for test chambers. The use of large ($ 20 L) glass carboys is discouraged for safety reasons.

5.1.4 New plastic products of a type not previously used should be tested for toxicity before initial use by exposing the test organisms in the test system where the material is used. Equipment (pumps, valves, etc.) which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below (see Section 5, Facilities, Equipment and Supplies, Subsection 5.3.2). Fiberglass and stainless steel, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system, but once contaminated with pollutants the fiberglass should not be reused. All material should be flushed or rinsed thoroughly with the test media before using in the test.

5.1.5 Copper, galvanized material, rubber, brass, and lead must not come in contact with culturing, holding, acclimation, or dilution water, or with effluent samples and test solutions. Some materials, such as several types of neoprene rubber (commonly used for stoppers), may be toxic and should be tested before use.

5.1.6 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

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5.2 TEST CHAMBERS

5.2.1 Test chamber size and shape are varied according to size of the test organism. Requirements are specified in each toxicity test method.

5.3 CLEANING TEST CHAMBERS AND LABORATORY APPARATUS

5.3.1 New plasticware used for sample collection or organism exposure vessels does not require thorough cleaning before use. It is sufficient to rinse new sample containers once with dilution water before use. New glassware must be soaked overnight in 10% acid (see below) and rinsed well in deionized water and dilution water.

5.3.2 All non-disposable sample containers, test vessels, tanks, and other equipment that have come in contact with effluent must be washed after use to remove contaminants as described below.

1. Soak 15 min in tap water and scrub with detergent, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh, dilute (10%, V:V) hydrochloric or nitric acid to remove scale, metals and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

5.3.3 Special requirements for cleaning glassware used in the green alga, *Selenastrum capricornutum*, toxicity tests (Method 1003.0, Section 14). Prepare all graduated cylinders, test flasks, bottles, volumetric flasks, centrifuge tubes and vials used in algal assays as follows:

1. Wash with non-phosphate detergent solution, preferably heated to $ 50°C. Brush the inside of flasks with a stiff-bristle brush to loosen any attached material. The use of a commercial laboratory glassware washer or heavy-duty kitchen dishwasher (under-counter type) is highly recommended.
2. Rinse with tap water.
3. Test flasks should be thoroughly rinsed with acetone and a 10% solution (by volume) of reagent grade hydrochloric acid (HCl). It may be advantageous to soak the flasks in 10% HCl for several days. Fill vials and centrifuge tubes with the 10% HCl solution and allow to stand a few minutes; fill all larger containers to about one-tenth capacity with HCl solution and swirl so that the entire surface is bathed.
4. Rinse twice with MILLIPORE® MILLI-Q® OR QPAK™$_2$, or equivalent, water.
5. New test flasks, and all flasks which through use may become contaminated with toxic organic substances, must be rinsed with pesticide-grade acetone or heat-treated before use. To thermally degrade organics, place glassware in a high temperature oven at 400°C for 30 min. After cooling, go to 7. If acetone is used, go to 6.
6. Rinse thoroughly with MILLIPORE® MILLI-Q® or QPAK™$_2$, or equivalent water, and dry in an 105°C oven. All glassware should be autoclaved before use and between uses.
7. Cover the mouth of each chamber with aluminum foil or other closure, as appropriate, before storing.

5.3.4 The use of sterile, disposable pipets will eliminate the need for pipet washing and minimize the possibility of contaminating the cultures with toxic substances.

5.3.5 All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

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5.4 APPARATUS AND EQUIPMENT FOR CULTURING AND TOXICITY TESTS

5.4.1 Apparatus and equipment requirements for culturing and testing are specified in each toxicity test method. Also, see USEPA, 2002a.

5.4.2 WATER PURIFICATION SYSTEM

5.4.2.1 A good quality, laboratory grade deionized water, providing a resistance of 18 megaohm-cm, must be available in the laboratory and in sufficient quantity for laboratory needs. Deionized water may be obtained from MILLIPORE® Milli-Q®, MILLIPORE® QPAK™$_2$ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade deionizer with preconditioned water from a Culligan®, Continental®, or equivalent mixed-bed water treatment system.

5.5 REAGENTS AND CONSUMABLE MATERIALS

5.5.1 SOURCES OF FOOD FOR CULTURE AND TOXICITY TESTS

1. Brine shrimp, *Artemia* sp., cysts -- Many commercial sources of brine shrimp cysts are available.
2. Frozen adult brine shrimp, *Artemia* -- Available from most pet supply shops or other commercial sources.
3. Flake fish food -- TETRAMIN® and BIORIL® are available from most pet shops.
4. Trout chow -- Available from commercial sources.
5. Cereal leaves, CEROPHYLL® or equivalent -- Available from commercial sources.
6. Yeast -- Packaged dry yeast, such as Fleischmann's, or equivalent, can be purchased at the local grocery store or commercial sources.
7. Alfalfa Rabbit Pellets -- Available from feed stores as Purina rabbit chow.
8. Algae - Available from commercial sources.

5.5.1.1 All food should be tested for nutritional suitability and chemically analyzed for organochlorine pesticides, PCBs, and toxic metals (see Section 4, Quality Assurance).

5.5.2 Reagents and consumable materials are specified in each toxicity test method section. Also, see Section 4, Quality Assurance.

5.6 TEST ORGANISMS

5.6.1 Test organisms should be obtained from inhouse cultures or from commercial suppliers (see specific test method; Section 4, Quality Assurance; and Section 6, Test Organisms).

5.7 SUPPLIES

5.7.1 See test methods (see Sections 11-14) for specific supplies.

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SECTION 6

TEST ORGANISMS

6.1 TEST SPECIES

6.1.1 The species used in characterizing the chronic toxicity of effluents and/or receiving waters will depend on the requirements of the regulatory authority and the objectives of the test. It is essential that good quality test organisms be readily available throughout the year from inhouse or commercial sources to meet NPDES monitoring requirements. The organisms used in the toxicity tests must be identified to species. If there is any doubt as to the identity of the test organism, representative specimens should be sent to a taxonomic expert to confirm the identification.

6.1.2 Toxicity test conditions and culture methods for the species listed in Subsection 6.1.3 are provided in this manual also, see USEPA, 2002a.

6.1.3 The organisms used in the short-term chronic toxicity tests described in this manual are the fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia* (Berner, 1986), and the green alga, *Selenastrum capricornutum*.

6.1.4 Some states have developed culturing and testing methods for indigenous species that may be as sensitive, or more sensitive, than the species recommended in Subsection 6.1.3. However, USEPA allows the use of indigenous species only where state regulations require their use or prohibit importation of the recommended species in Subsection 6.1.3. Where state regulations prohibit importation of non-native fishes or the use of recommended test species, permission must be requested from the appropriate state agency prior to their use.

6.1.5 Where states have developed culturing and te sting methods for indigenous species other than those recommended in this manual, data comparing the sensitivity of the substitute species and the one or more recommended species must be obtained in side-by-side toxicity tests with reference toxicants and/or effluents, to ensure that the species selected are at least as sensitive as the recommended species. These data must be submitted to the permitting authority (State or Region) if required. USEPA acknowledges that reference toxicants prepared from pure chemicals may not always be representative of effluents. However, because of the observed and/or potential variability in the quality and toxicity of effluents, it is not possible to specify a representative effluent.

6.1.6 Guidance for the selection of test organisms where the salinity of the effluent and/or receiving water requires special consideration is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991a).

1. Where the salinity of the receiving water is < 1‰, freshwater organisms are used regardless of the salinity of the effluent.
2. Where the salinity of the receiving water is $ 1‰, the choice of organisms depends on state water quality standards and/or permit requirements.

6.2 SOURCES OF TEST ORGANISMS

6.2.1 The test organisms recommended in this manual can be cultured in the laboratory using culturing and handling methods for each organism described in the respective test method sections. The fathead minnow, *Pimephales promelas,* culture method is given in Section 11 and not repeated in Section 12. Also, see USEPA (2002a).

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6.2.2 Inhouse cultures should be established wherever it is cost effective. If inhouse cultures cannot be maintained or it is not cost effective, test organisms or starter cultures should be purchased from experienced commercial suppliers (see USEPA, 2002a).

6.2.3 Starter cultures of the green algae, *Selenastrum capricornutum, S. minutum,* and *Chlamydomonas reinhardti* are available from commercial suppliers.

6.2.4 Because the daphnid, *Ceriodaphnia dubia*, must be cultured individually in the laboratory for at least seven days before the test begins, it will be necessary to obtain a starter culture from a commercial source at least three weeks before the test is to begin if they are not being cultured inhouse.

6.2.5 If, because of their source, there is any uncertainty concerning the identity of the organisms, it is advisable to have them examined by a taxonomic specialist to confirm their identification. For detailed guidance on identification, see the individual test methods.

6.2.6 FERAL (NATURAL OCCURRING, WILD CAUGHT) ORGANISMS

6.2.6.1 The use of test organisms taken from the receiving water has strong appeal, and would seem to be a logical approach. However, it is generally impractical and not recommended for the following reasons:

1. Sensitive organisms may not be present in the receiving water because of previous exposure to the effluent or other pollutants.
2. It is often difficult to collect organisms of the required age and quality from the receiving water.
3. Most states require collecting permits, which may be difficult to obtain. Therefore, it is usually more cost effective to culture the organisms in the laboratory or obtain them from private, state, or Federal sources. The fathead minnow, *Pimephales promelas*, the daphnid, *Ceriodaphnia dubia*, and the green alga, *Selenastrum capricornutum*, are easily cultured in the laboratory or readily available commercially.
4. The required QA/QC records, such as the single laboratory precision data, would not be available.
5. Since it is mandatory that the identity of the test organism be known to species level, it would be necessary to examine each organism caught in the wild to confirm its identity. This would usually be impractical or, at the least, very stressful to the organisms.
6. Test organisms obtained from the wild must be observed in the laboratory for a minimum of one week prior to use, to assure that they are free of signs of parasitic or bacterial infections and other adverse effects. Fish captured by electroshocking must not be used in toxicity testing.

6.2.6.2 Guidelines for collecting natural occurring organisms are provided in USEPA (1973), USEPA (1990) and USEPA (1993b).

6.2.7 Regardless of their source, test organisms should be carefully observed to ensure that they are free of signs of stress and disease, and in good physical condition. Some species of test organisms can be obtained from commercial stock certified as "disease-free".

6.3 **LIFE STAGE**

6.3.1 Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as larval fish, is required for all tests. In a given test, all organisms should be approximately the same age and should be taken from the same source. Since age may affect the results of the tests, it would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility.

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6.4 LABORATORY CULTURING

6.4.1 Instructions for culturing and/or holding the recommended test organisms are included in the respective test methods (also, see USEPA, 2002a).

6.5 HOLDING AND HANDLING TEST ORGANISMS

6.5.1 Test organisms should not be subjected to changes of more than 3°C in water temperature in any 12 h period or 2 units of pH in any 24-h period.

6.5.2 Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch a dry surface or are injured during handling must be discarded. Dipnets are best for handling larger organisms. These nets are commercially available or can be made from small-mesh nylon netting, silk batting cloth, plankton netting, or similar material. Wide-bore, smooth glass tubes (4 to 8 mm ID) with rubber bulbs or pipettors (such as PROPIPETTE®) should be used for transferring smaller organisms such as larval fish.

6.5.3 Holding tanks for fish are supplied with good quality water (see Section 5, Facilities, Equipment, and Supplies) with flow-through rate of at least two tank volumes per day. Otherwise use a recirculation system where water flows through an activated carbon or undergravel filter to remove dissolved metabolites. Culture water can also be piped through high intensity ultraviolet light sources for disinfection, and to photodegrade dissolved organics.

6.5.4 Crowding must be avoided because it will stress the organisms and lower the DO concentrations to unacceptable levels. The solution of oxygen depends on temperature and altitude. The DO must be maintained at a minimum of 4.0 mg/L. Aerate gently if necessary.

6.5.5 The organisms should be observed carefully each day for signs of disease, stress, physical damage, or mortality. Dead and abnormal organisms should be removed as soon as observed. It is not uncommon for some fish mortality (5-10%) to occur during the first 48 h in a holding tank because of individuals that refuse to feed on artificial food and die of starvation. Organisms in the holding tanks should generally be fed as in the cultures (see culturing methods in the respective methods).

6.5.6 Fish should be fed as much as they will eat at least once a day with live brine shrimp nauplii, *Artemia,* or frozen adult brine shrimp, or dry food (frozen food should be completely thawed before use). Adult brine shrimp can be supplemented with commercially prepared food such as TETRAMIN® or BIORIL® flake food, or equivalent. Excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning.

6.5.7 A daily record of feeding, behavioral observations, and mortality should be maintained.

6.6 TRANSPORTATION TO THE TEST SITE

6.6.1 Organisms are transported from the base or supply laboratory to a remote test site in culture water or standard dilution water in plastic bags or large-mouth screw-cap (500 mL) plastic bottles in styrofoam coolers. Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags or by use of an airstone supplied by a portable pump. The DO concentration must not fall below 4.0 mg/L.

6.6.2 Upon arrival at the test site, the organisms are transferred to receiving water if receiving water is to be used as the test dilution water. All but a small volume of the holding water (approximately 5%) is removed by siphoning and replaced slowly over a 10 to 15 minute period with dilution water. If receiving water is to be used as the dilution water, caution must be exercised in exposing the test organisms to it, because of the possibility that it might be toxic. For this reason, it is recommended that only approximately 10% of the test organisms be exposed initially

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to the dilution water. If this group does not show excessive mortality or obvious signs of stress in a few hours, the remainder of the test organisms may be transferred to the dilution water.

6.6.3 A group of organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed, or if mortality appears to exceed 10% preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. The mortality may be due to the presence of toxicity, if the receiving water is used as dilution water, rather than a diseased condition of the test organisms. If the acclimation process is repeated with a new group of test organisms and excessive mortality occurs, it is recommended that an alternative source of dilution water be used.

6.7 TEST ORGANISM DISPOSAL

6.7.1 When the toxicity test(s) is concluded, all test organisms (including controls) should be humanely destroyed and disposed of in an appropriate manner.

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SECTION 7

DILUTION WATER

7.1 TYPES OF DILUTION WATER

7.1.1 The type of dilution water used in effluent toxicity tests will depend largely on the objectives of the study.

7.1.1.1 If the objective of the test is to estimate the absolute chronic toxicity of the effluent, a synthetic (standard) dilution water is used. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.2 If the objective of the test is to estimate the chronic toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of receiving water (if non-toxic), collected either upstream and outside the influence of the outfall, or with other uncontaminated natural water (ground or surface water) or standard dilution water having approximately the same characteristics (hardness, alkalinity, and conductivity) as the receiving water. Seasonal variations in the quality of receiving waters may affect effluent toxicity. Therefore, the pH, alkalinity, hardness, and conductivity of receiving water samples should be determined before each use. If the test organisms have been cultured in water which is different from the test dilution water, a second set of controls, using culture water, should be included in the test.

7.1.1.3 If the objective of the test is to determine the additive or mitigating effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected immediately upstream or outside the influence of the outfall. A second set of controls, using culture water, should be included in the test.

7.1.2 An acceptable dilution water is one which is appropriate for the objectives of the test; supports adequate performance of the test organisms with respect to survival, growth, reproduction, or other responses that may be measured in the test (i.e., consistently meets test acceptability criteria for control responses); is consistent in quality; and does not contain contaminants that could produce toxicity. Receiving waters, synthetic waters, or synthetic waters adjusted to approximate receiving water characteristics may be used for dilution provided that the water meets the above listed qualifications for an acceptable dilution water. USEPA (2000a) provides additional guidance on selecting appropriate dilution waters.

7.1.3 When dual controls (one control using culture water and one control using dilution water) are used (see Subsections 7.1.1.1 - 7.1.1.3 above), the dilution water control should be used to determine test acceptability. It is also the dilution water control that should be compared to effluent treatments in the calculation and reporting of test results. The culture water control should be used to evaluate the appropriateness of the dilution water source. Significant differences between organism responses in culture water and dilution water controls could indicate toxicity in the dilution water and may suggest an alternative dilution water source. USEPA (2000a) provides additional guidance on dual controls.

7.2 STANDARD, SYNTHETIC DILUTION WATER

7.2.1 Standard, synthetic dilution water is prepared with deionized water and reagent grade chemicals or mineral water (Tables 3 and 4). The source water for the deionizer can be ground water or tap water.

7.2.2 DEIONIZED WATER USED TO PREPARE STANDARD, SYNTHETIC, DILUTION WATER

7.2.2.1 Deionized water is obtained from a MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$ or equivalent system. It is advisable to provide a preconditioned (deionized) feed water by using a Culligan®, Continental®, or

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equivalent system in front of the MILLIPORE® System to extend the life of the MILLIPORE® cartridges (see Section 5, Facilities, Equipment, and Supplies).

7.2.2.2 The recommended order of the cartridges in a four-cartridge deionizer (i.e., MILLI-Q® System or equivalent) is (1) ion exchange, (2) ion exchange, (3) carbon, and (4) organic cleanup (such as ORGANEX-Q®, or equivalent) followed by a final bacteria filter. The QPAK™$_2$ water system is a sealed system which does not allow for the rearranging of the cartridges. However, the final cartridge is an ORGANEX-Q® filter, followed by a final bacteria filter. Commercial laboratories using this system have not experienced any difficulty in using the water for culturing or testing. Reference to the MILLI-Q® systems throughout the remainder of the manual includes all MILLIPORE® or equivalent systems.

7.2.3 STANDARD, SYNTHETIC FRESHWATER

7.2.3.1 To prepare 20 L of synthetic, moderately hard, reconstituted water, use the reagent grade chemicals in Table 3 as follows:

1. Place 19 L of MILLI-Q®, or equivalent, water in a properly cleaned plastic carboy.
2. Add 1.20 g of $MgSO_4$, 1.92 g $NaHCO_3$, and 0.080g KCl to the carboy.
3. Aerate overnight.
4. Add 1.20 g of $CaSO_4 \cdot 2H_20$ to 1 L of MILLI-Q® or equivalent deionized water in a separate flask. Stir on magnetic stirrer until calcium sulfate is dissolved, add to the 19 L above, and mix well.
5. For *Ceriodaphnia dubia* culturing and testing, add sufficient sodium selenate (Na_2SeO_4) to provide 2 mg selenium per liter of final dilution water.
6. Aerate the combined solution vigorously for an additional 24 h to dissolve the added chemicals and stabilize the medium.
7. The measured pH, hardness, etc., should be as listed in Table 3.

TABLE 3. PREPARATION OF SYNTHETIC FRESHWATER USING REAGENT GRADE CHEMICALS[1]

Water Type	Reagent Added (mg/L)[2]				Approximate Final Water Quality		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	pH[3]	Hardness[4]	Alkalinity[4]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	57-64
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

1 Taken in part from Marking and Dawson (1973).
2 Add reagent grade chemicals to deionized water.
3 Approximate equilibrium pH after 24 h of aeration.
4 Expressed as mg $CaCO_3$/L.

7.2.3.2 If large volumes of synthetic reconstituted water will be needed, it may be advisable to mix 1 L portions of concentrated stock solutions of $NaHCO_3$, $MgSO_4$, and KCl for use in preparation of the reconstituted waters.

7.2.3.3 To prepare 20 L of standard, synthetic, moderately hard, reconstituted water, using mineral water such as PERRIER® Water, or equivalent (Table 4), follow the instructions below.

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1. Place 16 L of MILLI-Q® or equivalent water in a properly cleaned plastic carboy.
2. Add 4 L of PERRIER® Water, or equivalent.
3. Aerate vigorously for 24 h to stabilize the medium.
4. The measured pH, hardness and alkalinity of the aerated water will be as indicated in Table 4.
5. This synthetic water is referred to as diluted mineral water (DMW) in the toxicity test methods.

TABLE 4. PREPARATION OF SYNTHETIC FRESHWATER USING MINERAL WATER[1]

Water Type	Volume of Mineral Water Added (mL/L)[2]	Proportion of Mineral Water (%)	Approximate Final Water Quality		
			pH[3]	Hardness[4]	Alkalinity[4]
Very soft	50	2.5	7.2-8.1	10-13	10-13
Soft	100	10.0	7.9-8.3	40-48	30-35
Moderately Hard	200	20.0	7.9-8.3	80-100	57-64
Hard	400	40.0	7.9-8.3	160-180	110-120
Very hard[5]	---	---	---	---	---

[1] From Mount et al. (1987), and data provided by Philip Lewis, EMSL-Cincinnati, OH.
[2] Add mineral water to Milli-Q® water, or equivalent, to prepare Diluted Mineral Water (DMW).
[3] Approximate equilibrium pH after 24 h of aeration.
[4] Expressed as mg $CaCO_3$/L.
[5] Dilutions of PERRIER® Water form a precipitate when concentrations equivalent to "very hard water" are aerated.

7.3 USE OF RECEIVING WATER AS DILUTION WATER

7.3.1 If the objectives of the test require the use of uncontaminated receiving water as dilution water, and the receiving water is uncontaminated, it may be possible to collect a sample of the receiving water upstream of, or close to, but outside of the zone influenced by the effluent. However, if the receiving water is contaminated, it may be necessary to collect the sample in an area "remote" from the discharge site, matching as closely as possible the physical and chemical characteristics of the receiving water near the outfall.

7.3.2 The sample should be collected immediately prior to the test, but never more than 96 h before the test begins. Except where it is used within 24 h, or in the case where large volumes are required for flow through tests, the sample should be chilled to 0-6°C during or immediately following collection, and maintained at that temperature prior to use in the test.

7.3.3 Receiving water containing debris or indigenous organisms that may be confused with or attack the test organisms should be filtered through a sieve having 60 mm mesh openings prior to use.

7.3.4 Where toxicity-free dilution water is required in a test, the water is considered acceptable if test organisms show the required survival, growth, and reproduction in the controls during the test.

7.3.5 The regulatory authority may require that the hardness of the dilution water be comparable to the receiving water at the discharge site. This requirement can be satisfied by collecting an uncontaminated receiving water with a suitable hardness, or adjusting the hardness of an otherwise suitable receiving water by addition of reagents as indicated in Table 3.

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7.4 USE OF TAP WATER AS DILUTION WATER

7.4.1 The use of tap water as dilution water is discouraged unless it is dechlorinated and passed through a deionizer and carbon filter. Tap water can be dechlorinated by deionization, carbon filtration, or the use of sodium thiosulfate. Use of 3.6 mg/L (anhydrous) sodium thiosulfate will reduce 1.0 mg chlorine/L (APHA, 1992). Following dechlorination, total residual chlorine should not exceed 0.01 mg/L. Because of the possible toxicity of thiosulfate to test organisms, a control lacking thiosulfate should be included in toxicity tests utilizing thiosulfate-dechlorinated water.

7.4.2 To be adequate for general laboratory use following dechlorination, the tap water is passed through a deionizer and carbon filter to remove toxic metals and organics, and to control hardness and alkalinity.

7.5 DILUTION WATER HOLDING

7.5.1 A given batch of dilution water should not be used for more than 14 days following preparation because of the possible build-up of bacterial, fungal, or algal slime growth and the problems associated with it. The container should be kept covered and the contents should be protected from light.

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SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING, SAMPLE HANDLING, AND SAMPLE PREPARATION FOR TOXICITY TESTS

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point should be the same as that specified in the NPDES discharge permit (USEPA, 1988a). Conditions for exception would be: (l) better access to a sampling point between the final treatment and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants or separate wastewater streams in industrial facilities prior to their being combined with other wastewater streams or non-contact cooling water, additional sampling points may be chosen.

8.1.2 The decision on whether to collect grab or composite samples is based on the objectives of the test and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitively large number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and average the quality of the effluent over the sampling period. Sampling recommendations are provided below (also see USEPA, 2002a).

8.1.3 Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

8.1.4 Details of date, time, location, duration, and procedures used for effluent sample and dilution water collection should be recorded.

8.2 EFFLUENT SAMPLE TYPES

8.2.1 The advantages and disadvantages of effluent grab and composite samples are listed below:

8.2.1.1 GRAB SAMPLES

Advantages:

1. Easy to collect; require a minimum of equipment and on-site time.
2. Provide a measure of instantaneous toxicity. Toxicity spikes are not masked by dilution.

Disadvantages:

1. Samples are collected over a very short period of time and on a relatively infrequent basis. The chances of detecting a spike in toxicity would depend on the frequency of sampling and the probability of missing a spike is high.

8.2.1.2 COMPOSITE SAMPLES

Advantages:

1. A single effluent sample is collected over a 24-h period.

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2. The sample is collected over a much longer period of time than a single grab sample and contains all toxicity spikes.

Disadvantages:

1. Sampling equipment is more sophisticated and expensive, and must be placed on-site for at least 24 h.
2. Toxicity spikes may not be detected because they are masked by dilution with less toxic wastes.

8.3 EFFLUENT SAMPLING RECOMMENDATIONS

8.3.1 When tests are conducted on-site, test solutions can be renewed daily with freshly collected samples, except for the green alga, *Selenastrum capricornutum*, test which is not renewed.

8.3.2 When tests are conducted off-site, a minimum of three samples are collected. If these samples are collected on Test Days 1, 3, and 5, the first sample would be used for test initiation, and for test solution renewal on Day 2. The second sample would be used for test solution renewal on Days 3 and 4. The third sample would be used for test solution renewal on Days 5, 6, and 7.

8.3.3 Sufficient sample volume must be collected to perform the required toxicity and chemical tests. A 4-L (1-gal) CUBITAINER® will provide sufficient sample volume for most tests.

8.3.4 THE FOLLOWING EFFLUENT SAMPLING METHODS ARE RECOMMENDED:

8.3.4.1 Continuous Discharges

8.3.4.1.1 If the facility discharge is continuous, a single 24-h composite sample is to be taken.

8.3.4.2 Intermittent discharges

8.3.4.2.1 If the facility discharge is intermittent, a composite sample is to be collected for the duration of the discharge but not more than 24 hours.

8.4 RECEIVING WATER SAMPLING

8.4.1 Logistical problems and difficulty in securing sampling equipment generally preclude the collection of composite receiving water samples for toxicity tests. Therefore, based on the requirements of the test, a single grab sample or daily grab sample of receiving water is collected for use in the test.

8.4.2 The sampling point is determined by the objectives of the test. In rivers, samples should be collected from mid-stream and at mid-depth, if accessible. In lakes the samples are collected at mid-depth.

8.4.3 To determine the extent of the zone of toxicity in the receiving water downstream from the outfall, receiving water samples are collected at several distances downstream from the discharge. The time required for the effluent-receiving-water mixture to travel to sampling points downstream from the outfall, and the rate and degree of mixing, may be difficult to ascertain. Therefore, it may not be possible to correlate downstream toxicity with effluent toxicity at the discharge point unless a dye study is performed. The toxicity of receiving water samples from five stations downstream from the discharge point can be evaluated using the same number of test vessels and test organisms as used in one effluent toxicity test with five effluent dilutions.

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8.5 EFFLUENT AND RECEIVING WATER SAMPLE HANDLING, PRESERVATION, AND SHIPPING

8.5.1 Unless the samples are used in an on-site toxicity test the day of collection (or hand delivered to the testing laboratory for use on the day of collection), they should be chilled and maintained at 0-6°C until used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances.

8.5.2 Composite samples should be chilled as they are collected. Grab samples should be chilled immediately following collection.

8.5.3 If the effluent has been chlorinated, total residual chlorine must be measured immediately following sample collection.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of each grab or composite sample must not exceed 36 h. EPA believes that 36 h is adequate time to deliver the samples to the laboratories performing the test in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the USEPA Regional Administrator under 40 CFR 136.3(e) should include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However, in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, each grab or composite sample may also be used to prepare test solutions for renewal at 24 h, 48 h, and/or 72 h after first use, if stored at 0-6°C, with minimum head space, as described in Subsection 8.5. If shipping problems (e.g., unsuccessful Saturday delivery) are encountered with renewal samples after a test has been initiated, the permitting authority may allow the continued use of the most recently used sample for test renewal. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

8.5.5 To minimize the loss of toxicity due to volatilization of toxic constituents, all sample containers should be "completely" filled, leaving no air space between the contents and the lid.

8.5.6 SAMPLES USED IN ON-SITE TESTS

8.5.6.1 Samples collected for on-site tests should be used within 24 h.

8.5.7 SAMPLES SHIPPED TO OFF-SITE FACILITIES

8.5.7.1 Samples collected for off-site toxicity testing are to be chilled to 0-6°C during or immediately after collection, and shipped iced to the performing laboratory. Sufficient ice should be placed with the sample in the shipping container to ensure that ice will still be present when the sample arrives at the laboratory and is unpacked. Insulating material should not be placed between the ice and the sample in the shipping container unless required to prevent breakage of glass sample containers.

8.5.7.2 Samples may be shipped in one or more 4-L (l-gal) CUBITAINERS® or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. After use with receiving water or effluents, CUBITAINERS® and plastic jugs are punctured to prevent reuse.

8.5.7.3 Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. Saturday and Sunday shipping and receiving schedules of private carriers vary with the carrier.

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8.6 SAMPLE RECEIVING

8.6.1 Upon arrival at the laboratory, samples are logged in and the temperature is measured and recorded. If the samples are not immediately prepared for testing, they are stored at 0-6°C until used.

8.6.2 Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory, and the sample holding time should not exceed 36 h unless a variance has been granted by the NPDES permitting authority.

8.7 PERSISTENCE OF EFFLUENT TOXICITY DURING SAMPLE SHIPMENT AND HOLDING

8.7.1 The persistence of the toxicity of an effluent prior to its use in a toxicity test is of interest in assessing the validity of toxicity test data, and in determining the possible effects of allowing an extension of the holding time. Where a variance in holding time (> 36 h, but # 72 h) is requested by a permittee, (see Subsection 8.5.4 above), information on the effects of the extension in holding time on the toxicity of samples must be obtained by comparing the results of multi-concentration chronic toxicity tests performed on effluent samples held 36 h with toxicity test results using the same samples after they were held for the requested, longer period. The portion of the sample set aside for the second test should be held under the same conditions as during shipment and holding.

8.8 PREPARATION OF EFFLUENT AND RECEIVING WATER SAMPLES FOR TOXICITY TESTS

8.8.1 When aliquots are removed from the sample container, the head space above the remaining sample should be held to a minimum. Air which enters a container upon removal of sample should be expelled by compressing the container before reclosing, if possible (i.e., where a CUBITAINER® is used), or by using an appropriate discharge valve (spigot).

8.8.2 With the daphnid, *Ceriodaphnia dubia*, and fathead minnow, *Pimephales promelas*, tests, effluents and receiving waters should be filtered through a 60-µm plankton net to remove indigenous organisms that may attack or be confused with the test organisms (see the daphnid, *Ceriodaphnia dubia*, test method for details). Receiving waters used in green alga, *Selenastrum capricornutum*, toxicity tests must be filtered through a 0.45-µm pore diameter filter before use. It may be necessary to first coarse-filter the dilution and/or waste water through a nylon sieve having 2- to 4-mm mesh openings to remove debris and/or break up large floating or suspended solids. Because filtration may increase the dissolved oxygen (DO) in the effluent, the DO should be checked both before and after filtering. Low dissolved oxygen concentrations will indicate a potential problem in performing the test. **Caution:** filtration may remove some toxicity.

8.8.3 If the samples must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved oxygen and nitrogen may become a problem. To avoid this problem, samples may be warmed slowly in open test containers. If DO is still above 100% saturation after warming to test temperature, samples should be aerated moderately (approximately 500 mL/min) for a few minutes using an airstone. If DO is below 4.0 mg/L after warming to test temperature, the solutions must be aerated moderately (approximately 500 mL/min) for a few minutes, using an airstone, until the DO is within the prescribed range ($4.0 mg/L). **Caution:** avoid excessive aeration.

8.8.4 The DO concentration in the samples should be near saturation prior to use. Aeration may be used to bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH. However, aeration during collection, transfer, and preparation of samples should be minimized to reduce the loss of volatile chemicals.

8.8.4.1 Aeration during the test may alter the results and should be used only as a last resort to maintain the required DO. Aeration can reduce the apparent toxicity of the test solutions by stripping them of highly volatile toxic substances, or increase their toxicity by altering pH. However, the DO in the test solutions should not be allowed to fall below 4.0 mg/L.

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8.8.4.2 In static tests (renewal or non-renewal), low DOs may commonly occur in the higher concentrations of wastewater. Aeration is accomplished by bubbling air through a pipet at a rate of 100 bubbles/min. If aeration is necessary, all test solutions must be aerated. It is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test is started. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.

8.8.5 At a minimum, pH, conductivity, and total residual chlorine are measured in the undiluted effluent or receiving water, and pH and conductivity are measured in the dilution water.

8.8.5.1 It is recommended that total alkalinity and total hardness also be measured in the undiluted effluent test water, receiving water, and the dilution water.

8.8.6 Total ammonia is measured in effluent and receiving water samples where toxicity may be contributed by un-ionized ammonia (i.e., where total ammonia $ 5 mg/L). The concentration (mg/L) of un-ionized (free) ammonia in a sample is a function of temperature and pH, and is calculated using the percentage value obtained from Table 5, under the appropriate pH and temperature, and multiplying it by the concentration (mg/L) of total ammonia in the sample.

8.8.7 Effluents and receiving waters can be dechlorinated using 6.7 mg/L anhydrous sodium thiosulfate to reduce 1 mg/L chlorine (APHA, 1992). Note that the amount of thiosulfate required to dechlorinate effluents is greater than the amount needed to dechlorinate tap water (see Section 7, Dilution Water, Subsection 7.4.1). Since thiosulfate may contribute to sample toxicity, a thiosulfate control should be used in the test in addition to the normal dilution water control.

8.8.8 Mortality or impairment of growth or reproduction due to pH alone may occur if the pH of the sample falls outside the range of 6.0 - 9.0. Thus, the presence of other forms of toxicity (metals and organics) in the sample may be masked by the toxic effects of low or high pH. The question about the presence of other toxicants can be answered only by performing two parallel tests, one with an adjusted pH, and one without an adjusted pH. Freshwater samples are adjusted to pH 7.0 by adding 1N NaOH or 1N HCl dropwise, as required, being careful to avoid overadjustment.

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TABLE 5. PERCENT UNIONIZED NH_3 IN AQUEOUS AMMONIA SOLUTIONS: TEMPERATURES 15-26°C AND pH 6.0-8.9[1]

pH	TEMPERATURE (°C)											
	15	16	17	18	19	20	21	22	23	24	25	26
6.0	0.0274	0.0295	0.0318	0.0343	0.0369	0.0397	0.0427	0.0459	0.0493	0.0530	0.0568	0.0610
6.1	0.0345	0.0372	0.0400	0.0431	0.0464	0.0500	0.0537	0.0578	0.0621	0.0667	0.0716	0.0768
6.2	0.0434	0.0468	0.0504	0.0543	0.0584	0.0629	0.0676	0.0727	0.0781	0.0901	0.0901	0.0966
6.3	0.0546	0.0589	0.0634	0.0683	0.0736	0.0792	0.0851	0.0915	0.0983	0.1134	0.1134	0.1216
6.4	0.0687	0.0741	0.0799	0.0860	0.0926	0.0996	0.107	0.115	0.124	0.133	0.143	0.153
6.5	0.0865	0.0933	0.1005	0.1083	0.1166	0.1254	0.135	0.145	0.156	0.167	0.180	0.193
6.6	0.109	0.117	0.127	0.136	0.147	0.158	0.170	0.182	0.196	0.210	0.226	0.242
6.7	0.137	0.148	0.159	0.171	0.185	0.199	0.214	0.230	0.247	0.265	0.284	0.305
6.8	0.172	0.186	0.200	0.216	0.232	0.250	0.269	0.289	0.310	0.333	0.358	0.384
6.9	0.217	0.234	0.252	0.271	0.292	0.314	0.338	0.363	0.390	0.419	0.450	0.482
7.0	0.273	0.294	0.317	0.342	0.368	0.396	0.425	0.457	0.491	0.527	0.566	0.607
7.1	0.343	0.370	0.399	0.430	0.462	0.497	0.535	0.575	0.617	0.663	0.711	0.762
7.2	0.432	0.466	0.502	0.540	0.581	0.625	0.672	0.722	0.776	0.833	0.893	0.958
7.3	0.543	0.586	0.631	0.679	0.731	0.786	0.845	0.908	0.975	1.05	1.12	1.20
7.4	0.683	0.736	0.793	0.854	0.918	0.988	1.061	1.140	1.224	1.31	1.41	1.51
7.5	0.858	0.925	0.996	1.07	1.15	1.24	1.33	1.43	1.54	1.65	1.77	1.89
7.6	1.08	1.16	1.25	1.35	1.45	1.56	1.67	1.80	1.93	2.07	2.21	2.37
7.7	1.35	1.46	1.57	1.69	1.82	1.95	2.10	2.25	2.41	2.59	2.77	2.97
7.8	1.70	1.83	1.97	2.12	2.28	2.44	2.62	2.82	3.02	3.24	3.46	3.71
7.9	2.13	2.29	2.46	2.65	2.85	3.06	3.28	3.52	3.77	4.04	4.32	4.62
8.0	2.66	2.87	3.08	3.31	3.56	3.82	4.10	4.39	4.70	5.03	5.38	5.75
8.1	3.33	3.58	3.85	4.14	4.44	4.76	5.10	5.46	5.85	6.25	6.68	7.14
8.2	4.16	4.47	4.80	5.15	5.52	5.92	6.34	6.78	7.25	7.75	8.27	8.82
8.3	5.18	5.56	5.97	6.40	6.86	7.34	7.85	8.39	8.96	9.56	10.2	10.9
8.4	6.43	6.90	7.40	7.93	8.48	9.07	9.69	10.3	11.0	11.7	12.5	13.3
8.5	7.97	8.54	9.14	9.78	10.45	11.16	11.90	12.7	13.5	14.4	15.2	16.2
8.6	9.83	10.5	11.2	12.0	12.8	13.6	14.5	15.5	16.4	17.4	18.5	19.5
8.7	12.07	12.9	13.8	14.7	15.6	16.6	17.6	18.7	19.8	21.0	22.2	23.4
8.8	14.7	15.7	16.7	17.8	18.9	20.0	21.2	22.5	23.7	25.1	26.4	27.8
8.9	17.9	19.0	20.2	21.4	22.7	24.0	25.3	26.7	28.2	29.6	31.1	32.6

[1] Table provided by Teresa Norberg-King, ERL, Duluth, Minnesota. Also see Emerson et al. (1975), Thurston et al. (1974), and USEPA (1985a).

8.9 PRELIMINARY TOXICITY RANGE-FINDING TESTS

8.9.1 USEPA Regional and State personnel generally have observed that it is not necessary to conduct a toxicity range-finding test prior to initiating a static, chronic, definitive toxicity test. However, when preparing to perform a static test with a sample of completely unknown quality, or before initiating a flow-through test, it is advisable to conduct a preliminary toxicity range-finding test.

8.9.2 A toxicity range-finding test ordinarily consists of a down-scaled, abbreviated static acute test in which groups of five organisms are exposed to several widely-spaced sample dilutions in a logarithmic series, such as

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100%, 10.0%, 1.00%, and 0.100%, and a control, for 8-24 h. **Caution**: if the sample must also be used for the full-scale definitive test, the 36-h limit on holding time (see Subsection 8.5.4) must not be exceeded before the definitive test is initiated.

8.9.3 It should be noted that the toxicity (LC50) of a sample observed in a range-finding test may be significantly different from the toxicity observed in the follow-up chronic definitive test because: (1) the definitive test is longer; and (2) the test may be performed with a sample collected at a different time, and possibly differing significantly in the level of toxicity.

8.10 MULTI-CONCENTRATION (DEFINITIVE) EFFLUENT TOXICITY TESTS

8.10.1 The tests recommended for use in determining discharge permit compliance in the NPDES program are multi-concentration, or definitive, tests which provide (1) a point estimate of effluent toxicity in terms of an IC25, IC50, or LC50, or (2) a no-observed-effect-concentration (NOEC) defined in terms of mortality, growth, reproduction, and/or teratogenicity and obtained by hypothesis testing. The tests may be static renewal or static non-renewal.

8.10.2 The tests consist of a control and a minimum of five effluent concentrations. USEPA recommends the use of a $0.5 dilution factor for selecting effluent test concentrations. Effluent test concentrations of 6.25%, 12.5%, 25%, 50%, and 100% are commonly used, however, test concentrations should be selected independently for each test based on the objective of the study, the expected range of toxicity, the receiving water concentration, and any available historical testing information on the effluent. USEPA (2000a) provides additional guidance on choosing appropriate test concentrations.

8.10.3 When these tests are used in determining compliance with permit limits, effluent test concentrations should be selected to bracket the receiving water concentration. This may be achieved by selecting effluent test concentrations in the following manner: (1) 100% effluent, (2) [RWC + 100]/2, (3) RWC, (4) RWC/2, and (5) RWC/4. For example, where the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

8.10.4 If acute/chronic ratios are to be determined by simultaneous acute and short-term chronic tests with a single species, using the same sample, both types of tests must use the same test conditions, i.e., pH, temperature, water hardness, salinity, etc.

8.11 RECEIVING WATER TESTS

8.11.1 Receiving water toxicity tests generally consist of 100% receiving water and a control. The total hardness of the control should be comparable to the receiving water.

8.11.2 The data from the two treatments are analyzed by hypothesis testing to determine if test organism survival in the receiving water differs significantly from the control. Four replicates and 10 organisms per replicate are required for each treatment (see Summary of Test Conditions and Test Acceptability Criteria in the specific test method).

8.11.3 In cases where the objective of the test is to estimate the degree of toxicity of the receiving water, a multi-concentration test is performed by preparing dilutions of the receiving water, using a $ 0.5 dilution series, with a suitable control water.

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SECTION 9

CHRONIC TOXICITY TEST ENDPOINTS AND DATA ANALYSIS

9.1 ENDPOINTS

9.1.1 The objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe" or "no-effect concentration" of these substances. For practical reasons, the responses observed in these tests are usually limited to hatchability, gross morphological abnormalities, survival, growth, and reproduction, and the results of the tests are usually expressed in terms of the highest toxicant concentration that has no statistically significant observed effect on these responses, when compared to the controls. The terms currently used to define the endpoints employed in the rapid, chronic and sub-chronic toxicity tests have been derived from the terms previously used for full life-cycle tests. As shorter chronic tests were developed, it became common practice to apply the same terminology to the endpoints. The terms used in this manual are as follows:

9.1.1.1 Safe Concentration - The highest concentration of toxicant that will permit normal propagation of fish and other aquatic life in receiving waters. The concept of a "safe concentration" is a biological concept, whereas the "no-observed-effect concentration" (below) is a statistically defined concentration.

9.1.1.2 No-Observed-Effect-Concentration (NOEC) - The highest concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle (short-term) test, that causes no observable adverse effects on the test organisms (i.e., the highest concentration of toxicant in which the values for the observed responses are not statistically significantly different from the controls). This value is used, along with other factors, to determine toxicity limits in permits.

9.1.1.3 Lowest-Observed-Effect-Concentration (LOEC) - The lowest concentration of toxicant to which organisms are exposed in a life-cycle or partial life-cycle (short-term) test, which causes adverse effects on the test organisms (i.e., where the values for the observed responses are statistically significantly different from the controls).

9.1.1.4 Effective Concentration (EC) - A point estimate of the toxicant concentration that would cause an observable adverse affect on a quantal, "all or nothing," response (such as death, immobilization, or serious incapacitation) in a given percent of the organisms, calculated by point estimation techniques. If the observable effect is death or immobility, the term, Lethal Concentration (LC), should be used (see Subsection 9.1.1.5). A certain EC or LC value might be judged from a biological standpoint to represent a threshold concentration, or lowest concentration that would cause an adverse effect on the observed response.

9.1.1.5 Lethal Concentration (LC) - The toxicant concentration that would cause death in a given percent of the test population. Identical to EC when the observed adverse effect is death. For example, the LC50 is the concentration of toxicant that would cause death in 50% of the test population.

9.1.1.6 Inhibition Concentration (IC) - The toxicant concentration that would cause a given percent reduction in a non-quantal biological measurement for the test population. For example, the IC25 is the concentration of toxicant that would cause a 25% reduction in mean young per female or in growth for the test population, and the IC50 is the concentration of toxicant that would cause a 50% reduction.

9.2 RELATIONSHIP BETWEEN ENDPOINTS DETERMINED BY HYPOTHESIS TESTING AND POINT ESTIMATION TECHNIQUES

9.2.1 If the objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe or no-effect concentration" of these substances, it is imperative to understand how the statistical endpoints of these tests are related to the "safe" or "no-effect" concentration. NOECs and LOECs are determined by hypothesis testing (Dunnett's Test, a t test with the Bonferroni adjustment, Steel's Many-one Rank Test, or the Wilcoxon Rank

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Sum Test with the Bonferroni adjustment), whereas LCs, ICs, and ECs are determined by point estimation techniques (Probit Analysis, Spearman-Karber Method, Trimmed Spearman-Karber Method, Graphical Method or Linear Interpolation Method). There are inherent differences between the use of a NOEC or LOEC derived from hypothesis testing to estimate a "safe" concentration, and the use of a LC, EC, IC, or other point estimates derived from curve fitting, interpolation, etc.

9.2.2 Most point estimates, such as the LC, IC, or EC, are derived from a mathematical model that assumes a continuous dose-response relationship. By definition, any LC, IC, or EC value is an estimate of some amount of adverse effect. Thus the assessment of a "safe" concentration must be made from a biological standpoint rather than with a statistical test. In this instance, the biologist must determine some amount of adverse effect that is deemed to be "safe", in the sense that from a practical biological viewpoint it will not affect the normal propagation of fish and other aquatic life in receiving waters.

9.2.3 The use of NOECs and LOECs, on the other hand, assumes either (1) a continuous dose-response relationship, or (2) a non-continuous (threshold) model of the dose-response relationship.

9.2.3.1 In the case of a continuous dose-response relationship, it is also assumed that adverse effects that are not "statistically observable" are also not important from a biological standpoint, since they are not pronounced enough to test as statistically significant against some measure of the natural variability of the responses.

9.2.3.2 In the case of non-continuous dose-response relationships, it is assumed that there exists a true threshold, or concentration below which there is no adverse effect on aquatic life, and above which there is an adverse effect. The purpose of the statistical analysis in this case is to estimate as closely as possible where that threshold lies.

9.2.3.3 In either case, it is important to realize that the amount of adverse effect that is statistically observable (LOEC) or not observable (NOEC) is highly dependent on all aspects of the experimental design, such as the number of concentrations of toxicant, number of replicates per concentration, number of organisms per replicate, and use of randomization. Other factors that affect the sensitivity of the test include the choice of statistical analysis, the choice of an alpha level, and the amount of variability between responses at a given concentration.

9.2.3.4 Where the assumption of a continuous dose-response relationship is made, by definition some amount of adverse effect might be present at the NOEC, but is not great enough to be detected by hypothesis testing.

9.2.3.5 Where the assumption of a non-continuous dose-response relationship is made, the NOEC would indeed be an estimate of a "safe" or "no-effect" concentration if the amount of adverse effect that appears at the threshold is great enough to test as statistically significantly different from the controls in the face of all aspects of the experimental design mentioned above. If, however, the amount of adverse effect at the threshold were not great enough to test as statistically different, some amount of adverse effect might be present at the NOEC. In any case, the estimate of the NOEC with hypothesis testing is always dependent on the aspects of the experimental design mentioned above. For this reason, the reporting and examination of some measure of the sensitivity of the test (either the minimum significant difference or the percent change from the control that this minimum difference represents) is extremely important.

9.2.4 In summary, the assessment of a "safe" or "no-effect" concentration cannot be made from the results of statistical analysis alone, unless (1) the assumptions of a strict threshold model are accepted, and (2) it is assumed that the amount of adverse effect present at the threshold is statistically detectable by hypothesis testing. In this case, estimates obtained from a statistical analysis are indeed estimates of a "no-effect" concentration. If the assumptions are not deemed tenable, then estimates from a statistical analysis can only be used in conjunction with an assessment from a biological standpoint of what magnitude of adverse effect constitutes a "safe" concentration. In this instance, a "safe" concentration is not necessarily a truly "no-effect" concentration, but rather a concentration at which the effects are judged to be of no biological significance.

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9.2.5 A better understanding of the relationship between endpoints derived by hypothesis testing (NOECs) and point estimation techniques (LCs, ICs, and ECs) would be very helpful in choosing methods of data analysis. Norberg-King (1991) reported that the IC25s were comparable to the NOECs for 23 effluent and reference toxicant data sets analyzed. The data sets included short-term chronic toxicity tests for the fathead minnow, *Pimephales promelas*, and the daphnid, *Ceriodaphnia dubia*. Birge et al. (1985) reported that LC1s derived from Probit Analysis of data from short-term embryo-larval tests with reference toxicants were comparable to NOECs for several organisms. Similarly, USEPA (1988d) reported that the IC25s were comparable to the NOECs for a set of daphnid, *Ceriodaphnia dubia*, chronic tests with a single reference toxicant. However, the scope of these comparisons was very limited, and sufficient information is not yet available to establish an overall relationship between these two types of endpoints, especially when derived from effluent toxicity test data.

9.3 **PRECISION**

9.3.1 HYPOTHESIS TESTS

9.3.1.1 When hypothesis tests are used to analyze toxicity test data, it is not possible to express precision in terms of a commonly used statistic. The results of the test are given in terms of two endpoints, the No-Observed- Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC). The NOEC and LOEC are limited to the concentrations selected for the test. The width of the NOEC-LOEC interval is a function of the dilution series, and differs greatly depending on whether a dilution factor of 0.3 or 0.5 is used in the test design. Therefore, **USEPA recommends the use of the $ 0.5 dilution factor** (see Section 4, Quality Assurance). It is not possible to place confidence limits on the NOEC and LOEC derived from a given test, and it is difficult to quantify the precision of the NOEC-LOEC endpoints between tests. If the data from a series of tests performed with the same toxicant, toxicant concentrations, and test species, were analyzed with hypothesis tests, precision could only be assessed by a qualitative comparison of the NOEC-LOEC intervals, with the understanding that maximum precision would be attained if all tests yielded the same NOEC-LOEC interval. In practice, the precision of results of repetitive chronic tests is considered acceptable if the NOECs vary by no more than one concentration interval above or below a central tendency. Using these guidelines, the "normal" range of NOECs from toxicity tests using a 0.5 dilution factor (two-fold difference between adjacent concentrations), would be four-fold.

9.3.2 POINT ESTIMATION TECHNIQUES

9.3.2.1 Point estimation techniques have the advantage of providing a point estimate of the toxicant concentration causing a given amount of adverse (inhibiting) effect, the precision of which can be quantitatively assessed (1) within tests by calculation of 95% confidence limits, and (2) across tests by calculating a standard deviation and coefficient of variation.

9.3.2.2 It should be noted that software used to calculate point estimates occasionally may not provide associated 95% confidence intervals. This situation may arise when test data do not meet specific assumptions required by the statistical methods, when point estimates are outside of the test concentration range, and when specific limitations imposed by the software are encountered. USEPA (2000a) provides guidance on confidence intervals under these circumstances.

9.4 **DATA ANALYSIS**

9.4.1 ROLE OF THE STATISTICIAN

9.4.1.1 The use of the statistical methods described in this manual for routine data analysis does not require the assistance of a statistician. However, the interpretation of the results of the analysis of the data from any of the toxicity tests described in this manual can become problematic because of the inherent variability and sometimes unavoidable anomalies in biological data. If the data appear unusual in any way, or fail to meet the necessary assumptions, a statistician should be consulted. Analysts who are not proficient in statistics are strongly advised to seek the assistance of a statistician before selecting the method of analysis and using any of the results.

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9.4.1.2 The statistical methods recommended in this manual are not the only possible methods of statistical analysis. Many other methods have been proposed and considered. Certainly there are other reasonable and defensible methods of statistical analysis for this kind of toxicity data. Among alternative hypothesis tests some, like Williams' Test, require additional assumptions, while others, like the bootstrap methods, require computer-intensive computations. Alternative point estimation approaches most probably would require the services of a statistician to determine the appropriateness of the model (goodness of fit), higher order linear or nonlinear models, confidence intervals for estimates generated by inverse regression, etc. In addition, point estimation or regression approaches would require the specification by biologists or toxicologists of some low level of adverse effect that would be deemed acceptable or safe. The statistical methods contained in this manual have been chosen because they are (1) applicable to most of the different toxicity test data sets for which they are recommended, (2) powerful statistical tests, (3) hopefully "easily" understood by nonstatisticians, and (4) amenable to use without a computer, if necessary.

9.4.2 PLOTTING THE DATA

9.4.2.1 The data should be plotted, both as a preliminary step to help detect problems and unsuspected trends or patterns in the responses, and as an aid in interpretation of the results. Further discussion and plotted sets of data are included in the methods and the Appendices.

9.4.3 DATA TRANSFORMATIONS

9.4.3.1 Transformations of the data, (e.g., arc sine square root and logs), are used where necessary to meet assumptions of the proposed analyses, such as the requirement for normally distributed data.

9.4.4 INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

9.4.4.1 Statistical independence among observations is a critical assumption in all statistical analysis of toxicity data. One of the best ways to insure independence is to properly follow rigorous randomization procedures. Randomization techniques should be employed at the start of the test, including the randomization of the placement of test organisms in the test chambers and randomization of the test chamber location within the array of chambers. Discussions of statistical independence, outliers and randomization, and a sample randomization scheme, are included in Appendix A.

9.4.5 REPLICATION AND SENSITIVITY

9.4.5.1 The number of replicates employed for each toxicant concentration is an important factor in determining the sensitivity of chronic toxicity tests. Test sensitivity generally increases as the number of replicates is increased, but the point of diminishing returns in sensitivity may be reached rather quickly. The level of sensitivity required by a hypothesis test or the confidence interval for a point estimate will determine the number of replicates, and should be based on the objectives for obtaining the toxicity data.

9.4.5.2 In a statistical analysis of toxicity data, the choice of a particular analysis and the ability to detect departures from the assumptions of the analysis, such as the normal distribution of the data and homogeneity of variance, is also dependent on the number of replicates. More than the minimum number of replicates may be required in situations where it is imperative to obtain optimal statistical results, such as with tests used in enforcement cases or when it is not possible to repeat the tests. For example, when the data are analyzed by hypothesis testing, the nonparametric alternatives cannot be used unless there are at least four replicates at each toxicant concentration.

9.4.6 RECOMMENDED ALPHA LEVELS

9.4.6.1 The data analysis examples included in the manual specify an alpha level of 0.01 for testing the assumptions of hypothesis tests and an alpha level of 0.05 for the hypothesis tests themselves. These levels are

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common and well accepted levels for this type of analysis and are presented as a recommended minimum significance level for toxicity test data analysis.

9.5 CHOICE OF ANALYSIS

9.5.1 The recommended statistical analysis of most data from chronic toxicity tests with aquatic organisms follows a decision process illustrated in the flowchart in Figure 2. An initial decision is made to use point estimation techniques (the Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, the Graphical Method, or Linear Interpolation Method) and/or to use hypothesis testing (Dunnett's Test, the t test with the Bonferroni adjustment, Steel's Many-one Rank Test, or the Wilcoxon Rank Sum Test with the Bonferroni adjustment). **NOTE: For the NPDES Permit Program, the point estimation techniques are the preferred statistical methods in calculating end points for effluent toxicity tests.** If hypothesis testing is chosen, subsequent decisions are made on the appropriate procedure for a given set of data, depending on the results of the tests of assumptions, as illustrated in the flowchart. A specific flow chart is included in the analysis section for each test.

9.5.2 Since a single chronic toxicity test might yield information on more than one parameter (such as survival, growth, and reproduction), the lowest estimate of a "no-observed-effect concentration" for any of the responses would be used as the "no-observed-effect concentration" for each test. It follows logically that in the statistical analysis of the data, concentrations that had a significant toxic effect on one of the observed responses would not be subsequently tested for an effect on some other response. This is one reason for excluding concentrations that have shown a statistically significant reduction in survival from a subsequent hypothesis test for effects on another parameter such as reproduction. A second reason is that the exclusion of such concentrations usually results in a more powerful and appropriate statistical analysis. In performing the point estimation techniques recommended in this manual, an all-data approach is used. For example, data from concentrations above the NOEC for survival are included in determining ICp estimates using the Linear Interpolation Method.

9.5.3 ANALYSIS OF GROWTH AND REPRODUCTION DATA

9.5.3.1 Growth data from the fathead minnow, *Pimephales promelas*, larval survival and growth test are analyzed using hypothesis testing or point estimation techniques according to the flowchart in Figure 2. The above mentioned growth data may also be analyzed by generating a point estimate with the Linear Interpolation Method. Data from effluent concentrations that have tested significantly different from the control for survival are excluded from further hypothesis tests concerning growth effects. Growth is defined as the dry weight per original number of test organisms when group weights are obtained. When analyzing the data using point estimation techniques, data from all concentrations are included in the analysis.

9.5.3.2 Reproduction data from the daphnid, *Ceriodaphnia dubia*, survival and reproduction test are analyzed using hypothesis testing or point estimation techniques according to the flowchart in Figure 2. In hypothesis testing, data from effluent concentrations that have significantly lower survival than the control, as determined by Fisher's Exact test, are not included in the hypothesis tests for reproductive effects. Data from all concentrations are included when using point estimation techniques.

9.5.4 ANALYSIS OF ALGAL GROWTH RESPONSE DATA

9.5.4.1 The growth response data from the green alga, *Selenastrum capricornutum*, toxicity test, after an appropriate transformation, if necessary, to meet the assumptions of normality and homogeneity of variance, may be analyzed by hypothesis testing according to the flowchart in Figure 2. Point estimates, such as the IC25 and IC50, would also be appropriate in analyzing algal growth data.

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9.5.5 ANALYSIS OF MORTALITY DATA

9.5.5.1 Mortality data are analyzed by Probit Analysis, if appropriate, or other point estimation techniques (i.e., the Spearman-Karber Method, the Trimmed Spearman-Karber Method, or the Graphical Method) (see Appendices I-L and the discussion below). The mortality data can also be analyzed by hypothesis testing, after an arc sine square root transformation (see Appendix B-F), according to the flowchart in Figure 2.

9.5.5.2 Mortality data from the daphnid, *Ceriodaphnia dubia*, survival and reproduction test are analyzed by Fisher's Exact Test (Appendix G) prior to the analysis of the reproduction data. The mortality data may also be analyzed by Probit Analysis, if appropriate or other methods (see Subsection 9.5.5.1).

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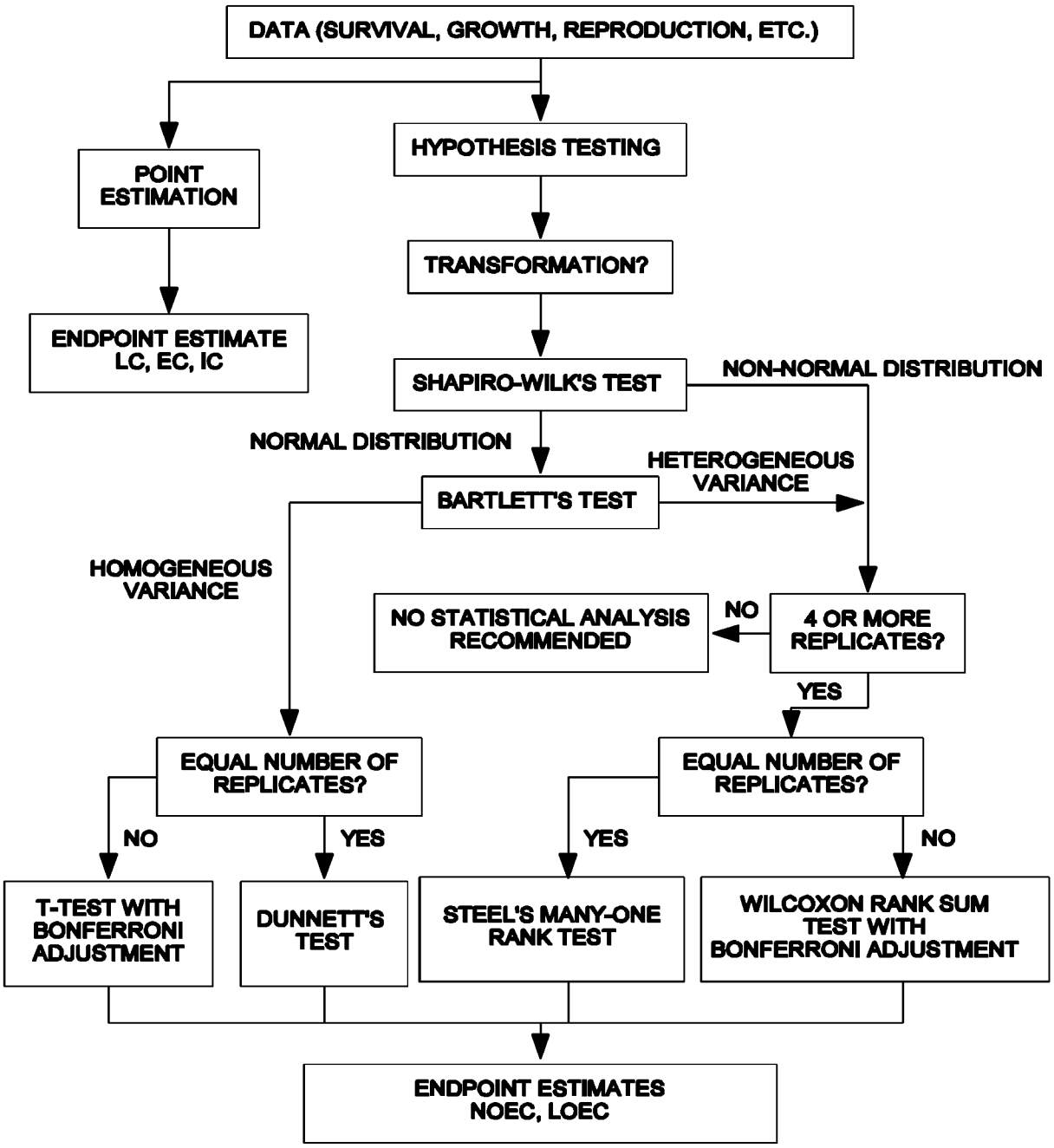

Figure 2. Flowchart for statistical analysis of test data

9.6 **HYPOTHESIS TESTS**

9.6.1 DUNNETT'S PROCEDURE

9.6.1.1 Dunnett's Procedure is used to determine the NOEC. The procedure consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison procedure for comparing each of the treatment means with the control mean, in a series of paired tests (see Appendix C). Use of Dunnett's Procedure requires at least three replicates per treatment to check the assumptions of the test. In cases where the numbers of data points (replicates) for each concentration are not equal, a t test may be performed with Bonferroni's adjustment for multiple comparisons (see Appendix D), instead of using Dunnett's Procedure.

9.6.1.2 The assumptions upon which the use of Dunnett's Procedure is contingent are that the observations within treatments are normally distributed, with homogeneity of variance. Before analyzing the data, these assumptions must be tested using the procedures provided in Appendix B.

9.6.1.3 If, after suitable transformations have been carried out, the normality assumptions have not been met, Steel's Many-one Rank Test should be used if there are four or more data points (replicates) per toxicant concentration. If the numbers of data points for each toxicant concentration are not equal, the Wilcoxon Rank Sum Test with Bonferroni's adjustment should be used (see Appendix F).

9.6.1.4 Some indication of the sensitivity of the analysis should be provided by calculating (1) the minimum difference between means that can be detected as statistically significant, and (2) the percent change from the control mean that this minimum difference represents for a given test.

9.6.1.5 A step-by-step example of the use of Dunnett's Procedure is provided in Appendix C.

9.6.2 T TEST WITH THE BONFERRONI ADJUSTMENT

9.6.2.1 A t test with Bonferroni's adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus Dunnett's Procedure is a more powerful test.

9.6.2.2 The assumptions upon which the use of the t test with Bonferroni's adjustment is contingent are that the observations within treatments are normally distributed, with homogeneity of variance. These assumptions must be tested using the procedures provided in Appendix B.

9.6.2.3 The estimate of the safe concentration derived from this test is reported in terms of the NOEC. A step-by-step example of the use of the t test with Bonferroni's adjustment is provided in Appendix D.

9.6.3 STEEL'S MANY-ONE RANK TEST

9.6.3.1 Steel's Many-one Rank Test is a multiple comparison procedure for comparing several treatments with a control. This method is similar to Dunnett's Procedure, except that it is not necessary to meet the assumption of normality. The data are ranked, and the analysis is performed on the ranks rather than on the data themselves. If the data are normally or nearly normally distributed, Dunnett's Procedure would be more sensitive (would detect smaller differences between the treatments and control). For data that are not normally distributed, Steel's Many-one Rank Test can be much more efficient (Hodges and Lehmann, 1956).

9.6.3.2 It is necessary to have at least four replicates per toxicant concentration to use Steel's test. Unlike Dunnett's procedure, the sensitivity of this test cannot be stated in terms of the minimum difference between treatment means and the control mean that can be detected as statistically significant.

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9.6.3.3 The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of Steel's Many-one Rank Test is provided in Appendix E.

9.6.4 WILCOXON RANK SUM TEST WITH THE BONFERRONI ADJUSTMENT

9.6.4.1 The Wilcoxon Rank Sum Test with the Bonferroni Adjustment is a nonparametric test for comparing treatments with a control. The data are ranked and the analysis proceeds exactly as in Steel's Test except that Bonferroni's adjustment for multiple comparisons is used instead of Steel's tables. When Steel's test can be used (i.e., when there are equal numbers of data points per toxicant concentration), it will be more powerful (able to detect smaller differences as statistically significant) than the Wilcoxon Rank Sum Test with Bonferroni's adjustment.

9.6.4.2 The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of the Wilcoxon Rank Sum Test with Bonferroni Adjustment is provided in Appendix F.

9.6.5 A CAUTION IN THE USE OF HYPOTHESIS TESTING

9.6.5.1 If in the calculation of an NOEC by hypothesis testing, two tested concentrations cause statistically significant adverse effects, but an intermediate concentration did not cause statistically significant effects, the results should be used with extreme caution.

9.7 POINT ESTIMATION TECHNIQUES

9.7.1 PROBIT ANALYSIS

9.7.1.1 Probit Analysis is used to estimate the LC1, LC50, EC1, or EC50 and the associated 95% confidence interval. The analysis consists of adjusting the data for mortality in the control, and then using a maximum likelihood technique to estimate the parameters of the underlying log tolerance distribution, which is assumed to have a particular shape.

9.7.1.2 The assumption upon which the use of Probit Analysis is contingent is a normal distribution of log tolerances. If the normality assumption is not met, and at least two partial mortalities are not obtained, Probit Analysis should not be used. It is important to check the results of Probit Analysis to determine if use of the analysis is appropriate. The chi-square test for heterogeneity provides one good test of appropriateness of the analysis. The computer program (see Appendix I) checks the chi-square statistic calculated for the data set against the tabular value, and provides an error message if the calculated value exceeds the tabular value.

9.7.1.3 A discussion of Probit Analysis, and examples of computer program input and output, are found in Appendix I.

9.7.1.4 In cases where Probit Analysis is not appropriate, the LC50 and associated confidence interval may be estimated by the Spearman-Karber Method (Appendix J) or the Trimmed Spearman-Karber Method (Appendix K). If the test results in 100% survival and 100% mortality in adjacent treatments (all or nothing effect), the LC50 may be estimated using the Graphical Method (Appendix L).

9.7.2 LINEAR INTERPOLATION METHOD

9.7.2.1 The Linear Interpolation Method (see Appendix M) is a procedure to calculate a point estimate of the effluent or other toxicant concentration [Inhibition Concentration, (IC)] that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms. The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests.

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9.7.2.2 Use of the Linear Interpolation Method is based on the assumptions that the responses (1) are monotonically non-increasing (the mean response for each higher concentration is less than or equal to the mean response for the previous concentration), (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. The assumption for piecewise linear response cannot be tested statistically, and no defined statistical procedure is provided to test the assumption for monotonicity. Where the observed means are not strictly monotonic by examination, they are adjusted by smoothing. In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean.

9.7.2.3 The inability to test the monotonicity and piecewise linear response assumptions for this method makes it difficult to assess when the method is, or is not, producing reliable results. Therefore, the method should be used with caution when the results of a toxicity test approach an "all or nothing" response from one concentration to the next in the concentration series, and when it appears that there is a large deviation from monotonicity. See Appendix M for a more detailed discussion of the use of this method and a computer program available for performing calculations.

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SECTION 10

REPORT PREPARATION AND TEST REVIEW

10.1 REPORT PREPARATION

The following general format and content are recommended for the report:

10.1.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contract Laboratory (if the tests are performed under contract)
   a Name of firm
   b. Phone number
   c. Address
6. Objective of test

10.1.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of waste flow (MGD, CFS, GPM)
8. Design flow of treatment facility at time of sampling

10.1.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Mean daily discharge on sample collection date
   f. Lapsed time from sample collection to delivery
   g. Sample temperature when received at the laboratory

2. Receiving Water Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Streamflow (at time of sampling)
   f. Sample temperature when received at the laboratory
   g Lapsed time from sample collection to delivery

3. Dilution Water Samples
   a. Source

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b. Collection date(s) and time(s)
c. Pretreatment
d. Physical and chemical characteristics

10.1.4 TEST METHODS

1. Toxicity test method used (title, number, source)
2. Endpoint(s) of test
3. Deviation(s) from reference method, if any, and the reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type and volume of test chambers
7. Volume of solution used per chamber
8. Number of organisms per test chamber
9. Number of replicate test chambers per treatment
10. Acclimation of test organisms (temperature mean and range)
11. Test temperature (mean and range)
12. Specify if aeration was needed
13. Feeding frequency, and amount and type of food
14. Specify if (and how) pH control measures were implemented

10.1.5 TEST ORGANISMS

1. Scientific name and how determined
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)
7. Taxonomic key used for species identification

10.1.6 QUALITY ASSURANCE

1. Reference toxicant used routinely; source
2. Date and time of most recent reference toxicant test, test results, and current control chart
3. Dilution water used in reference toxicant test
4. Results (NOEC or, where applicable, LOEC, LC50, EC50, IC25 and/or IC50); report percent minimum significant difference (PMSD) calculated for sublethal endpoints determined by hypothesis testing in reference toxicant test
5. Physical and chemical methods used

10.1.7 RESULTS

1. Provide raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls) and replicate, and in graphical form (plots of toxicity data)
2. Provide table of LC50s, NOECs, IC25, IC50, etc. (as required in the applicable NPDES permit)
3. Indicate statistical methods used to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data
6. Provide percent minimum significant difference (PMSD) calculated for sublethal endpoints

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10.1.8 CONCLUSIONS AND RECOMMENDATIONS

1. Relationship between test endpoints and permit limits
2. Actions to be taken

10.2 TEST REVIEW

10.2.1 Test review is an important part of an overall quality assurance program (Section 4) and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the regulatory authority.

10.2.2 SAMPLING AND HANDLING

10.2.2.1 The collection and handling of samples are reviewed to verify that the sampling and handling procedures given in Section 8 were followed. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times (Subsection 8.5.4). Any deviations from the procedures given in Section 8 should be documented and described in the data report (Subsection 10.1).

10.2.3 TEST ACCEPTABILITY CRITERIA

10.2.3.1 Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. Any test not meeting the minimum test acceptability criteria is considered invalid. All invalid tests must be repeated with a newly collected sample.

10.2.4 TEST CONDITIONS

10.2.4.1 Test conditions are reviewed and compared to the specifications listed in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) also are reviewed and compared to specified ranges. Any deviations from specifications should be documented and described in the data report (Subsection 10.1).

10.2.4.2 The summary of test condition tables presented for each method identify test conditions as required or recommended. For WET test data submitted under NPDES permits, all required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. Deviations from recommended test conditions must be evaluated on a case-by-case basis to determine the validity of test results. Deviations from recommended test conditions may or may not invalidate a test result depending on the degree of the departure and the objective of the test. The reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result as valid. For example, if dissolved oxygen is measured below 4.0 mg/L in one test chamber, the reviewer should consider whether any observed mortality in that test chamber corresponded with the drop in dissolved oxygen.

10.2.4.3 Whereas slight deviations in test conditions may not invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicate the need for improved quality control in that laboratory.

10.2.5 STATISTICAL METHODS

10.2.5.1 The statistical methods used for analyzing test data are reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report. Statistical methods other than those recommended in the statistical flowcharts may be appropriate (see Subsection 9.4.1.2), however, the laboratory must document the use of and provide the rationale for the use of any alternate statistical method. In all cases (flowchart recommended

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methods or alternate methods), reviewers should verify that the necessary assumptions are met for the statistical method used.

10.2.6 CONCENTRATION-RESPONSE RELATIONSHIPS

10.2.6.1 The concept of a concentration-response, or more classically, a dose-response relationship is "the most fundamental and pervasive one in toxicology" (Casarett and Doull, 1975). This concept assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response. A response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant. The classical concentration-response relationship is depicted as a sigmoidal shaped curve, however, the particular shape of the concentration-response curve may differ for each coupled toxicant and response pair. In general, more severe responses (such as acute effects) occur at higher concentrations of the toxicant, and less severe responses (such as chronic effects) occur at lower concentrations. A single toxicant also may produce multiple responses, each characterized by a concentration-response relationship. A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results.

10.2.6.2 The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. USEPA (2000a) provides guidance on evaluating concentration-response relationships to assist in determining the validity of WET test results. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to USEPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a). This guidance provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations: that calculated effect concentrations are reliable and should be reported, that calculated effect concentrations are anomalous and should be explained, or that the test was inconclusive and the test should be repeated with a newly collected sample. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting.

10.2.7 REFERENCE TOXICANT TESTING

10.2.7.1 Test review of a given effluent or receiving water test should include review of the associated reference toxicant test and current control chart. Reference toxicant testing and control charting is required for documenting the quality of test organisms (Subsection 4.7) and ongoing laboratory performance (Subsection 4.16). The reviewer should verify that a quality control reference toxicant test was conducted according to the specified frequency required by the permitting authority or recommended by the method (e.g., monthly). The test acceptability criteria, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant test are reviewed to verify that the reference toxicant test conducted was a valid test. The results of the reference toxicant test are then plotted on a control chart (see Subsection 4.16) and compared to the current control chart limits (± 2 standard deviations).

10.2.7.2 Reference toxicant tests that fall outside of recommended control chart limits are evaluated to determine the validity of associated effluent and receiving water tests (see Subsection 4.16). An out of control reference toxicant test result does not necessarily invalidate associated test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increasing test organism sensitivity or toward decreasing test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test. More frequent and/or concurrent reference toxicant testing may be advantageous if recent problems (e.g., invalid tests, reference toxicant test results outside of control chart limits, reduced health of organism cultures, or increased within-test variability) have been identified in testing.

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10.2.8 TEST VARIABILITY

10.2.8.1 The within-test variability of individual tests should be reviewed. Excessive within-test variability may invalidate a test result and warrant retesting. For evaluating within-test variability, reviewers should consult EPA guidance on upper and lower percent minimum significant difference (PMSD) bounds (USEPA, 2000b).

10.2.8.2 When NPDES permits require sublethal hypothesis testing endpoints from Methods 1000.0,1002.0, or 1003.0 (e.g., growth or reproduction NOECs and LOECs), within-test variability must be reviewed and variability criteria must be applied as described in this section (10.2.8.2). When the methods are used for non-regulatory purposes, the variability criteria herein are recommended but are not required, and their use (or the use of alternative variability criteria) may depend upon the intended uses of the test results and the requirements of any applicable data quality objectives and quality assurance plan.

10.2.8.2.1 To measure test variability, calculate the percent minimum significant difference (PMSD) achieved in the test. The PMSD is the smallest percentage decrease in growth or reproduction from the control that could be determined as statistically significant in the test. The PMSD is calculated as 100 times the minimum significant difference (MSD) divided by the control mean. The equation and examples of MSD calculations are shown in Appendix C. PMSD may be calculated legitimately as a descriptive statistic for within-test variability, even when the hypothesis test is conducted using a non-parametric method. The PMSD bounds were based on a representative set of tests, including tests for which a non-parametric method was required for determining the NOEC or LOEC. The conduct of hypothesis testing to determine test results should follow the statistical flow charts provided for each method. That is, when test data fail to meet assumptions of normality or heterogeneity of variance, a non-parametric method (determined following the statistical flowchart for the method) should be used to calculate test results, but the PMSD may be calculated as described above (using parametric methods) to provide a measure of test variability.

10.2.8.2.2 Compare the PMSD measured in the test with the upper PMSD bound variability criterion listed in Table 6. When the test PMSD exceeds the upper bound, the variability among replicates is unusually large for the test method. Such a test should be considered insufficiently sensitive to detect toxic effects on growth or reproduction of substantial magnitude. A finding of toxicity at a particular concentration may be regarded as trustworthy, but a finding of "no toxicity" or "no statistically significant toxicity" at a particular concentration should not be regarded as a reliable indication that there is no substantial toxic effect on growth or reproduction at that concentration.

10.2.8.2.3 If the PMSD measured for the test is less than or equal to the upper PMSD bound variability criterion in Table 6, then the test's variability measure lies within normal bounds and the effect concentration estimate (e.g., NOEC or LOEC) would normally be accepted unless other test review steps raise serious doubts about its validity.

10.2.8.2.4 If the PMSD measured for the test exceeds the upper PMSD bound variability criterion in Table 6, then one of the following two cases applies (10.2.8.2.4.1, 10.2.8.2.4.2).

10.2.8.2.4.1 If toxicity is found at the permitted receiving water concentration (RWC) based upon the value of the effect concentration estimate (NOEC or LOEC), then the test shall be accepted and the effect concentration estimate may be reported, unless other test review steps raise serious doubts about its validity.

10.2.8.2.4.2 If toxicity is not found at the permitted RWC based upon the value of the effect concentration estimate (NOEC or LOEC) and the PMSD measured for the test exceeds the upper PMSD bound, then the test shall not be accepted, and a new test must be conducted promptly on a newly collected sample.

10.2.8.2.5 To avoid penalizing laboratories that achieve unusually high precision, lower PMSD bounds shall also be applied when a hypothesis test result (e.g., NOEC or LOEC) is reported. Lower PMSD bounds, which are based on the 10th percentiles of national PMSD data, are presented in Table 6. The 10th percentile PMSD represents a practical limit to the sensitivity of the test method because few laboratories are able to achieve such precision on a

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regular basis and most do not achieve it even occasionally. In determining hypothesis test results (e.g., NOEC or LOEC), a test concentration shall not be considered toxic (i.e., significantly different from the control) if the relative difference from the control is less than the lower PMSD bounds in Table 6. See USEPA, 2000b for specific examples of implementing lower PMSD bounds.

10.2.8.3 To assist in reviewing within-test variability, EPA recommends maintaining control charts of PMSDs calculated for successive effluent tests (USEPA, 2000b). A control chart of PMSD values characterizes the range of variability observed within a given laboratory, and allows comparison of individual test PMSDs with the laboratory's typical range of variability. Control charts of other variability and test performance measures, such as the MSD, standard deviation or CV of control responses, or average control response, also may be useful for reviewing tests and minimizing variability. The log of PMSD will provide an approximately normal variate useful for control charting.

TABLE 6. VARIABILITY CRITERIA (UPPER AND LOWER PMSD BOUNDS) FOR SUBLETHAL HYPOTHESIS TESTING ENDPOINTS SUBMITTED UNDER NPDES PERMITS.[1]

Test Method	Endpoint	Lower PMSD Bound	Upper PMSD Bound
Method 1000.0, Fathead Minnow Larval Survival and Growth Test	growth	12	30
Method 1002.0, *Ceriodaphnia dubia* Survival and Reproduction Test	reproduction	13	47
Method 1003.0, *Selenastrum capricornutum* Growth Test	growth	9.1	29

[1] Lower and upper PMSD bounds were determined from the 10th and 90th percentile, respectively, of PMSD data from EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

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SECTION 11

TEST METHOD

FATHEAD MINNOW, *PIMEPHALES PROMELAS,* LARVAL SURVIVAL AND GROWTH TEST METHOD 1000.0

11.1 SCOPE AND APPLICATION

11.1.1 This method estimates the chronic toxicity of effluents and receiving water to the fathead minnow, *Pimephales promelas*, using newly hatched larvae in a seven-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

11.1.2 Daily observations on mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

11.1.3 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

11.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly degradable or highly volatile toxicants present in the source may not be detected in the test.

11.1.5 This test method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

11.2 SUMMARY OF METHOD

11.2.1 Fathead minnow, *Pimephales promelas*, larvae are exposed in a static renewal system for seven days to different concentrations of effluent or to receiving water. Test results are based on the survival and weight of the larvae.

11.3. INTERFERENCES

11.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment and Supplies).

11.3.2 Adverse effects of low dissolved oxygen (DO) concentrations, high concentrations of suspended and/or dissolved solids, and extremes of pH, alkalinity, or hardness, may mask the presence of toxic substances.

11.3.3 Improper effluent sampling and sample handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.3.4 Pathogenic and/or predatory organisms in effluent samples or receiving water that is used for dilution may affect test organism survival and confound test results. When pathogen interference is suggested by observation (11.3.4.1) and data evaluations (11.3.4.2) and confirmed by parallel testing (11.3.4.4), steps should be taken to minimize pathogen interference to the extent that test results are not confounded by mortality due to pathogens. Pathogen control techniques that do not require modification of effluent samples, such as use of the modified test design described in Subsection 11.3.4.5, are recommended for controlling pathogen interference. Upon approval by the regulatory authority, analysts also may use additional pathogen control techniques that require sample modification (11.3.4.6) provided that parallel testing of altered and unaltered samples further confirms the presence

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of pathogen interference and demonstrates successful pathogen control (11.3.4.6).

11.3.4.1 A typical indication that pathogen interference has occurred in a WET test is when test organisms exhibit "sporadic mortality". This sporadic morality phenomenon is characterized by an unexpected concentration-response relationship (i.e., effects that do not increase with increasing effluent concentration) and organism survival that varies greatly among replicates and among effluent dilutions (USEPA, 2000a). The observed sporadic mortality among replicates may occur in receiving water controls, lower effluent concentrations, and occasionally in full-strength effluent on day 3 or day 4 of the chronic test. When sporadic mortality occurs, a fungal growth may appear directly on the fish, especially in the gill area. The fungus has not been definitively identified, but the fungal growth appears to be compatible with *Saprolegnea sp.* (Downey *et al.*, 2000). Microbiological evaluations on receiving waters, the fish, and the food indicated the ubiquitous nature of pathogenic organisms (e.g., *Flexibacter spp.*, *Aeromonas hydrophila*), and eradicating them from the test through the decontamination of the fish and their food has not been practical (Geis *et al.*, 2000).

11.3.4.2 When pathogen interference is suspected, a series of data evaluations are required. The test data must be reviewed to determine a cause for any unexpected concentration-response pattern and subsequently to determine the validity of calculated results (USEPA, 2000a). USEPA (2000a) provides guidance on reviewing concentration-response relationships including specific response patterns that may indicate pathogen effects. Each treatment (including the control) should be evaluated for an unusually high mortality response and unevenness of mortalities among replicates. Within-treatment coefficient of variation (CVs) for survival of >40% in effluent or receiving water treatments but relatively small for control replicates in a standard reconstituted water may be an indication of pathogen interference. Receiving water controls from improper preparation or collection also should be evaluated.

11.3.4.3 Because of the ubiquitous nature of the pathogens or predatory organisms, all test equipment, glassware, and pipettes must be kept clean and dry when not in use. Use of separate glassware, pipettes, and siphons for each concentration is recommended to minimize cross contaminating replicates of all treatments. Care also should be taken to properly clean test chambers by removing excess food, dead fish larvae, and other debris prior to daily renewal (see Subsection 11.10.7). When proper laboratory hygiene and filtration through a 2-4 mm mesh opening (Subsection 8.8.2) do not eliminate the sporadic mortality, the analyst should determine the source and confirm pathogen interference using parallel testing (11.3.4.4).

11.3.4.4 Parallel tests should be conducted using reconstituted water and receiving water as diluents with the effluent to confirm that the test results are due to pathogen interference and to determine the source of pathogens in the test. This determination is an important step in controlling pathogen interference. When the dilution water exhibits the interference (i.e., pathogen interference is not observed in the test using reconstituted laboratory water for dilution), reconstituted laboratory water instead of receiving waters should be used to eliminate the interference. However, if receiving water is required, the analyst may modify the test design to control pathogen interference (Subsection 11.3.4.5) or treat the dilution water prior to testing to remove the interference (Subsection 11.3.4.6). If pathogen interference is due to pathogens in the effluent (i.e., pathogen interference is still observed in the test using reconstituted laboratory water for dilution), it is recommended that the analyst modify the test design to control pathogen interference (Subsection 11.3.4.5). Upon approval by the regulatory authority, analysts also may use various sample sterilization techniques to control pathogen interference (11.3.4.6) provided that parallel testing of altered and unaltered samples further confirms the presence of pathogen interference and demonstrates successful pathogen control.

11.3.4.5 When data evaluation indicates that sporadic mortality has occurred as described in Subsections 11.3.4.1 - 11.3.4.2, the test design can be modified as described below to control pathogen interference. The use of 2 fish per 20 ml in each 1 ounce plastic cup test solution or 2 fish per 50 ml in each 4 ounce plastic cup can be used rather than 10 fish per test chamber. The total number of fish tested remains unchanged (i.e., 40 per treatment). At test initiation, for each test concentration and replicate, the test cups must be labeled to easily recombine the fish to the original replicate at the end of the test. For example, for replicate A, each of the five plastic test cups would be identified as subreplicate A1, A2, A3, A4, and A5 repeating the pattern for subsequent replicates (e.g., for replicate B, each cup would be identified as subreplicate B1, B2, B3, B4, and B5). At test termination, all test organisms

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from the five A subreplicates are combined for a survival and weight determination. Document the recombination of replicates in records.

11.3.4.5.1 All test chambers must be randomized using a template for randomization or by using a table of random numbers. Test chambers are randomized once at the beginning of the test (see Subsection 11.10.2.3). When using templates, a number of different templates should be prepared, so that the same template is not used for every test. Randomization procedures must be documented with daily records.

11.3.4.5.2 When adding or transferring the larvae to test chambers, the amount of excess water added to the chambers should be kept to a minimum to avoid unnecessary dilution of the test concentrations. The fish in each test chamber should be fed 0.1 mL of a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4 h intervals, or 0.15 mL should be fed twice daily at an interval of 6 h. (NOTE: to prevent low dissolved oxygen levels, the amount of food added to cups should be adjusted to account for the modified test design that uses smaller test chambers). Dead test organisms should be removed as soon as they are observed.

11.3.4.5.3 Fish are transferred to new or clean test chambers daily. At the time of the daily renewal of the test solutions, the fish are transferred to a new test chamber containing fresh test solution using a pipette which has at least a 5mm bore diameter. Separate pipettes should be used for each treatment. Water transfer is kept to a minimum by allowing the fish to swim out of the pipette into the new test chamber. Any potential injury to individual fish should be recorded on the test sheets.

11.3.4.5.4 At test termination, the surviving larvae in each chamber must be counted and all subreplicates within a replicate (e.g., A1, A2, A3, A4, and A5) combined. For example, all test cups (within a treatment) labeled A would be combined for a survival and dry weight determination.

11.3.4.6 When parallel testing has confirmed pathogen interference, the regulatory authority may allow modifications of the effluent samples or receiving water diluent to remove or inactivate the pathogens (Subsection 11.3.4.6.1 - 11.3.4.6.4). Techniques that control pathogen interference without modifying the effluent sample (11.3.4.5) are recommended, but they may not always be able to minimize pathogen interference to the extent that test results are not confounded by mortality due to pathogens. Therefore, regulatory authorities may allow appropriate pathogen control techniques (including those that modify the effluent sample) on a case-by-case basis. TIE approaches (USEPA, 1991b; USEPA, 1992) and the following procedures (Subsection 11.3.4.6.1 - 11.3.4.6.4) can be used alone or in combination to ascertain the adverse influence on tests caused by pathogens. Prior to routine use of pathogen control techniques that modify the sample, the effects of pathogenic bacteria and the effectiveness of the selected pathogen control technique must be confirmed by parallel and simultaneous testing of the technique with altered and unaltered samples.

11.3.4.6.1 Use of ultra-violet light to irradiate the sample. The rate of pumping specified by the manufacturer of the apparatus should be used (provided that adequate disinfection is achieved), and the life of the UV light source must follow manufacturers' recommendations and be documented. For example, one liter of water can be irradiated for 20 min using an 8 watt UV light (Aquatic Ecosystems, Apopka, FL) prior to use each day of the test. Light sources have limited lifetimes and their effectiveness will decrease with age. The delivery pump and the light source should be on the same electrical circuit to ensure that when power is interrupted both terminate operation. QA/QC procedures should be put into place to assure that the light source is on at the beginning and at the end of the procedure. Treatment of the large volumes of water necessary for test dilution also may be impractical. Caution: Since the effluent or receiving water samples must be passed through the UV sterilizer and then test treatments prepared, there may be potential effects of UV light on the sample. UV exposure may increase or decrease toxicity from other pollutants in the sample. UV treatment is known to cause photoactivation of some organic compounds, which may increase toxicity. UV treatment also is known to cause the photochemical breakdown of certain organic compounds, which could decrease toxicity (if the parent compound is toxic) or increase toxicity (if reaction products are toxic). These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. The effectiveness of UV for

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sterilization may decrease with turbid or stained samples. Bacteria can escape exposure by being lodged in crevices of particulate matter in the sample. All toxicity tests using a sterilized sample must include a blank preparation consisting of similarly sterilized laboratory water.

11.3.4.6.2 Ultra-filtration through a 0.22 µm pore diameter filter (such as Gelman Suprocap®) may be conducted on sample aliquots before daily use. Samples may need to be filtered through a glass fiber filter prior to the 0.22 µm filter. This is time consuming and volume restricted. Treatment of the large volumes of water necessary for test dilution may be impractical. Caution: Since the effluent or receiving water samples must be passed through the filter, the effect of filtering must be evaluated. Filtration can remove toxicity if toxic components of the sample are bound to particles (USEPA, 1991b; 1992). The removal of suspended solids also may influence the bioavailability of chemical pollutants. These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. The removal of toxicity by filtration must be evaluated for each sample by testing samples before and after filtration. All toxicity tests using a sterilized sample also must include a blank preparation consisting of similarly sterilized reconstituted laboratory water.

11.3.4.6.3 Use of chlorination and dechlorination. In some cases, pathogens can survive the chlorination/ dechlorination process and the pathogenic effects may increase due to lack of competition from other organisms. Sufficient data must be collected and documented to determine the effective dosage required. Caution: Chlorination of effluent samples could cause unknown effects on the sample. Chlorination could increase or decrease sample toxicity by oxidizing organic compounds or forming chlorination by-products. These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. Toxicity tests conducted with the addition of chlorine and subsequent dechlorination (USEPA, 1991b; 1992) to either effluent or receiving water samples also must include a blank preparation consisting of similarly treated laboratory water.

11.3.4.6.4 Use of antibiotics. The addition of wide spectrum antibiotics has been effective in removing the pathogen effect (Downey *et al.*, 2000). Antibacterial treatment such as those commonly used in aquaculture or home aquarium maintenance (e.g., oxytetracycline, chloramphenicol, and actinomycin) may be effective. Sufficient data must be collected to determine the effective dosage required. Caution: While antibiotics are effective, easy to use, inexpensive, and readily available, the antibiotic treatment may alter the sample in unknown or undesirable ways and may make the sample too cloudy. Large volumes of a sample may need to be treated. These effects should be considered in the selection of pathogen control strategies, and the analyst should attempt to minimize these effects to the extent reasonably practicable. All toxicity tests using antibiotic treatments also must include treatment blanks of similarly prepared laboratory water.

11.3.5 Food added during the test may sequester metals and other toxic substances and confound test results. Daily renewal of solutions, however, will reduce the probability of reduction of toxicity caused by feeding.

11.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 11.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 11.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

11.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 11.3.6.2.

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The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

11.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ± 0.2 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.2 pH units in pH-controlled tests (USEPA, 1992).

11.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

11.3.6.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 11.3.6.1.1).

11.3.6.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 11.3.6.2) is applied routinely to subsequent testing of the effluent.

11.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992); or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, CO_2 is injected to maintain the test pH at the pH of the sample upon completion of collection. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

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11.4 SAFETY

11.4.1 See Section 3, Health and Safety.

11.5 APPARATUS AND EQUIPMENT

11.5.1 Fathead minnow and brine shrimp culture units -- see USEPA, 1985a and USEPA, 2002a. This test requires 240-360 larvae. It is preferable to obtain larvae from an in-house fathead minnow culture unit. If it is not feasible to culture fish in-house, embryos or newly hatched larvae can be shipped in well oxygenated water in insulated containers.

11.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L.

11.5.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.5.4 Environmental chamber or equivalent facility with temperature control (25 ± 1°C).

11.5.5 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

11.5.6 Balance -- analytical, capable of accurately weighing to 0.00001 g.

11.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus fish.

11.5.8 Test chambers -- four borosilicate glass or non-toxic disposable plastic test chambers are required for each concentration and control. Test chambers may be 1 L, 500 mL or 250 mL beakers, 500 mL plastic cups, or fabricated rectangular (0.3 cm thick) glass chambers, 15 cm x 7.5 cm x 7.5 cm. To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick).

11.5.9 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions. 5.10

11.5.10 Volumetric pipets -- Class A, 1-100 mL.

11.5.11 Serological pipets -- 1-10 mL, graduated.

11.5.12 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

11.5.13 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring larvae.

11.5.14 Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

11.5.15 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

11.5.16 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

11.5.17 Thermometers, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979b) -- to calabrate laboratory themometers.

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11.5.18 Meters, pH, DO, and specific conductivity -- for routine physical and chemical measurements.

11.5.19 Drying oven -- 50-105° C range for drying larvae.

11.6 REAGENTS AND CONSUMABLE MATERIALS

11.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.6.2 Data sheets (one set per test) -- for recording data.

11.6.3 Vials, marked -- 24 per test, containing 4% formalin or 70% ethanol to preserve larvae (optional).

11.6.4 Weighing boats, aluminum -- 24 per test.

11.6.5 Tape, colored -- for labeling test chambers.

11.6.6 Markers, waterproof -- for marking containers, etc.

11.6.7 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA, 1979b.

11.6.8 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for instrument calibration (see USEPA Method 150.1, USEPA, 1979b).

11.6.9 Specific conductivity standards -- see USEPA Method 120.1, USEPA, 1979b.

11.6.10 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

11.6.11 Laboratory quality control samples and standards -- for calibration of the above methods.

11.6.12 Reference toxicant solutions (see Section 4, Quality Assurance).

11.6.13 Ethanol (70%) or formalin (4%) -- for use as a preservative for the fish larvae.

11.6.14 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

11.6.15 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

11.6.16 Brine Shrimp, *Artemia*, Nauplii -- for feeding cultures and test organisms

11.6.16.1 Newly-hatched *Artemia* nauplii are used as food (see USEPA, 2002a) for fathead minnow, *Pimephales promelas*, larvae in toxicity tests and frozen brine shrimp and flake food are used in the maintenance of continuous stock cultures. Although there are many commercial sources of brine shrimp cysts, the Brazilian or Colombian strains are currently preferred because the supplies examined have had low concentrations of chemical residues and produce nauplii of suitably small size.

11.6.16.2 Each new batch of brine shrimp, *Artemia*, cysts must be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (see Leger et al., 1985; Leger et al., 1986) against known suitable reference cysts by performing a side by side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be a previously tested and acceptable batch of cysts. A

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sample of newly-hatched *Artemia* nauplii from each new batch of cysts should be chemically analyzed. The *Artemia* cysts should not be used if the concentration of total organochlorine exceeds 0.15 µg/g wet weight or the total concentration of organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight. (For analytical methods see USEPA, 1982).

11.6.16.3 *Artemia* nauplii are obtained as follows:

1. Add 1 L of seawater, or a solution prepared by adding 35.0 g uniodized salt (NaCl) or artificial sea salts to 1 L deionized water, to a 2-L separatory funnel, or equivalent.
2. Add 10 mL *Artemia* cysts to the separatory funnel and aerate for 24-h at 27EC. (Hatching time varies with incubation temperature and the geographic strain of *Artemia* used) (see USEPA, 1991b; USEPA, 2002a and ASTM, 1993).
3. After 24 h, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic, and will concentrate at the bottom of the funnel if it is covered for 5-10 min. To prevent mortality, do not leave the concentrated nauplii at the bottom of the funnel more than 10 min without aeration.
4. Drain the nauplii into a beaker or funnel fitted with a # 150 µm Nitex® or stainless steel screen, and rinse with deionized water, or equivalent, before use.

11.6.16.4 Testing *Artemia* nauplii as food for toxicity test organisms.

11.6.16.4.1 The primary criterion for acceptability of each new supply of brine shrimp cysts is the ability of the nauplii to support good survival and growth of the fathead minnow larvae (see Subsection 11.12). The larvae used to evaluate the suitability of the brine shrimp nauplii must be of the same geographical origin, species, and stage of development as those used routinely in the toxicity tests. Sufficient data to detect differences in survival and growth should be obtained by using three replicate test vessels, each containing a minimum of 15 larvae, for each type of food.

11.6.16.4.2 The feeding rate and frequency, test vessels, volume of control water, duration of the test, and age of the nauplii at the start of the test, should be the same as used for the routine toxicity tests.

11.6.16.4.3 Results of the brine shrimp nutrition assay, where there are only two treatments, can be evaluated statistically by use of a t test. The "new" food is acceptable if there are no statistically significant differences in the survival and growth of the larvae fed the two sources of nauplii.

11.6.17 TEST ORGANISMS, FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

11.6.17.1 Newly hatched fish less than 24 h old should be used for the test. If organisms must be shipped to the testing site, fish up to 48 h old may be used, all hatched within a 24-h window.

11.6.17.2 If the fish are kept in a holding tank or container, most of the water should be siphoned off to concentrate the fish. The fish are then transferred one at a time randomly to the test chambers until each chamber contains ten fish. Alternately, fish may be placed one or two at a time into small beakers or plastic containers until they each contain five fish. Three (minimum of two) of these beakers/plastic containers are then assigned to randomly-arranged control and exposure chambers.

11.6.17.2.1 The fish are transferred directly to the test vessels or intermediate beakers/plastic containers, using a large-bore, fire-polished glass tube (6 mm to 9 mm I.D. X 30 cm long) equipped with a rubber bulb, or a large volumetric pipet with tip removed and fitted with a safety type bulb filler. The glass or plastic containers should only contain a small volume of dilution water.

11.6.17.2.2 It is important to note that larvae should not be handled with a dip net. Dipping small fish with a net may result in damage to the fish and cause mortality.

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11.6.17.3 The test is conducted with a minimum of four test chambers at each toxicant concentration and control. Fifteen (minimum of ten) embryos are placed in each replicate test chamber. Thus 60 (minimum of 40) fish are exposed at each test concentration.

11.6.17.4 Sources of organisms

11.6.17.4.1 Fathead minnows, *Pimephales promelas*, may be obtained from commercial biological supply houses. Fish obtained from outside sources for use as brood stock or in toxicity tests may not always be of suitable age and quality. Fish provided by supply houses should be guaranteed to be of (1) the correct species, (2) disease free, (3) in the requested age range, and (4) in good condition. This can be done by providing the record of the date on which the eggs were laid and hatched, and information on the sensitivity of contemporary fish to reference toxicants.

11.6.17.5 Inhouse Sources of Fathead Minnows, *Pimephales promelas*

11.6.17.5.1 Problems in obtaining suitable fish from outside laboratories can be avoided by developing an inhouse laboratory culture facility. Fathead minnows, *Pimephales promelas*, can be easily cultured in the laboratory from eggs to adults in static, recirculating, or flow-through systems. The larvae, juveniles, and adult fish should be kept in 60 L (15 gal) or 76 L (20 gal) rearing tanks supplied with reconstituted water, dechlorinated tap water, or natural water. The water should be analyzed for toxic metals and organics quarterly (see Section 4, Quality Assurance).

11.6.17.5.1.1 If a static or recirculating system is used, it is necessary to equip each tank with an outside activated carbon filter system, similar to those sold for tropical fish hobbyists (or one large activated carbon filter system for a series of tanks) to prevent the accumulation of toxic metabolic wastes (principally nitrite and ammonia) in the water.

11.6.17.5.2 Flow-through systems require large volumes of water and may not be feasible in some laboratories. The culture tanks should be shielded from extraneous disturbances using opaque curtains, and should be isolated from toxicity testing activities to prevent contamination.

11.6.17.5.3 To avoid the possibility of inbreeding of the inhouse brood stock, fish from an outside source should be introduced yearly into the culture unit.

11.6.17.5.4 Dissolved oxygen -- The DO concentration in the culture tanks should be maintained near saturation, using gentle aeration with 15 cm air stones if necessary. Brungs (1971), in a carefully controlled long-term study, found that the growth of fathead minnows was reduced significantly at all dissolved oxygen concentrations below 7.9 mg/L. Soderberg (1982) presented an analytical approach to the re-aeration of flowing water for culture systems.

11.6.17.5.5 Culture Maintenance

11.6.17.5.5.1 Adequate procedures for culture maintenance must be followed to avoid poor water quality in the culture system. The spawning and brood stock culture tanks should be kept free of debris (excess food, detritus, waste, etc.) by siphoning the accumulated materials (such as dead brine shrimp nauplii or cysts) from the bottom of the tanks daily with a glass siphon tube attached to a plastic hose leading to the floor drain. The tanks are more thoroughly cleaned as required. Algae, mostly diatoms and green algae, growing on the glass of the spawning tanks are left in place, except for the front of the tank, which is kept clean for observation. To avoid excessive build-up of algal growth, the walls of the tanks are periodically scraped. The larval culture tanks are cleaned once or twice a week to reduce the mass of fungus growing on the bottom of the tank.

11.6.17.5.5.2 Activated charcoal and floss in the tank filtration systems should be changed weekly, or more often if needed. Culture water may be maintained by preparation of reconstituted water or use of dechlorinated tap water. Distilled or deionized water is added as needed to compensate for evaporation.

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11.6.17.5.5.3 Before new fish are placed in tanks, salt deposits are removed by scraping or with 5% acid solution, the tanks are washed with detergent, sterilized with a hypochlorite solution, and rinsed well with hot tap water and then with laboratory water.

11.6.17.5.6 Obtaining Embryos for Toxicity Tests

11.6.17.5.6.1 Embryos can be shipped to the laboratory from an outside source or obtained from adults held in the laboratory as described below.

11.6.17.5.6.2 For breeding tanks, it is convenient to use 60 L (15 gal) or 76 L (20 gal) aquaria. The spawning unit is designed to simulate conditions in nature conducive to spawning, such as water temperature and photoperiod. Spawning tanks must be held at a temperature of 25 ± 2°C. Each aquarium is equipped with a heater, if necessary, a continuous filtering unit, and spawning substrates. The photoperiod for the culture system should be maintained at 16 h light and 8 h darkness. For the spawning tanks, this photoperiod must be rigidly controlled. A convenient photoperiod is 5:00 AM to 9:00 PM. Fluorescent lights should be suspended about 60 cm above the surface of the water in the brood and larval tanks. Both DURATEST® and cool-white fluorescent lamps have been used, and produce similar results. An illumination level of 50 to 100 ft-c is adequate.

11.6.17.5.6.3 To simulate the natural spawning environment, it is necessary to provide substrates (nesting territories) upon which the eggs can be deposited and fertilized, and which are defended and cared for by the males. The recommended spawning substrates consist of inverted half-cylinders, 7.6 cm × 7.6 cm (3 in × 3 in) of Schedule 40 PVC pipe. The substrates should be placed equi-distant from each other on the bottom of the tanks.

11.6.17.5.6.4 To establish a breeding unit, 15-20 pre-spawning adults six to eight months old are taken from a "holding" or culture tank and placed in a 76-L spawning tank. At this point, it is not possible to distinguish the sexes. However, after less than a week in the spawning tank, the breeding males will develop their distinct coloration and territorial behavior, and spawning will begin. As the breeding males are identified, all but two are removed, providing a final ratio of 5-6 females per male. The excess spawning substrates are used as shelter by the females.

11.6.17.5.6.5 Sexing of the fish to ensure a correct female/male ratio in each tank can be a problem. However, the task usually becomes easier as experience is gained (Flickinger, 1966). Sexually mature females usually have large bellies and a tapered snout. The sexually mature males are usually distinguished by their larger overall size, dark vertical color bands, and the spongy nuptial tubercles on the snout. Unless the males exhibit these secondary breeding characteristics, no reliable method has been found to distinguish them from females. However, using the coloration of the males and the presence of enlarged urogenital structures and other characteristics of the females, the correct selection of the sexes can usually be achieved by trial and error.

11.6.17.5.6.6 Sexually immature males are usually recognized by their aggressive behavior and partial banding. These undeveloped males must be removed from the spawning tanks because they will eat the eggs and constantly harass the mature males, tiring them and reducing the fecundity of the breeding unit. Therefore, the fish in the spawning tanks must be carefully checked periodically for extra males.

11.6.17.5.6.7 A breeding unit should remain in their spawning tank about four months. Thus, each brood tank or unit is stocked with new spawners about three times a year. However, the restocking process is rotated so that at any one time the spawning tanks contain different age groups of brood fish.

11.6.17.5.6.8 Fathead minnows spawn mostly in the early morning hours. They should not be disturbed except for a morning feeding (8:00 AM) and daily examination of substrates for eggs in late morning or early afternoon. In nature, the male protects, cleans, and aerates the eggs until they hatch. In the laboratory, however, it is necessary to remove the eggs from the tanks to prevent them from being eaten by the adults, for ease of handling, for purposes of recording embryo count and hatchability, and for the use of the newly hatched young fish for toxicity tests.

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11.6.17.5.6.9 Daily, beginning six to eight hours after the lights are turned on (11:00 AM - 1:00 PM), the substrates in the spawning tanks are each lifted carefully and inspected for embryos. Substrates without embryos are immediately returned to the spawning tank. Those with embryos are immersed in clean water in a collecting tray, and replaced with a clean substrate. A daily record is maintained of each spawning site and the estimated number of embryos on the substrate.

11.6.17.5.6.10 Three different methods are described for embryo incubation.

1. Incubation of Embryos on the Substrates: Several (2-4) substrates are placed on end in a circular pattern (with the embryos on the innerside) in 10 cm of water in a tray. The tray is then placed in a constant temperature water bath, and the embryos are aerated with a 2.5 cm airstone placed in the center of the circle. The embryos are examined daily, and the dead and fungused embryos are counted, recorded, and removed with forceps. At an incubation temperature of 25°C, 50% hatch occurs in five days. At 22°C embryos incubated on aerated tiles require 7 days for 50% hatch.

2. Incubation of Embryos in a Separatory Funnel: The embryos are removed from the substrates with a rolling action of the index finger ("rolled off") (Gast and Brungs, 1973), their total volume is measured, and the number of embryos is calculated using a conversion factor of approximately 430 embryos/mL. The embryos are incubated in about 1.5 L of water in a 2 L separatory funnel maintained in a water bath. The embryos are stirred in the separatory funnel by bubbling air from the tip of a plastic micro-pipette placed at the bottom, inside the separatory funnel. During the first two days, the embryos are taken from the funnel daily, those that are dead and fungused are removed, and those that are alive are returned to the separatory funnel in clean water. The embryos hatch in four days at a temperature of 25°C. However, usually on day three the eyed embryos are removed from the separatory funnel and placed in water in a plastic tray and gently aerated with an air stone. Using this method, the embryos hatch in five days. Hatching time is greatly influenced by the amount of agitation of the embryos and the incubation temperature. If on day three the embryos are transferred from the separatory funnel to a static, unaerated container, a 50% hatch will occur in six days (instead of five) and a 100% hatch will occur in seven days. If the culture system is operated at 22°C, embryos incubated on aerated tiles require seven days for 50% hatch.

3. Incubation in Embryo Incubation Cups: The embryos are "rolled off" the substrates, and the total number is estimated by determining the volume. The embryos are then placed in incubation cups attached to a rocker arm assembly (Mount, 1968). Both flow-through and static renewal incubation have been used. On day one, the embryos are removed from the cups and those that are dead and fungused are removed. After day one only dead embryos are removed from the cups. During the incubation period, the eggs are examined daily for viability and fungal growth, until they hatch. Unfertilized eggs, and eggs that have become infected by fungus, should be removed with forceps using a table top magnifier-illuminator. Non-viable eggs become milky and opaque, and are easily recognized. The non-viable eggs are very susceptible to fungal infection, which may then spread throughout the egg mass. Removal of fungus should be done quickly, and the substrates should be returned to the incubation tanks as rapidly as possible so that the good eggs are not damaged by desiccation. Hatching takes four to five days at an optimal temperature of 25°C. Hatching can be delayed several (two to four) days by incubating at lower temperatures. A large plastic tank receiving recirculating water from a temperature control unit, can be used as a water bath for incubation of embryos.

11.6.17.5.6.11 Newly-hatched larvae are transferred daily from the egg incubation apparatus to small rearing tanks, using a large bore pipette, until the hatch is complete. New rearing tanks are set up on a daily basis to separate fish by age group. Approximately 1500 newly hatched larvae are placed in a 60-L (15 gal) or 76-L (20 gal) all-glass aquarium for 30 days. A density of 150 fry per liter is suitable for the first four weeks. The water temperature in the rearing tanks is allowed to follow ambient laboratory temperatures of 20-25°C, but sudden, extreme variations in temperature must be avoided.

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11.6.17.5.7 Food and Feeding

11.6.17.5.7.1 The amount of food and feeding schedule affects both growth and egg production. The spawning fish and pre-spawners in holding tanks usually are fed all the adult frozen brine shrimp and tropical fish flake food or dry commercial fish food (No. l or No. 2 granules) that they can eat (*ad libitum*) at the beginning of the work day and in the late afternoon (8:00 AM and 4:00 PM). The fish are fed twice a day (twice a day with dry food and once a day with adult shrimp) during the week and once a day on weekends.

11.6.17.5.7.2 Fathead minnow larvae are fed freshly-hatched brine shrimp (*Artemia*) nauplii twice daily until they are four weeks old. Utilization of older (larger) brine shrimp nauplii may result in starvation of the young fish because they are unable to ingest the larger food organisms (see Subsection 11.6.16 or USEPA, 2002a for instructions on the preparation of brine shrimp nauplii).

11.6.17.5.7.3 Fish older than four weeks are fed frozen brine shrimp and commercial fish starter (#l and #2), which is ground fish meal enriched with vitamins. As the fish grow, larger pellet sizes are used, as appropriate. (Starter, No. 1 and N. 2 granules, U.S. Fish and Wildlife Service Formulation Specification Diet SD9-30). Newly hatched brine shrimp nauplii, and frozen adult brine shrimp are fed to the fish cultures in volumes based on age, size, and number of fish in the tanks.

11.6.17.5.7.4 Fish in the larval tanks (from hatch to 30 days old) are fed commercial starter fish food at the beginning and end of the work day, and newly hatched brine shrimp nauplii (from the brine shrimp culture unit) once a day, usually mid-morning and mid-afternoon.

11.6.17.5.7.5 Attempts should be made to avoid introducing *Artemia* cysts and empty shells when the brine shrimp nauplii are fed to the fish larvae. Some of the mortality of the larval fish observed in cultures could be caused from the ingestion of these materials.

11.6.17.5.8 Disease Control

11.6.17.5.8.1 Fish are observed daily for abnormal appearance or behavior. Bacterial or fungal infections are the most common diseases encountered. However, if normal precautions are taken, disease outbreaks will rarely, if ever, occur. Hoffman and Mitchell (1980) have put together a list of some chemicals that have been used commonly for fish diseases and pests.

11.6.17.5.8.2 In aquatic culture systems where filtration is utilized, the application of certain antibacterial agents should be used with caution. A treatment with a single dose of antibacterial drugs can interrupt nitrate reduction and stop nitrification for various periods of time, resulting in changes in pH, and in ammonia, nitrite and nitrate concentrations (Collins et al., 1976). These changes could cause the death of the culture organisms.

11.6.17.5.8.3 Do not transfer equipment from one tank to another without first disinfecting tanks and nets. If an outbreak of disease occurs, any equipment, such as nets, airlines, tanks, etc., which has been exposed to diseased fish should be disinfected with sodium hypochlorite. Also to avoid the contamination of cultures or spread of disease, each time nets are used to remove live or dead fish from tanks, they are first sterilized with sodium hypochlorite or formalin, and rinsed in hot tap water. Before a new lot of fish is transferred to culture tanks, the tanks are cleaned and sterilized as described above.

11.6.17.5.8.4 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Newly hatched fathead minnow larvae less than 24 h old are used to monitor the chronic toxicity of the reference toxicant to the test fish produced by the culture unit (see Section 4, Quality Assurance).

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11.6.17.5.9 Record Keeping

11.6.17.5.9.1 Records, kept in a bound notebook, include: (l) type of food and time of feeding for all fish tanks; (2) time of examination of the tiles for embryos, the estimated number of embryos on the tile, and the tile position number; (3) estimated number of dead embryos and embryos with fungus observed during the embryonic development stages; (4) source of all fish; (5) daily observation of the condition and behavior of the fish; and (6) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

11.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

11.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

11.8 CALIBRATION AND STANDARDIZATION

11.8.1 See Section 4, Quality Assurance.

11.9 QUALITY CONTROL

11.9.1 See Section 4, Quality Assurance.

11.10 TEST PROCEDURES

11.10.1 TEST SOLUTIONS

11.10.1.1 Receiving Waters

11.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 250 mL, and 400 mL for chemical analyses, would require approximately 1.5 L or more of sample per test per day.

11.10.1.2 Effluents

11.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as the dilution factor is increased beyond 0.5, and declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the $ 0.5 dilution factor.**

11.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of effluent concentrations.

11.10.1.2.3 The volume of effluent required for daily renewal of four replicates per concentration, each containing 250 mL of test solution, is approximately 2.5 L. Sufficient test solution (approximately 1500 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses at the high, medium, and low test concentrations. If the sample is used for more than one daily renewal of test solutions, the volume must be increased proportionately.

11.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity

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tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

11.10.1.2.5 Just prior to test initiation (approximately 1 h) the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature and maintained at that temperature during the addition of dilution water.

11.10.1.2.6 The DO of the test solutions should be checked prior to the test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If any solution has a DO concentration below 4.0 mg/L, all of the solutions and the control must be gently aerated.

11.10.1.3 Dilution Water

11.10.1.3.1 Dilution water may be uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other uncontaminated natural water (see Section 7, Dilution Water).

11.10.2 START OF THE TEST

11.10.2.1 Label the test chambers with a marking pen. Use of color-coded tape to identify each treatment and replicate is helpful. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) must have a minimum of four replicates.

11.10.2.2 Tests performed in laboratories that have in-house fathead minnow breeding cultures should use larvae less than 24 h old. When eggs or larvae must be shipped to the test site from a remote location, it may be necessary to use larvae older than 24 h because of the difficulty in coordinating test organism shipments with field operations. However, in the latter case, the larvae must not be more than 48 h old at the start of the test and must all be within 24 h of the same age.

11.10.2.3 Randomize the position of test chambers at the beginning of the test (see Appendix A). Maintain the chambers in this configuration throughout the test. Preparation of a position chart may be helpful.

11.10.2.4 The larvae are pooled and placed one or two at a time into each randomly arranged test chamber or intermediate container in sequential order, until each chamber contains 15 (minimum of 10) larvae, for a total of 60 larvae (minimum of 40) for each concentration (see Appendix A). The test organisms should come from a pool of larvae consisting of at least three separate spawnings. The amount of water added to the chambers when transferring the larvae should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

11.10.2.4.1 The chambers may be placed on a light table to facilitate counting the larvae.

11.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

11.10.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at 25 ± 1°C.

11.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

11.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should be measured in the new solutions at the start of the test (Day 0) and before daily renewal of the test solutions on subsequent days. The DO concentrations should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all concentrations and the control should be aerated.

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The aeration rate should not exceed 100 bubbles/min, using a pipet with an orifice of approximately 1.5 mm, such as a 1-mL, KIMAX® serological pipet, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue physical stress to the fish.

11.10.5 FEEDING

11.10.5.1 The fish in each test chamber are fed 0.1 g (approximately 700 to 1000) of a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 g are fed twice daily at an interval of 6 h. Equal amounts of nauplii must be added to each replicate chamber to reduce variability in larval weight. Sufficient numbers of nauplii should be provided to assure that some remain alive in the test chambers for several hours, but not in excessive amounts which will result in depletion of DO below acceptable levels (below 4.0 mg/L).

11.10.5.2 The feeding schedule will depend on when the test solutions are renewed. If the test is initiated after 12:00 PM, the larvae may be fed only once the first day. On following days, the larvae normally would be fed at the beginning of the work day, at least 2 h before test solution renewal, and at the end of the work day, after test solution renewal. However, if the test solutions are changed at the beginning of the work day, the first feeding would be after test solution renewal in the morning, and the remaining feeding(s) would be at the appropriate intervals. The larvae are not fed during the final 12 h of the test.

11.10.5.3 The nauplii should be rinsed with freshwater to remove salinity before use (see USEPA, 2002a). At feeding time pipette about 5 mL (5 g) of concentrated newly hatched brine shrimp nauplii into a 120 mesh nylon net or plastic cup with nylon mesh bottom. Slowly run freshwater through the net or rinse by immersing the cup in a container of fresh water several times. Resuspend the brine shrimp in 10 mL of fresh water in a 30 mL beaker or simply set the cup of washed brine shrimp in ¼ inch of fresh water so that the cup contains about 10 mL of water. Allow the container to set for a minute or two to allow dead nauplii and empty cysts to settle or float to the surface before collecting the brine shrimp from just below the surface in a pipette for feeding. Distribute 2 drops (0.1 g) of the brine shrimp to each test chamber. If the survival rate in any test chamber falls below 50%, reduce the feeding in that chamber to 1 drop of brine shrimp at each subsequent feeding.

11.10.6 OBSERVATIONS DURING THE TEST

11.10.6.1 Routine Chemical and Physical Determinations

11.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period in at least one test chamber at each test concentration and in the control.

11.10.6.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test chamber at each test concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test vessels at least at the end of the test to determine the temperature variation in the environmental chamber.

11.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

11.10.6.1.4 Conductivity, alkalinity and hardness are measured in each new sample (100% effluent or receiving water) and in the control.

11.10.6.1.5 Record all the measurements on the data sheet (Figure 1)

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11.10.6.2 Routine Biological Observations

11.10.6.2.1 The number of live larvae in each test chamber are recorded daily (Figure 2) , and the dead larvae are discarded.

11.10.6.2.2 Protect the larvae from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of dead larvae, carefully. Make sure the larvae remain immersed during the performance of these operations.

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Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, larval survival and growth test. Routine chemical and physical determinations.

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Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, larval survival and growth test. Routine chemical and physical determinations (CONTINUED).

ED_001324_00000043-00085

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

No. Surviving Organisms									
Conc:	Rep. No.	Day							
Control:		1	2	3	4	5	6	7	Remarks
Conc:									
Conc:									
Conc:									
Conc:									
Conc:									
Conc:									
Conc:									

Comments:

Figure 2. Survival data for the fathead minnow, *Pimephales promelas*, larval survival and growth test.

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11.10.7 DAILY CLEANING OF TEST CHAMBERS

11.10.7.1 Before the daily renewal of test solutions, uneaten and dead *Artemia*, dead fish larvae, and other debris are removed from the bottom of the test chambers with a siphon hose. Alternately, a large pipet (50 mL) fitted with a rubber bulb can be used. Because of their small size during the first few days of the tests, larvae are easily drawn into the siphon tube or pipet when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of larvae can be greatly reduced because they can be more easily seen. If the water siphoned from the test chambers is collected in a white plastic tray, the larvae caught up in the siphon can be retrieved and returned to the chambers. Any incidence of removal of live larvae from the test chambers during cleaning, and subsequent return to the chambers, should be noted in the records.

11.10.8 TEST SOLUTION RENEWAL

11.10.8.1 Freshly prepared solutions are used to renew the tests daily immediately after cleaning the test chambers. For on-site toxicity studies, fresh effluent or receiving water samples should be collected daily, and no more than 24 h should elapse between collection of the samples and their use in the tests (see Section 8, Effluent and Receiving Water Sampling, Sample Holding, and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples are collected, preferably on days one, three, and five. Maintain the samples in the refrigerator at 0-6°C until used.

11.10.8.2 For test solution renewal, the water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution (250 mL) should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence and possible injury to the larvae.

11.10.9 TERMINATION OF THE TEST

11.10.9.1 The test is terminated after seven days of exposure. At test termination, dead larvae are removed and discarded. The surviving larvae in each test chamber (replicate) are counted and immediately prepared as a group for dry weight determination, or are preserved as a group in 70% ethanol or 4% formalin. Preserved organisms are dried and weighed within 7 days. For safety, formalin should be used under a hood.

11.10.9.2 For immediate drying and weighing, place live larvae onto a 500 µm mesh screen in a large beaker to wash away debris that might contribute to the dry weight. Each group of larvae is rinsed with deionized water to remove food particles, transferred to a tared weighing boat that has been properly labeled, and dried at 60°C, for 24 h or at 100°C for a minimum of 6 h. Immediately upon removal from the drying oven, the weighing boats are placed in a dessicator until weighed, to prevent the absorption of moisture from the air. All weights should be measured to the nearest 0.01 mg and recorded on data sheets (Figure 3). Subtract tare weight to determine the dry weight of the larvae in each replicate. For each test chamber, divide the final dry weight by the number of original larvae in the test chamber to determine the average individual dry weight and record on the data sheet (Figure 3). For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptability criteria (See Section 11.11). Average weights should be expressed to the nearest 0.001 mg.

11.10.9.3 Prepare a summary table as illustrated in Figure 4.

11.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

11.11.1 A summary of test conditions and test acceptability criteria is presented in Table 1.

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Discharge: _____ Test Date(s): _____ Drying Temperature (EC): _____
Location: _____ Weighing Date: _____ Drying Time (h): _____
Analyst: _____

Conc:	Rep. No.	A Wgt. of tare (mg)	B Dry wgt: tare and larvae (mg)	B-A Total dry wgt of larvae (mg)	C No. of original larvae (mg)	(B-A)/C Mean dry wgt of larvae (mg)	Remarks
Control							
Conc:							
Conc:							
Conc:							
Conc:							
Conc:							

Figure 3. Weight data for the fathead minnow, *Pimephales promelas*, larval survival and growth test. From USEPA (1989a).

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Discharger: _____ Test Dates: _____

Location: _____ Analyst: _____

TREATMENT	CONTROL					
NO. LIVE LARVAE						
SURVIVAL (%)						
MEAN DRY WGT OF LARVAE (MG) ± SD						
TEMPERATURE RANGE (EC)						
DISSOLVED OXYGEN RANGE (MG/L)						
HARDNESS						
CONDUCTIVITY						

COMMENTS:

Figure 4. Summary data for the fathead minnow, *Pimephales promelas*, larval survival and growth test.

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TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL SURVIVAL AND GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1000.0)[1]

1.	Test type:	Static renewal (required)
2.	Temperature (°C):	25 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
3.	Light quality:	Ambient laboratory illumination (recommended)
4.	Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c)(ambient laboratory levels) (recommended)
5.	Photoperiod:	16 h light, 8 h darkness (recommended)
6.	Test chamber size:	500 mL (recommended minimum)
7.	Test solution volume:	250 mL (recommended minimum)
8.	Renewal of test solutions:	Daily (required)
9.	Age of test organisms:	Newly hatched larvae less than 24 h old. If shipped, not more than 48 h old, 24 h range in age (required)
10.	No. larvae per test chamber:	10 (recommended)
11.	No. replicate chambers per concentration:	4 (required minimum)
12.	No. larvae per concentration:	40 (required minimum)
13.	Source of food:	Newly hatched *Artemia* nauplii (less than 24 h old) (required)
14.	Feeding regime:	On days 0-6, feed 0.1 g newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 g twice daily at 6-h intervals (at the beginning of the work day prior to renewal, and at the end of the work day following renewal). Sufficient nauplii are added to provide an excess. (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

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TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL SURVIVAL AND GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1000.0) (CONTINUED)

15.	Cleaning:	Siphon daily, immediately before test solution renewal (required)
16.	Aeration:	None, unless DO concentration falls below 4.0 mg/L. Rate should not exceed 100 bubbles/minimum (recommended)
17.	Dilution water:	Uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals, or DMW (see Section 7, Dilution Water) (available options)
18.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended)
19.	Dilution factor:	Effluents: $ 0.5 (recommended) Receiving waters: None or $ 0.5 (recommended)
20.	Test duration:	7 days (required)
21.	Endpoints:	Survival and growth (weight) (required)
22.	Test acceptability criteria:	80% or greater survival in controls; average dry weight per surviving organism in control chambers equals or exceeds 0.25 mg (required)
23.	Sampling requirements:	For on-site tests, samples collected daily, and used within 24 h of the time they are removed from the sampling device; For off-site tests, a minimum of three samples (e.g., collected on days one, three and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
24.	Sample volume required:	2.5 L/day (recommended)

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11.12 ACCEPTABILITY OF TEST RESULTS

11.12.1 For the test results to be acceptable, survival in the controls must be at least 80%. The average dry weight per surviving control larvae at the end of the test must equal or exceed 0.25 mg.

11.13 DATA ANALYSIS

11.13.1 GENERAL

11.13.1.1 Tabulate and summarize the data. A sample set of survival and growth response data is shown in Table 2.

TABLE 2. SUMMARY OF SURVIVAL AND GROWTH DATA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAE EXPOSED TO A REFERENCE TOXICANT FOR SEVEN DAYS[1]

NaPCP Conc. (µg/L)	Proportion of Survival in Replicate Chambers A	B	C	D	Mean Prop. Surv	Avg Dry Wgt (mg) In Replicate Chambers A	B	C	D	Mean Dry Wgt (mg)
0	1.0	1.0	0.9	0.9	0.95	0.711	0.662	0.646	0.690	0.677
32	0.8	0.8	1.0	0.8	0.85	0.517	0.501	0.723	0.560	0.575
64	0.9	1.0	1.0	1.0	0.975	0.602	0.669	0.694	0.676	0.660
128	0.9	0.9	0.8	1.0	0.90	0.566	0.612	0.410	0.672	0.565
256	0.7	0.9	1.0	0.5	0.775	0.455	0.502	0.606	0.254	0.454
512	0.4	0.3	0.4	0.2	0.325	0.143	0.163	0.195	0.099	0.150

[1] Four replicates of 10 larvae each.

11.13.1.2 The endpoints of toxicity tests using the fathead minnow, *Pimephales promelas*, larvae are based on the adverse effects on survival and growth. The LC50, the IC25, and the IC50 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for survival and growth are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50, IC25 and IC50. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for survival and growth, but included in the estimation of the LC50, IC25, and IC50. See the Appendices for examples of the manual computations, and examples of data input and program output.

11.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

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11.13.2 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW, *PIMEPHALES PROMELAS*, SURVIVAL DATA

11.13.2.1 Formal statistical analysis of the survival data is outlined in Figures 5 and 6. The response used in the analysis is the proportion of animals surviving in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the LC50, EC50, and IC endpoints. Concentrations at which there is no survival in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC, EC, and LC endpoints.

11.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

11.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

11.13.2.4 Probit Analysis (Finney, 1971; see Appendix I) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, or the Graphical Method may be used (see Appendices I-L).

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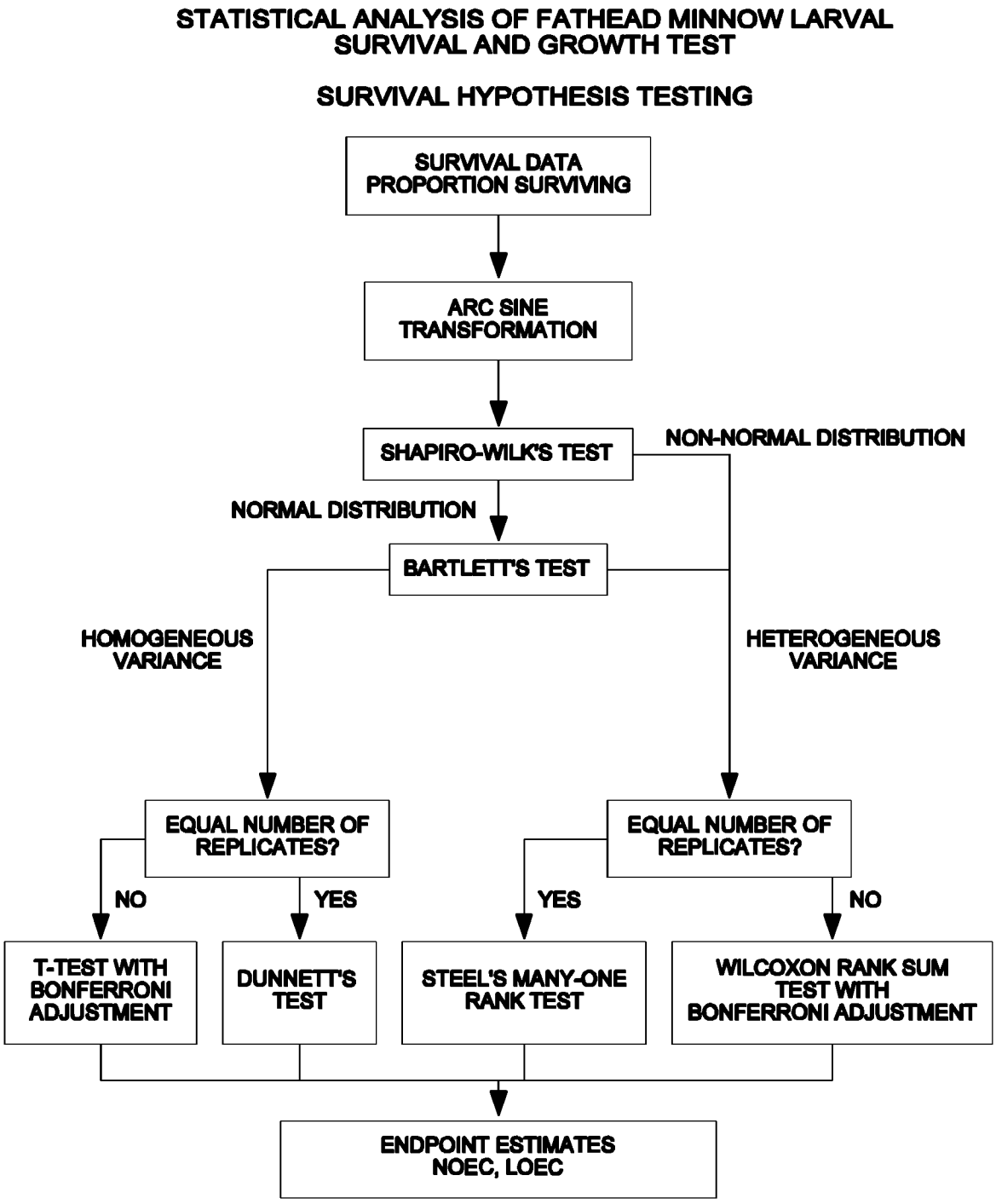

Figure 5. Flowchart for statistical analysis of the fathead minnow, *Pimephales promelas*, larval survival data by hypothesis testing.

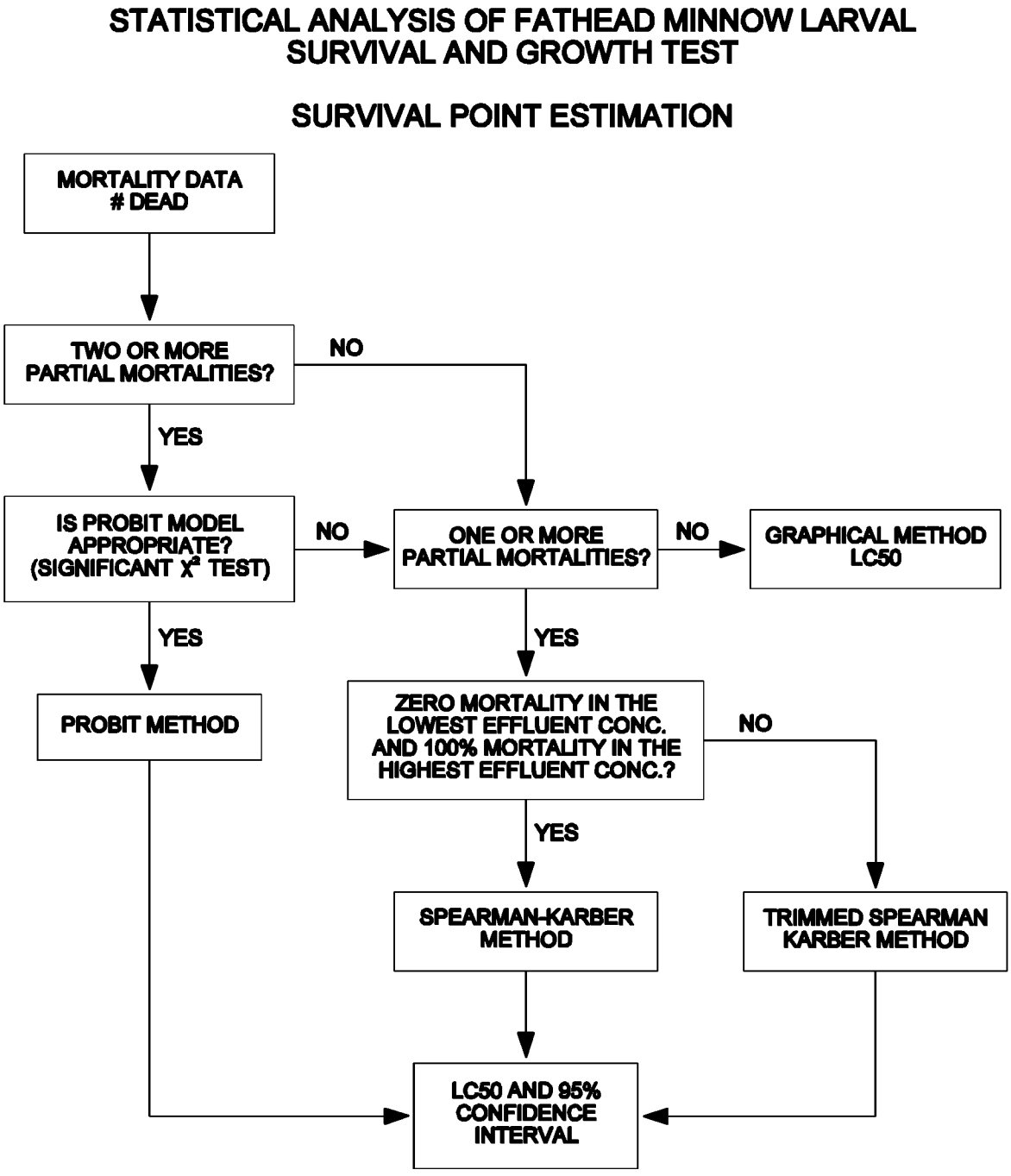

Figure 6. Flowchart for statistical analysis of the fathead minnow, *Pimephales promelas,* larval survival data by point estimation.

11.13.2.5 Example of Analysis of Survival Data

11.13.2.5.1 This example uses the survival data from the Fathead Minnow Larval Survival and Growth Test (Table 2). The proportion surviving in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each toxicant concentration and control are listed in Table 3. A plot of the survival proportions is provided in Figure 7.

TABLE 3. FATHEAD MINNOW, *PIMEPHALES PROMELAS*, SURVIVAL DATA

			NaPCP Concentration (µg/L)				
	Replicate	Control	32	64	128	256	512
	A	1.0	0.8	0.9	0.9	0.7	0.4
RAW	B	1.0	0.8	1.0	0.9	0.9	0.3
	C	0.9	1.0	1.0	0.8	1.0	0.4
	D	0.9	0.8	1.0	1.0	0.5	0.2
ARC SINE	A	1.412	1.107	1.249	1.249	0.991	0.685
TRANS-	B	1.412	1.107	1.412	1.249	1.249	0.580
FORMED	C	1.249	1.412	1.412	1.107	1.412	0.685
	D	1.249	1.107	1.412	1.412	0.785	0.464
Mean($\bar{Y}_i$)		1.330	1.183	1.371	1.254	1.109	0.604
S_i^2		0.0088	0.0232	0.0066	0.0155	0.0768	0.0111
i		1	2	3	4	5	6

11.13.2.6 Test for Normality

11.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 4.

11.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

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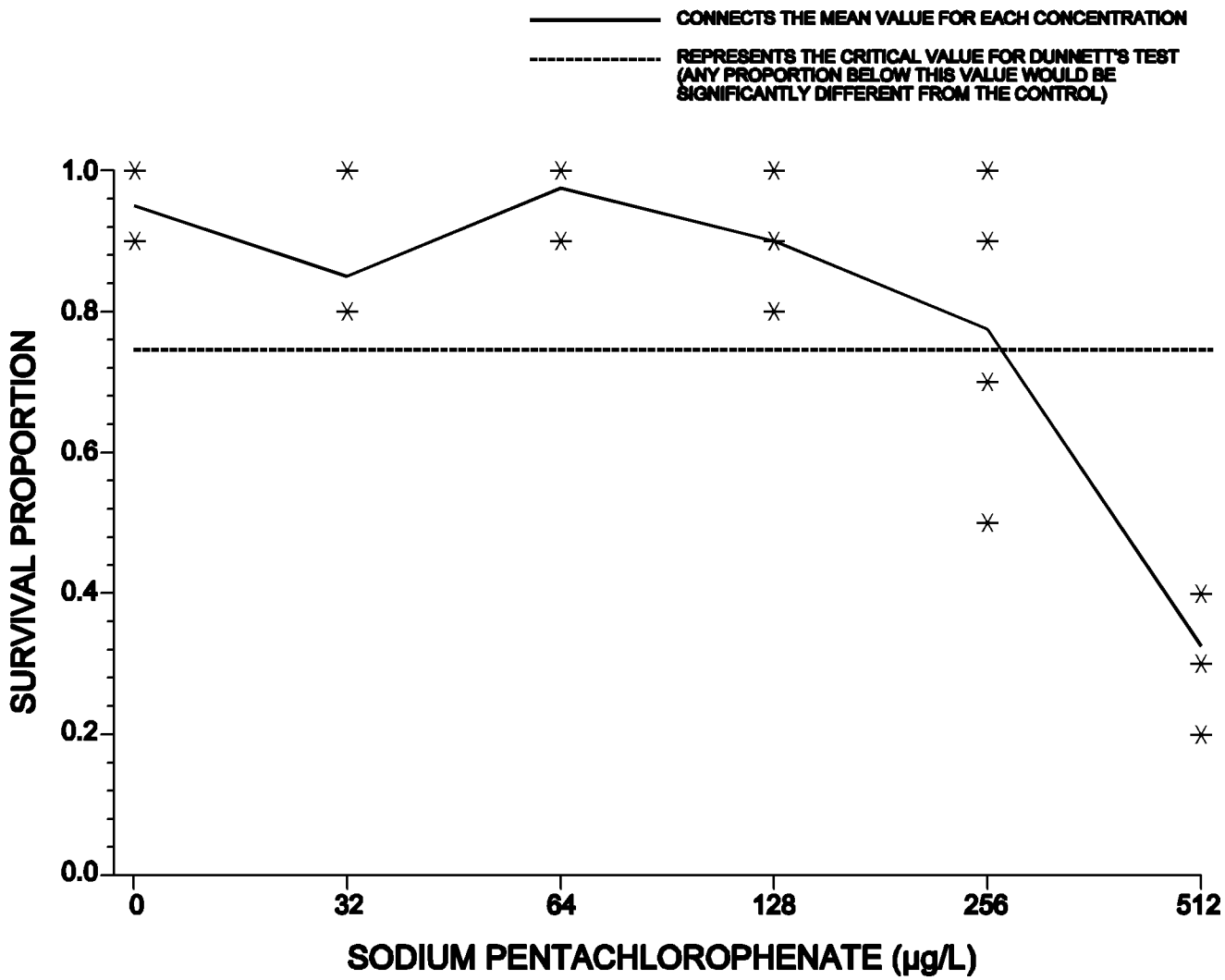

Figure 7. Plot of survival proportion data in Table 3.

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TABLE 4. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	NaPCP Concentration (µg/L) 32	64	128	256	512
A	0.082	-0.076	-0.122	-0.005	-0.118	0.081
B	0.082	-0.076	0.041	-0.005	0.140	-0.024
C	-0.081	0.229	0.041	-0.147	0.303	0.081
D	-0.081	-0.076	0.041	0.158	-0.324	-0.140

11.13.2.6.3 For this set of data: n = 24

$$\bar{X} = \frac{1}{24}(0.000) = 0.000$$

D = 0.4265

11.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 5.

TABLE 5. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.324	13	-0.005
2	-0.147	14	0.041
3	-0.140	15	0.041
4	-0.122	16	0.041
5	-0.118	17	0.081
6	-0.081	18	0.081
7	-0.081	19	0.082
8	-0.076	20	0.082
9	-0.076	21	0.140
10	-0.076	22	0.158
11	-0.024	23	0.229
12	-0.005	24	0.303

11.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 24 and k = 12. The a_i values are listed in Table 6.

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TABLE 6. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.627	$X^{(24)} - X^{(1)}$
2	0.3098	0.376	$X^{(23)} - X^{(2)}$
3	0.2554	0.298	$X^{(22)} - X^{(3)}$
4	0.2145	0.262	$X^{(21)} - X^{(4)}$
5	0.1807	0.200	$X^{(20)} - X^{(5)}$
6	0.1512	0.163	$X^{(19)} - X^{(6)}$
7	0.1245	0.162	$X^{(18)} - X^{(7)}$
8	0.0997	0.157	$X^{(17)} - X^{(8)}$
9	0.0764	0.117	$X^{(16)} - X^{(9)}$
10	0.0539	0.117	$X^{(15)} - X^{(10)}$
11	0.0321	0.065	$X^{(14)} - X^{(11)}$
12	0.0107	0.000	$X^{(13)} - X^{(12)}$

1.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 6. For the data in this example,

$$W = \frac{1}{0.4265}(0.6444)^2 = 0.974$$

11.13.2.6.7 The decision rule for this test is to compare W as calculated in Section 13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.974 is greater than the critical value, conclude that the data are normally distributed.

11.13.2.7 Test for Homogeneity of Variance

11.13.2.7.1 The test used to examine whether the variation in mean proportion surviving is the same across all toxicant concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{\left[\left(\sum_{i=1}^{P} V_i\right)\ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2\right]}{C}$$

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Where: V_i = degrees of freedom for each toxicant concentration and control, $V_i = (n_i - 1)$

n_i = the number of replicates for concentration i

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

$$\bar{S}^2 = \frac{\left(\sum_{i=1}^{p} V_i S_i^2\right)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + (3(p-1))^{-1}\left[\sum_{i=1}^{p} \frac{1}{V_i} - \left(\sum_{i=1}^{p} V_i\right)^{-1}\right]$$

11.13.2.7.2 For the data in this example (see Table 3), all toxicant concentrations including the control have the same number of replicates (n_i = 4 for all i). Thus, V_i = 3 for all i.

11.13.2.7.3 Bartlett's statistic is therefore:

$$B = [(18)\ln(0.0236) - 3\sum_{i=1}^{p} \ln(S_i^2)]/1.1296$$

$$= [18(-3.7465) - 3(-24.7516)]/1.1296$$

$$= 6.8178/1.1296$$

$$= 6.036$$

11.13.2.7.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test (from a table of chi-square distribution), at a significance level of 0.01 with five degrees of freedom, is 15.086. Since B = 6.036 is less than the critical value of 15.086, conclude that the variances are not different.

11.13.2.8 Dunnett's Procedure

11.13.2.8.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 7.

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TABLE 7. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number toxicant concentrations including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the proportion surviving for toxicant concentration i in test chamber j)

11.13.2.8.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 4$

N = 24

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 5.322$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 4.733$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 5.485$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 5.017$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 4.437$
$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} = 2.414$

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$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 27.408$

$$SSB = \sum_{i=1}^{p} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$

$$= \frac{1}{4}(131.495) - \frac{(27.408)^2}{24} = 1.574$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$

$$= 33.300 - \frac{(27.408)^2}{24} = 2.000$$

$SSW = SST - SSB = 2.000 - 1.574 = 0.4260$

$S_B^2 = SSB/(p-1) = 1.574/(6-1) = 0.3150$

$S_W^2 = SSW/(N-p) = 0.426/(24-6) = 0.024$

11.13.2.8.3 Summarize these calculations in the ANOVA table (Table 8).

TABLE 8. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	5	1.574	0.315
Within	18	0.426	0.024
Total	23	2.002	

11.13.2.8.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean proportion surviving for concentration i

$\bar{Y}_1$ = mean proportion surviving for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

11.13.2.8.5 Table 9 includes the calculated t values for each concentration and control combination. In this example, comparing the 32 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.330 - 1.183)}{[0.155\sqrt{(1/4) + (1/4)}]} = 1.341$$

TABLE 9. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.341
64	3	-0.374
128	4	0.693
256	5	2.016
512	6	6.624

11.13.2.8.6 Since the purpose of this test is to detect a significant reduction in proportion surviving, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 18 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.41. The mean proportion surviving for concentration i is considered significantly less than the mean proportion surviving for the control if t_i is greater than the critical value. Since t_6 is greater than 2.41, the 512 µg/L concentration has significantly lower survival than the control. Hence the NOEC and the LOEC for survival are 256 µg/L and 512 µg/L, respectively.

11.13.2.8.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be detected statistically may be calculated.

$$MSD = d\, S_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for Dunnett's procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

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11.13.2.8.8 In this example:

$$MSD \text{ ' } 2.41(0.155)\sqrt{(1/4) \% (1/4)}$$

$$= 2.41\ (0.155)(0.707)$$

$$= 0.264$$

11.13.2.8.9 The MSD (0.264) is in transformed units. To determine the MSD in terms of percent survival, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

   1.330 - 0.264 = 1.066

2. Obtain the untransformed values for the control mean and the difference calculated in 1.

   $[\text{Sine}\ (1.330)]^2 = 0.943$

   $[\text{Sine}\ (1.066)]^2 = 0.766$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from 2.

   $MSD_u = 0.943 - 0.766 = 0.177$

11.13.2.8.10 Therefore, for this set of data, the minimum difference in mean proportion surviving between the control and any toxicant concentration that can be detected as statistically significant is 0.177.

11.13.2.8.11 This represents a decrease in survival of 19% from the control.

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11.13.2.9 Calculation of the LC50

11.13.2.9.1 The data used for the Probit Analysis is summarized in Table 10. To perform the Probit Analysis, run the USEPA Probit Analysis Program. An example of the program input and output is supplied in Appendix I.

TABLE 10. DATA FOR PROBIT ANALYSIS

		NaPCP Concentration (µg/L)				
	Control	32	64	128	256	512
Number Dead	2	6	1	4	9	27
Number Exposed	40	40	40	40	40	40

11.13.2.9.2 For this example, the chi-square test for heterogeneity was not significant, thus Probit Analysis appears appropriate for this data.

11.13.2.9.3 Figure 8 shows the output data for the Probit Analysis of the data in Table 10 using the USEPA Probit Program.

11.13.3 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW, *PIMEPHALES PROMELAS,* GROWTH DATA

11.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 9. The response used in the statistical analysis is mean weight per original organism for each replicate. Because this measurement is based on the number of original organisms exposed (rather than the number surviving), the measured response is a combined survival and growth endpoint that can be termed biomass. An IC estimate can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain the NOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

11.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

11.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

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Probit Analysis of Fathead Minnow Larval Survival Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	40	2	0.0500	0.0000
32.0000	40	6	0.1500	0.0779
64.0000	40	1	0.0250	-.0577
128.0000	40	4	0.1000	0.0237
256.0000	40	9	0.2250	0.1593
512.0000	40	27	0.6750	0.6474

Chi - Square for Heterogeneity (calculated) = 4.522
Chi - Square for Heterogeneity
(Tabular value at 0.05 level) = 7.815

Probit Analysis of Fathead Minnow Larval Survival Data

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
LC/EC 1.00	127.637	34.590	195.433
LC/EC 50.00	422.696	345.730	531.024

Figure 8. Output for USEPA Probit Analysis Program, Version 1.5

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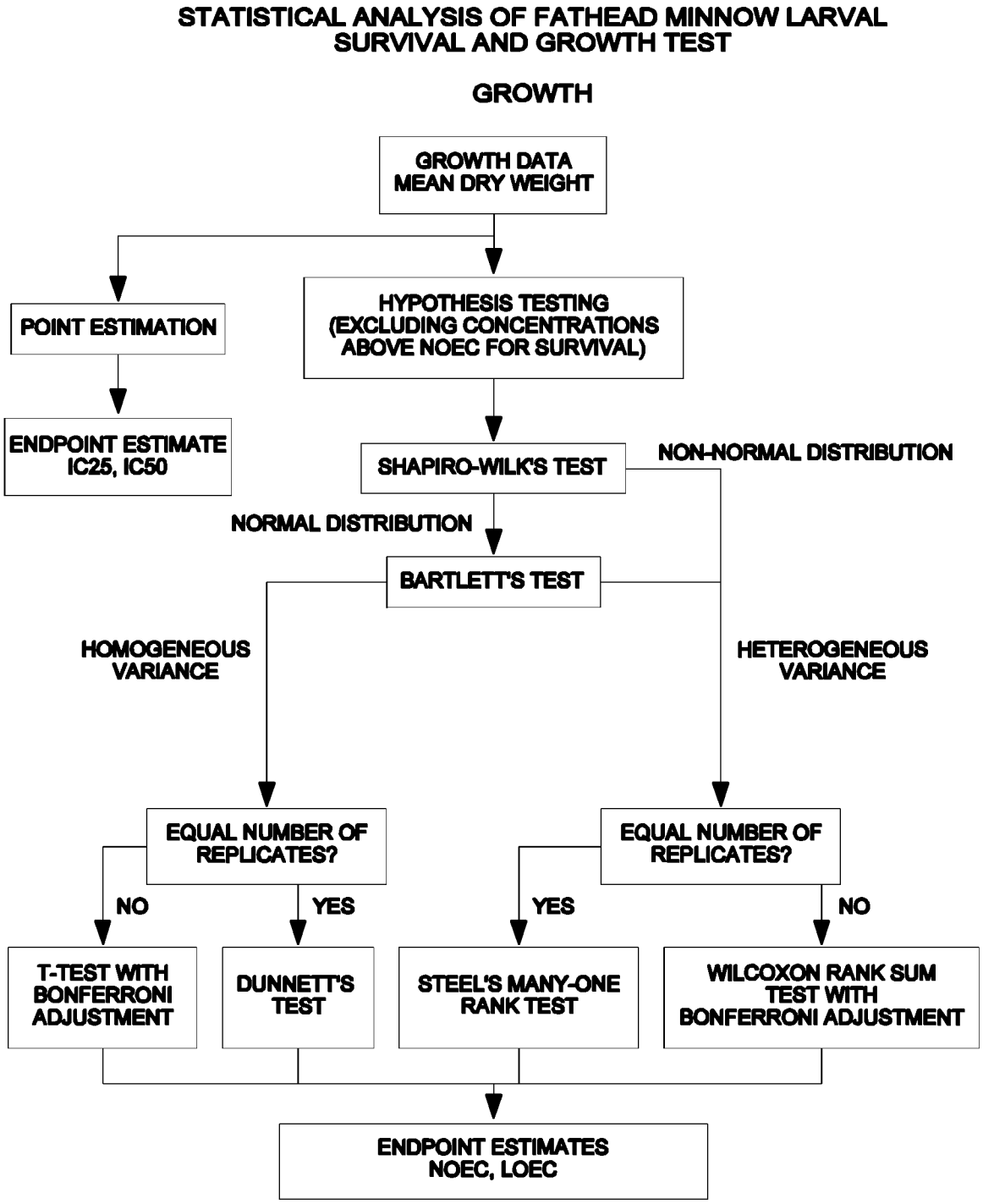

Figure 9. Flowchart for statistical analysis of fathead minnow, *Pimephales promelas,* larval growth data.

11.13.3.4 The data, mean and variance of the observations at each concentration including the control are listed in Table 11. A plot of the weight data for each treatment is provided in Figure 10. Since there is significant mortality in the 512 µg/L concentration, its effect on growth is not considered.

TABLE 11. FATHEAD MINNOW, *PIMEPHALES PROMELAS,* GROWTH DATA

		NaPCP Concentration (µg/L)				
Replicate	Control	32	64	128	256	512
A	0.711	0.517	0.602	0.566	0.455	-
B	0.662	0.501	0.669	0.612	0.502	-
C	0.646	0.723	0.694	0.410	0.606	-
D	0.690	0.560	0.676	0.672	0.254	-
Mean($\bar{Y}_i$)	0.677	0.575	0.660	0.565	0.454	-
S_i^2	0.00084	0.01032	0.00162	0.01256	0.0218	-
i	1	2	3	4	5	6

11.13.3.5 Test for Normality

11.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 12.

TABLE 12. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.034	-0.058	-0.058	0.001	0.001
B	-0.015	-0.074	0.009	0.047	0.048
C	-0.031	0.148	0.034	-0.155	0.152
D	0.013	-0.015	0.016	0.107	-0.200

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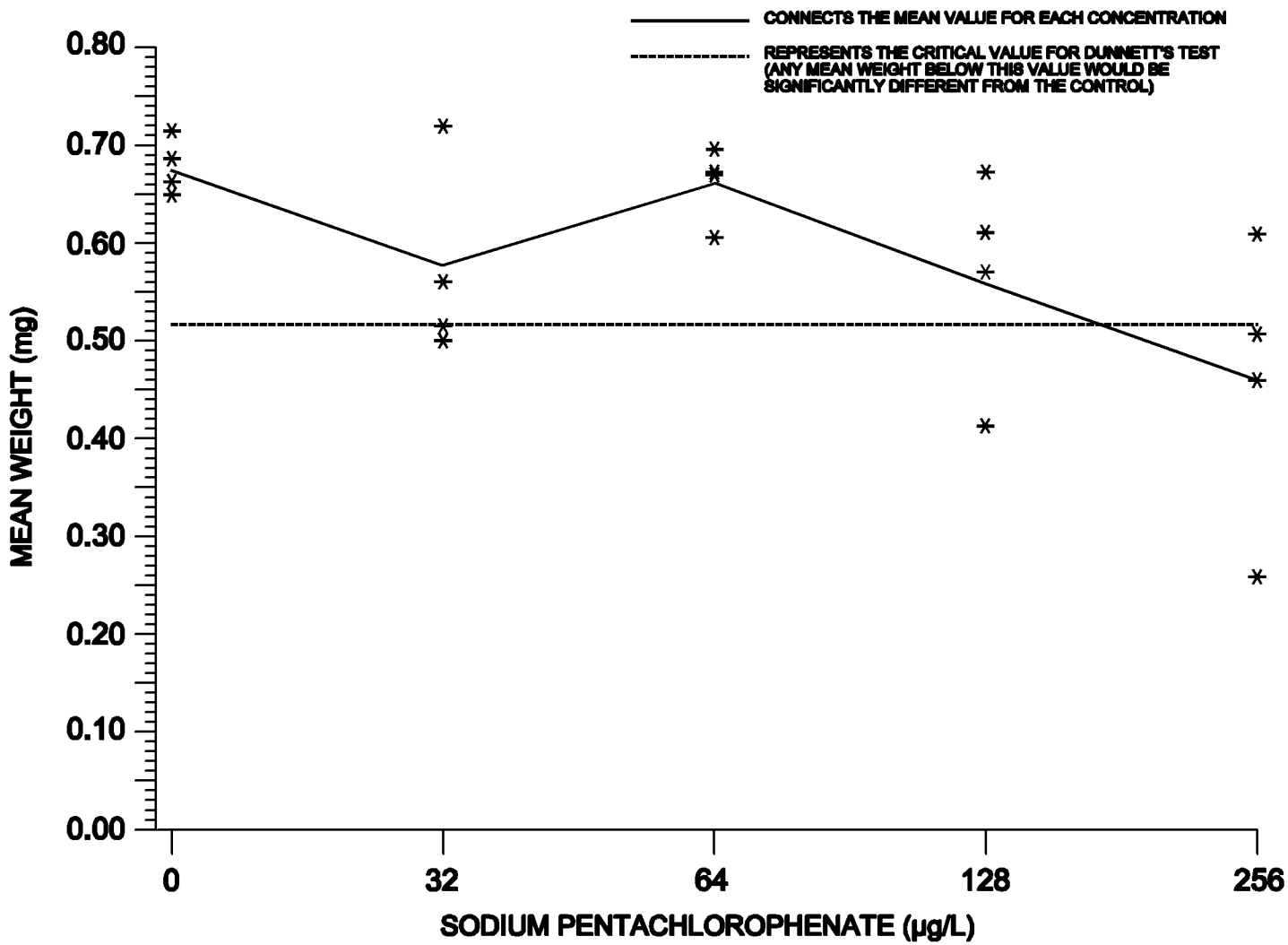

Figure 10. Plot of weight data from fathead minnow, *Pimephales promelas*, larval survival and growth test for point estimate testing.

11.13.3.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the <u>overall</u> mean of the centered observations

n = the total number of centered observations

For this set of data, n = 20

$$\bar{X} = \frac{1}{20}(0.004) = 0.000$$

D = 0.1414

11.13.3.5.3 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 13.

TABLE 13. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.200	11	0.009
2	-0.155	12	0.013
3	-0.074	13	0.016
4	-0.058	14	0.034
5	-0.058	15	0.034
6	-0.031	16	0.047
7	-0.015	17	0.048
8	-0.015	18	0.107
9	0.001	19	0.148
10	0.001	20	0.152

11.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 14.

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TABLE 14. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.352	$X^{(20)} - X^{(1)}$
2	0.3211	0.303	$X^{(19)} - X^{(2)}$
3	0.2565	0.181	$X^{(18)} - X^{(3)}$
4	0.2085	0.106	$X^{(17)} - X^{(4)}$
5	0.1686	0.105	$X^{(16)} - X^{(5)}$
6	0.1334	0.065	$X^{(15)} - X^{(6)}$
7	0.1013	0.049	$X^{(14)} - X^{(7)}$
8	0.0711	0.031	$X^{(13)} - X^{(8)}$
9	0.0422	0.012	$X^{(12)} - X^{(9)}$
10	0.0140	0.008	$X^{(11)} - X^{(10)}$

11.13.3.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

the differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 14. For this set of data:

$$W = \frac{1}{0.1414}(0.3666)^2 = 0.9505$$

11.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 20 observations (n) is 0.868. Since W = 0.9505 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

11.13.3.6 Test for Homogeneity of Variance

11.13.3.6.1 The test used to examine whether the variation in mean dry weight is the same across all toxicant concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{\left[\left(\sum_{i=1}^{P} V_i\right)\ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2\right]}{C}$$

Where: V_i = degrees of freedom for each toxicant concentration and control, $V_i = (n_i - 1)$

n_i = the number of replicates for concentration i.

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ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

$$\bar{S}^2 = \frac{\left(\sum_{i=1}^{p} V_i S_i^2\right)}{\sum_{i=1}^{p} V_i}$$

$$C = 1+(3(p-1))^{-1}\left[\sum_{i=1}^{p} 1/V_i - \left(\sum_{i=1}^{p} V_i\right)^{-1}\right]$$

11.13.3.6.2 For the data in this example, (see Table 11) all toxicant concentrations including the control have the same number of replicates (n_i = 4 for all i). Thus, V_i = 3 for all i.

11.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(15)\ln(0.00947) - 3\sum_{i=1}^{p} \ln(S_i^2)]/1.133$$

$$= [15(-5.9145) - 3(-26.2842]/1.133$$

$$= 8.8911/1.133$$

$$= 7.847$$

11.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with four degrees of freedom, is 13.277. Since B = 7.847 is less than the critical value of 13.277, conclude that the variances are not different.

11.13.3.7 Dunnett's Procedure

11.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 15.

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TABLE 15. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number toxicant concentrations including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean dry weight of the fish for toxicant concentration i in test chamber j)

11.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 4$

N = 20

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 2.709$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 2.301$

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$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.641$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.260$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 1.817$
$G = T_1 + T_2 + T_3 + T_4 + T_5 = 11.728$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$

$$= \frac{1}{4}(28.017) - \frac{(11.728)^2}{20} = 0.1270$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 7.146 - \frac{(11.728)^2}{20} = 0.2687$$

$$SSW = SST - SSB = 0.2687 - 0.1270 = 0.1417$$

$S_B^2 = SSB/(p-1) = 0.1270/(5-1) = 0.0318$

$S_W^2 = SSW/(N-p) = 0.041/(20-5) = 0.0094$

11.13.3.7.3 Summarize these calculations in the ANOVA table (Table 16).

TABLE 16. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	0.1270	0.0318
Within	15	0.1417	0.0094
Total	19	0.2687	

11.13.3.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1)+(1/n_i)}}$$

Where: $\bar{Y}_i$ = mean dry weight for toxicant concentration i

$\bar{Y}_1$ = mean dry weight for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

11.13.3.7.5 Table 17 includes the calculated t values for each concentration and control combination. In this example, comparing the 32 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(0.677 - 0.575)}{[0.097\sqrt{(1/4)+(1/4)}]} = 1.487$$

TABLE 17. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.487
64	3	0.248
128	4	1.632
256	5	3.251

11.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 15 degrees of freedom for error and four concentrations (excluding the control) the critical value is 2.36. The mean weight for concentration "*i*" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since t_5 is greater than 2.36, the 256 µg/L concentration had significantly lower growth than the control. Hence the NOEC and the LOEC for growth are 128 µg/L and 256 µg/L, respectively.

11.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\, S_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for the Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

11.13.3.7.8 In this example:

$$MSD \; ' \; 2.36(0.052)\sqrt{(1/4) \% (1/4)}$$

$$= 2.36\ (0.097)\ (0.707)$$

$$= 0.162$$

11.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.162 mg.

11.13.3.7.10 This represents a 24% reduction in mean weight from the control.

11.13.3.8 Calculation of the IC

11.13.3.8.1 The growth data in Table 2 modified to be mean weights per original number of fish are utilized in this example. As seen in Table 2 and Figure 11, the observed means are not monotonically non-increasing with respect to concentration (the mean response for each higher concentration is not less than or equal to the mean response for the previous concentration, and the responses between concentrations do not follow a linear trend). Therefore, the means are smoothed prior to calculating the IC. In the following discussion, the observed means are represented by $\bar{Y}_i$ and the smoothed means by M_i.

11.13.3.8.2 Starting with the control mean, $\bar{Y}_1 = 0.677$, we see that $\bar{Y}_1 > \bar{Y}_2$. Set $M_1 = \bar{Y}_1$ Comparing $\bar{Y}_2$ to $\bar{Y}_3$, $\bar{Y}_2 < \bar{Y}_3$.

11.13.3.8.3 Calculate the smoothed means:

$$M_2 = M_3 = (\bar{Y}_2 + \bar{Y}_3)/2 = 0.618$$

11.13.3.8.4 For the remaining observed means, $M_3 > \bar{Y}_4 > \bar{Y}_5 > \bar{Y}_6$. Thus, M_4 becomes $\bar{Y}_4$, M_5 becomes $\bar{Y}_5$ etc., for the remaining concentrations. Table 18 contains the smoothed means, and Figure 11 provides a plot of the smoothed concentration response curve.

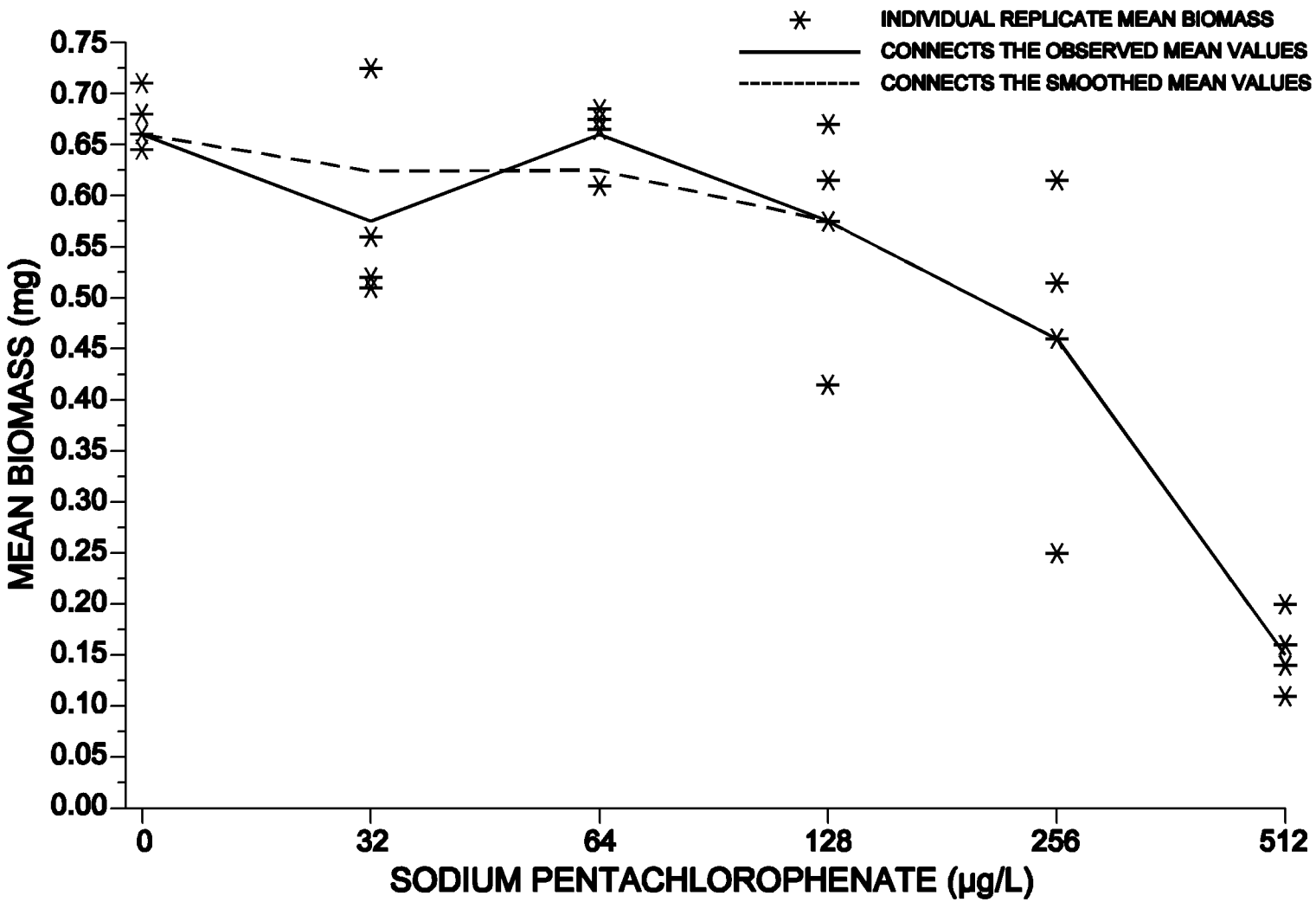

Figure 11. Plot of raw data, observed means, and smoothed means for the fathead minnow, *Pimephales promelas*, growth data in Tables 2 and 18.

TABLE 18. FATHEAD MINNOW, *PIMEPHALES PROMELAS*, MEAN GROWTH RESPONSE AFTER SMOOTHING

NaPCP Conc (µg/L)	i	Response means, $\bar{Y}_i$ (mg)	Smoothed means, M_i (mg)
Control	1	0.677	0.677
32	2	0.575	0.618
64	3	0.660	0.618
128	4	0.565	0.565
256	5	0.454	0.454
512	6	0.150	0.150

11.13.3.8.5 An IC25 and an IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean dry weight of 0.508 mg, where $M_1(1 - p/100) = 0.677(1 - 25/100)$. A 50% reduction in weight, compared to the controls, would result in a mean weight of 0.339 mg, where $M_1(1 - p/100) = 0.677(1 - 50/100)$. Examining the smoothed means and their associated concentrations (Table 18), the response 0.508 mg is bracketed by $C_4 = 128$ µg/L and $C_5 = 256$ µg/L. For the 50% reduction (0.339 mg), the response (0.339 µg) is bracketed by $C_5 = 256$ µg/L and $C_6 = 512$ µg/L.

11.13.3.8.6 Using the equation in Section 4.2 from Appendix M, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(p+1)} - C_j)}{(M_{(p+1)} - M_j)}$$

$$IC25 = 128 + [0.677(1 - 25/100) - 0.565]\frac{(256 - 128)}{(0.454 - 0.565)}$$

$$= 194 \text{ µg/L}$$

11.13.3.8.7 Using the equation in Section 4.2 of Appendix M the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\frac{(C_{(p+1)} - C_j)}{(M_{(p+1)} - M_j)}$$

$$IC50 = 256 + [0.677(1 - 50/100) - 0.454]\frac{(512 - 256)}{(0.150 - 0.454)}$$

$$= 353 \text{ µg/L}$$

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11.13.3.8.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 193.9503 µg/L. The empirical 95% confidence interval for the true mean was (54.9278 µg/L, 340.6617 µg/L). The computer program output for the IC25 for this data set is shown in Figure 12.

11.13.3.8.9 When the ICPIN program was used to analyze this set of data for the IC50, requesting 80 resamples, the estimate of the IC50 was 353.2884 µg/L. The empirical 95% confidence interval for the true mean was 208.4723 µg/L and 418.5276 µg/L. The computer program output is shown in Figure 13.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	32	64	128	256	512
Response 1	0.711	0.517	0.602	0.566	0.455	0.143
Response 2	0.662	0.501	0.669	0.612	0.502	0.163
Response 3	0.646	0.723	0.694	0.410	0.606	0.195
Response 4	0.690	0.560	0.676	0.672	0.254	0.099

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: NaPCP
Test Start Date: Example Test Ending Date:
Test Species: Fathead minnows
Test Duration: 7-d
DATA FILE: fhmanual.icp
OUTPUT FILE: fhmanual.i25

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	0.677	0.029	0.677
2	4	32.000	0.575	0.102	0.618
3	4	64.000	0.660	0.040	0.618
4	4	128.000	0.565	0.112	0.565
5	4	256.000	0.454	0.148	0.454
6	4	512.000	0.150	0.040	0.150

The Linear Interpolation Estimate: 193.9503 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 186.4935 Standard Deviation: 52.6094
Original Confidence Limits: Lower: 107.0613 Upper: 285.6449
Expanded Confidence Limits: Lower: 54.9278 Upper: 340.6617
Resampling time in Seconds: 1.81 Random Seed: 1272173518

Figure 12. ICPIN program output for the IC25.

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Conc. ID	1	2	3	4	5	6
Conc. Tested	0	32	64	128	256	512
Response 1	0.711	0.517	0.602	0.566	0.455	0.143
Response 2	0.662	0.501	0.669	0.612	0.502	0.163
Response 3	0.646	0.723	0.694	0.410	0.606	0.195
Response 4	0.690	0.560	0.676	0.672	0.254	0.099

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: NaPCP
Test Start Date: Example Test Ending Date:
Test Species: Fathead minnows
Test Duration: 7-d
DATA FILE: fhmanual.icp
OUTPUT FILE: fhmanual.i50

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	0.677	0.029	0.677
2	4	32.000	0.575	0.102	0.618
3	4	64.000	0.660	0.040	0.618
4	4	128.000	0.565	0.112	0.565
5	4	256.000	0.454	0.148	0.454
6	4	512.000	0.150	0.040	0.150

The Linear Interpolation Estimate: 353.2884 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 345.1108 Standard Deviation: 37.0938
Original Confidence Limits: Lower: 262.7783 Upper: 394.0629
Expanded Confidence Limits: Lower: 208.4723 Upper: 418.5276
Resampling time in Seconds: 1.87 Random Seed: 1126354766

Figure 13. ICPIN program output for the IC50.

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11.14 PRECISION AND ACCURACY

11.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 11.14.1.1 and 11.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

11.14.1.1 Single-Laboratory Precision

11.14.1.1.1 Information on the single-laboratory precision of the fathead minnow larval survival and growth test is presented in Table 19. The range of NOECs was only two concentration intervals, indicating good precision.

11.14.1.1.2 EPA evaluated within-laboratory precision of the Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test using a database of routine reference toxicant test results from 19 laboratories (USEPA, 2000b). The database consisted of 205 reference toxican t tests conducted in 19 laboratories using a variety of reference toxicants including: cadmium, chromium, copper, potassium chloride, sodium chloride, sodium pentachlorophenate, and sodium dodecyl sulfate. Among the 19 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 26% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 21%; and in 75% of laboratories, the within-laboratory CV was less than 38%.

TABLE 19. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, LARVAL SURVIVAL AND GROWTH TEST, USING NAPCP AS A REFERENCE TOXICANT[1,2]

Test	NOEC (µg/L)	LOEC (µg/L)	Chronic Value (µg/L)
1	256	512	362
2	128	256	181
3	256	512	362
4	128	256	181
5	128	256	181
n:	5	5	5
Mean:	NA	NA	253.4

[1] From Pickering, 1988.
[2] For a discussion of the precision of data from chronic toxicity tests, (see Section 4, Quality Assurance).

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11.14.1.2 Multilaboratory Precision

11.14.1.2.1 A multilaboratory study of Method 1000.0 described in the first edition of this manual (USEPA, 1985e), was performed using seven blind samples over an eight month period (DeGraeve et. al., 1988). In this study, each of the 10 participating laboratories was to conduct two tests simultaneous with each sample, each test having two replicates of 10 larvae for each of five concentrations and the control. Of the 140 tests planned, 135 were completed. Only nine of the 135 tests failed to meet the acceptance criterion of 80% survival in the controls. Of the 126 acceptable survival NOECs reported, an average of 41% were median values, and 89% were within one concentration interval of the median (Table 20). For the growth (weight) NOECs, an average of 32% were at the median, and 84% were within one concentration interval of the median (Table 21). Using point estimate techniques, the precision (CV) of the IC50 was 19.5% for the survival data and 19.8% for the growth data. If the mean weight acceptance criterion of 0.25 mg for the surviving control larvae, which is included in this revised edition of the method, had applied to the test results of the interlaboratory study, one third of the 135 tests would have failed to meet the test criteria (Norberg-King, personal communication and 1989 memorandum; DeGraeve et al., 1991).

11.14.1.2.2 In 2000, EPA conducted an interlaboratory variability study of the Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 27 participant laboratories tested 3 or 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Of the 101 Fathead Minnow Larval Survival and Growth tests conducted in this study, 98.0% were successfully completed and met the required test acceptability criteria. Of 24 tests that were conducted on blank samples, none showed false positive results for survival endpoints, and only one resulted in false positive results for the growth endpoint, yielding a false positive rate of 4.35%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 22 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 20.9% for IC25 results. Table 23 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned four concentrations for the reference toxicant sample type and two concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 97.2%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned five concentrations for the reference toxicant sample type and four concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 86.1%, 91.7%, and 76.9% for the reference toxicant, effluent, and receiving water sample types, respectively.

11.14.2 ACCURACY

11.14.2.1 The accuracy of toxicity tests cannot be determined.

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TABLE 20. COMBINED FREQUENCY DISTRIBUTION FOR SURVIVAL NOECs FOR ALL LABORATORIES[1]

Sample	NOEC Frequency (%) Distribution					
	Tests with Two Reps			Tests with Four Reps		
	Median	± 1[2]	> 2[3]	Median	± 1[2]	> 2[3]
1. Sodium Pentachlorophenate (A)	35	53	12	57	29	14
2. Sodium Pentachlorophenate (B)	42	42	16	56	44	0
3. Potassium Dichromate (A)	47	47	6	75	25	0
4. Potassium Dichromate (B)	41	41	18	50	50	0
5. Refinery Effluent 301	26	68	6	78	22	0
6. Refinery Effluent 401	37	53	10	56	44	0
7. Utility Waste 501	56	33	11	56	33	11

[1] From DeGraeve et al., 1988.
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.

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TABLE 21. COMBINED FREQUENCY DISTRIBUTION FOR WEIGHT NOECs FOR ALL LABORATORIES[1]

	NOEC Frequency (%) Distribution					
	Tests with Two Reps			Tests with Four Reps		
Sample	Median	± 1[2]	> 2[3]	Median	± 1[2]	> 2[3]
1. Sodium Pentachlorophenate (A)	59	41	0	57	43	0
2. Sodium Pentachlorophenate (B)	37	63	0	22	45	33
3. Potassium Dichromate (A)	35	47	18	88	0	12
4. Potassium Dichromate (B)	12	47	41	63	25	12
5. Refinery Effluent 301	35	53	12	75	25	0
6. Refinery Effluent 401	37	47	16	33	56	11
7. Utility Waste 501	11	61	28	33	56	11

[1] From DeGraeve et al., 1988.
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.

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TABLE 22. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	10.0	17.2	19.9
	Effluent	19.1	12.9	23.1
	Receiving water	-	-	19.8
	Average	14.6	15.0	20.9

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

3 The within-laboratory component of variability for duplicate samples tested at the same time in the same laboratory.

4 The between-laboratory component of variability for duplicate samples tested at different laboratories..

5 The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

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TABLE 23. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results $2[3]
Survival NOEC	Reference toxicant	50%	75.0	22.2	2.78
	Effluent	12.5%	76.9	23.1	0.00
	Receiving water	25%	69.2	30.8	0.00
Growth NOEC	Reference toxicant	50%	58.3	27.8	13.9
	Effluent	12.5%	66.7	25.0	8.33
	Receiving water	12.5%	30.8	46.1	23.1

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.

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SECTION 12

TEST METHOD

FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST METHOD 1001.0

12.1 SCOPE AND APPLICATION

12.1.1 This method estimates the chronic toxicity of whole effluents and receiving water to the fathead minnow, *Pimephales promelas*, using embryos in a seven-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms. The test is useful in screening for teratogens because organisms are exposed during embryonic development.

12.1.2 Daily observations on mortality make it possible to also calculate the acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

12.1.3 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

12.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly degradable and highly volatile toxicants, in the source may not be detected in the test.

12.1.5 This test method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

12.2 SUMMARY OF METHOD

12.2.1 Fathead minnow, *Pimephales promelas*, embryos are exposed in a static renewal system to different concentrations of effluent or to receiving water for seven days, starting shortly after fertilization of the eggs. Test results are based on the total frequency of both mortality and gross morphological deformities (terata).

12.3 INTERFERENCES

12.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

12.3.2 Adverse effects of low dissolved oxygen (DO), high concentrations of suspended and/or dissolved solids, and extremes of pH may mask the presence of toxic substances.

12.3.3 Improper effluent sampling and sample handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.3.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival and confound test results.

12.3.5 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with

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increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 12.3.5.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 12.3.5.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

12.3.5.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 12.3.5.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

12.3.5.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ±0.2 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.2 pH units in pH-controlled tests (USEPA, 1992).

12.3.5.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

12.3.5.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 12.3.6.1.1).

12.3.5.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 12.3.5.2) is applied routinely to subsequent testing of the effluent.

12.3.5.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992); or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents.

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If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, CO_2 is injected to maintain the test pH at the pH of the sample upon completion of collection. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

12.4 SAFETY

12.4.1 See Section 3, Health and Safety.

12.5 APPARATUS AND EQUIPMENT

12.5.1 Fathead minnow and brine shrimp culture units -- See Section 11, Fathead Minnow, *Pimephales Promelas*, Larval Survival and Growth Test, and USEPA, 2002a. To test effluent toxicity on-site or in the laboratory, sufficient numbers of newly fertilized eggs must be available, preferably from a laboratory fathead minnow culture unit. If necessary, embryos can be shipped in well oxygenated water in insulated containers. In cases where shipping is necessary, up to 48-h old embryos may be used for the test.

12.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L or more.

12.5.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.5.4 Environmental chamber or equivalent facility with temperature control (25 ± 1EC).

12.5.5 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

12.5.6 Balance -- analytical, capable of accurately weighing to 0.00001 g.

12.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of material to be weighed.

12.5.8 Test chambers -- four borosilicate glass or disposable, non-toxic plastic labware, per test solution, such as: 500-mL beakers; 100 mm x 15 mm or 100 mm x 20 mm glass or disposable polystyrene Petri dishes; or 12-cm OD, stackable "Carolina" culture dishes. The chambers should be covered with safety glass plates or sheet plastic during the test to avoid potential contamination from the air and excessive evaporation of the test solutions during the test.

12.5.9 Dissecting microscope, or long focal length magnifying lens, hand or stand supported -- for examining embryos and larvae in the test chambers.

12.5.10 Light box, microscope lamp, or flashlight -- for illuminating chambers during examination and observation of embryos and larvae.

12.5.11 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL, for making test solutions.

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12.5.12 Volumetric pipets -- Class A, 1-100 mL.

12.5.13 Serological pipets -- 1-10 mL, graduated.

12.5.14 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

12.5.15 Droppers, and glass tubing with fire polished edges, 2-mm ID -- for transferring embryos, and 4-mm ID -- for transferring larvae.

12.5.16 Wash bottles -- for washing embryos from substrates and containers and for rinsing small glassware and instrument electrodes and probes.

12.5.17 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

12.5.18 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

12.5.19 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA 1979b) -- to calibrate laboratory thermometers.

12.5.20 Meters, pH, DO, and specific conductivity -- for routine physical and chemical measurements.

12.6 REAGENTS AND CONSUMABLE MATERIALS

12.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling and Sample Preparation for Toxicity Tests).

12.6.2 Data sheets (one set per test) -- for recording data.

12.6.3 Tape, colored -- for labelling test chambers.

12.6.4 Markers, waterproof -- for marking containers, etc.

12.6.5 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA 1979b.

12.6.6 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA 1979b), or reagents -- for modified Winkler analysis.

12.6.7 Standard pH buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for instrument calibration (see USEPA Method 150.1, USEPA 1979b).

12.6.8 Specific conductivity standards -- see USEPA Method 120.1, USEPA 1979b.

12.6.9 Laboratory quality control samples and standards -- for calibration of the above methods.

12.6.10 Reference toxicant solutions -- see Section 4, Quality Assurance.

12.6.11 Reagent water -- defined as distilled or deionized water which does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

12.6.12 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

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12.6.13 TEST ORGANISMS, FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

12.6.13.1 Fathead minnow embryos, less than 36-h old, are used for the test. The test is conducted with four (minimum of three) test chambers at each toxicant concentration and control. Fifteen (minimum of ten) embryos are placed in each replicate test chamber. Thus 60 (minimum of 30) embryos are exposed at each test concentration and 360 (minimum of 180) embryos would be needed for a test consisting of five effluent concentrations and a control.

12.6.13.2 Sources of Organisms

12.6.13.2.1 It is recommended that the embryos be obtained from inhouse cultures or other local sources if at all possible, because it is often difficult to ship the embryos so that they will be less than 36 h old for beginning the test. Receipt of embryos via Express Mail, air express, or other carrier, from a reliable outside source is an acceptable alternative, but they must not be over 48 h old when used to begin the test.

12.6.13.2.2 Culturing methods for fathead minnows, *Pimephales promelas*, are described in Section 6, Section 11 and in USEPA, 2002a.

12.6.13.2.3 Fish obtained from outside sources (see Section 5, Facilities, Equipment, and Supplies) such as commercial biological supply houses for use as brood stock should be guaranteed to be (1) of the correct species, (2) disease free, (3) in the requested age range, and (4) in good condition. This can be done by providing the record of the date on which the eggs were laid and hatched, and information on the sensitivity of the contemporary fish to reference toxicants.

12.6.13.3 Obtaining Embryos for Toxicity Tests from Inhouse Cultures.

12.6.13.3.1 Spawning substrates with the newly-spawned, fertilized embryos are removed from the spawning tanks or ponds, and the embryos are separated from the spawning substrate by using the index finger and rolling the embryos gently with a circular movement of the finger (see Gast and Brungs, 1973). The embryos are then combined and washed from the spawning substrate onto a 400 µm NITEX® screen, sprayed with a stream of deionized water to remove detritus and food particles, and back-washed with dilution water into a crystallizing dish for microscopic examination. Damaged and infertile eggs are discarded.

12.6.13.3.2 The embryos from three or more spawns are pooled in a single container to provide a sufficient number to conduct the tests. These embryos may be used immediately to start a test inhouse or may be transported for use at a remote location. When transportation is required, embryos should be taken from the substrates within 12 h of spawning. This permits off-site tests to be started with less than 36-h old embryos. Embryos should be transported or shipped in clean, opaque, insulated containers, in well aerated or oxygenated fresh culture or dilution water, and should be protected from extremes of temperature and any other stressful conditions during transport. Instantaneous changes of water temperature when embryos are transferred from culture unit water to test dilution water, or from transport container water to on-site test dilution water, should be less than 2EC. Sudden changes in pH, dissolved ions, osmotic strength, and DO should be avoided.

12.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

12.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

12.8 CALIBRATION AND STANDARDIZATION

12.8.1 See Section 4, Quality Assurance.

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12.9 QUALITY CONTROL

12.9.1 See Section 4, Quality Assurance.

12.10 TEST PROCEDURES

12.10.1 TEST SOLUTIONS

12.10.1.1 Receiving Waters

12.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 100 mL, and 400 mL for chemical analysis, would require approximately one liter, or more, of sample per test day.

12.10.1.2 Effluents

12.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5 and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the $ 0.5 dilution factor.**

12.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of effluent concentrations.

12.10.1.2.3 The volume of effluent required for daily renewal of four replicates per concentration, each containing 100 mL of test solution, is 1.5 L. Sufficient test solution (approximately 1000 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses. If the sample is used for more than one daily renewal of test solutions, the volume must be increased proportionately.

12.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for the off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.10.1.2.5 Just prior to test initiation (approximately 1 h) the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature and maintained at that temperature during the addition of dilution water.

12.10.1.2.6 The DO of the test solutions should be checked prior to test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If any solution has a DO below 4.0 mg/L, all of the solutions and the control must be gently aerated.

12.10.1.3 Dilution Water

12.10.1.3.1 Dilution water may be uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other uncontaminated natural water (see Section 7, Dilution Water).

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12.10.1.3.2 If the hardness of the test solutions (including the control) does not equal or exceed 25 mg/L as $CaCO_3$, it may be necessary to adjust the hardness by adding reagents for synthetic softwater as listed in Table 3, Section 7. In this case parallel tests should be conducted, one with the hardness adjusted and one unadjusted.

12.10.2 START OF THE TEST

12.10.2.1 Label the test chambers with a marking pen and use color-coded tape to identify each treatment and replicate. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) should have four (minimum of three) replicates.

12.10.2.2 Tests performed in laboratories that have inhouse fathead minnow breeding cultures must initiate tests with embryos less than 36 h old. When the embryos must be shipped to the test site from a remote location, it may be necessary to use embryos older than 36 h because of the difficulty of coordinating test organism shipments with field operations. However, in the latter case, the embryos must not be more than 48 h old at the start of the test and should all be within 24 h of the same age.

12.10.2.3 Randomize the position of the test chambers at the beginning of the test (see Appendix A). Maintain the chambers in this configuration throughout the test. Preparation of a position chart may be helpful.

12.10.2.4 The test organisms should come from a pool of embryos consisting of at least three separate spawnings. Gently agitate and mix the embryos to be used in the test in a large container so that eggs from different spawns are thoroughly mixed.

12.10.2.5 Using a small bore (2 mm ID) glass tube, the embryos are placed one or two at a time into each randomly arranged test chamber or intermediate container in sequential order, until each chamber contains 15 (minimum of 10) embryos, for a total of 60 (minimum of 30) embryos for each concentration (see Appendix A). The amount of water added to the chambers when transferring the embryos to the compartments should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

12.10.2.6 After the embryos have been distributed to each test chamber, examine and count them. Remove and discard damaged or infertile eggs and replace with new undamaged embryos. Placing the test chambers on a light table may facilitate examining and counting the embryos.

12.10.3 LIGHT, PHOTOPERIOD AND TEMPERATURE

12.10.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at 25 ± 1EC.

12.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

12.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should be measured in the new solutions at the start of the test (Day 0) and before daily renewal of the new solutions on subsequent days. The DO concentrations should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all concentrations and the control should be aerated. The aeration rate should not exceed 100 bubbles/min, using a pipet with an orifice of approximately 1.5 mm, such as a l-mL KIMAX® serological Pipet, or equivalent. Care should be taken to ensure that turbulence resulting from the aeration does not cause undue physical stress to the embryos.

12.10.5 FEEDING

12.10.5.1 Feeding is not required.

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12.10.6 OBSERVATIONS DURING THE TEST

12.10.6.1 Routine Chemical and Physical Determinations

12.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period in at least one test chamber at each test concentrations and in the control.

12.10.6.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test chamber at each test concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test vessels, at least at the end of the test, to determine temperature variation in the environmental chamber.

12.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

12.10.6.1.4 Conductivity, alkalinity and hardness are measured in each new sample (100% effluent or receiving water) and in the control.

12.10.6.2 Record all the measurements on the data sheet (Figure 1).

12.10.6.3 Routine Biological Observations

12.10.6.3.1 At the end of the first 24 h of exposure, before renewing the test solutions, examine the embryos. Remove the dead embryos (milky colored and opaque) and record the number (Figure 2). If the rate of mortality (including those with fungal infection) exceeds 20% in the control chambers, or if excessive non-concentration-related mortality occurs, terminate the test and start a new test with new embryos.

12.10.6.3.2 At 25EC, hatching may begin on the fourth day. After hatching begins, count the number of dead and live embryos and the number of hatched, dead, live, and deformed larvae, daily. Deformed larvae are those with gross morphological abnormalities such as lack of appendages, lack of fusiform shape (non-distinct mass), lack of mobility, a colored, beating heart in an opaque mass, or other characteristics that preclude survival. Count and remove dead embryos and larvae as previously discussed and record the numbers for all of the test observations (Figure 2). Upon hatching, deformed larvae are counted as dead.

12.10.6.3.3 Protect the embryos and larvae from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of dead organisms carefully. Make sure that the test organisms remain immersed during the performance of the above operations.

12.10.7 DAILY CLEANING OF TEST CHAMBERS

12.10.7.1 Since feeding is not required, test chambers are not cleaned daily unless accumulation of particulate matter at the bottom of the chambers causes a problem.

12.10.8 TEST SOLUTION RENEWAL

12.10.8.1 Freshly prepared solutions are used to renew the tests daily. For on-site toxicity studies, fresh effluent or receiving water samples should be collected daily, and no more than 24 h should elapse between collection of the samples and their use in the tests (see Section 8, Effluent and Receiving Water Sampling, Sample Handling and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples are collected, preferably on days one, three, and five. Maintain the samples in the refrigerator at 0-6EC until used.

12.10.8.2 The test solutions are renewed immediately after removing dead embryos and/or larvae. During the daily renewal process, the water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence and possible injury to the embryos or larvae.

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Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Routine chemical and physical determinations.

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Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Conc:	Day 1	2	3	4	5	6	7	Remarks
Temp.								
D.O. Initial								
Final								
pH Initial								
Final								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Routine chemical and physical determinations (CONTINUED)

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Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Conc:	Rep. No.	Condition of Embryo/larvae	Day						
			1	2	3	4	5	6	7
Control	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							

Figure 2. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Survival and terata data.

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Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Conc:	Rep. No.	Condition of Embryo/larvae	Day						
			1	2	3	4	5	6	7
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							
Treatment	1	Live/dead							
		Terata							
	2	Live/dead							
		Terata							
	3	Live/dead							
		Terata							
	4	Live/dead							
		Terata							

Comments:

Figure 2. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Survival and terata data (CONTINUED).

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12.10.9 TERMINATION OF THE TEST

12.10.9.1 The test is terminated after seven days of exposure. Count the number of surviving, dead, and deformed larvae, and record the numbers of each (Figure 2). The deformed larvae are treated as dead in the analysis of the data. Keep a separate record of the total number and percent of deformed larvae for use in reporting the teratogenicity of the test solution.

12.10.9.2 Prepare a summary of the data as illustrated in Figure 3.

12.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

12.11.1 A summary of test conditions and test acceptability criteria is presented in Table 1.

12.12 ACCEPTABILITY OF TEST RESULTS

12.12.1 For the test results to be acceptable, survival in the controls must be at least 80%.

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Discharger: _____ Test Dates: _____

Location: _____ Analyst: _____

Treatment	Control					
No. dead embryos and larvae						
No. terata						
Total mortality (dead and deformed)						
Total mortality (%)						
Terata (%)						
Hatch (%)						

Comments:

Figure 3. Summary data for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test.

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TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS,* EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1001.0)[1]

1.	Test type:	Static renewal (required)
2.	Temperature:	25 ± 1EC (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3EC during the test (required)
3.	Light quality:	Ambient laboratory illumination (recommended)
4.	Light intensity:	10-20 $\mu E/m^2/s$ or 50-100 ft-c (ambient laboratory levels) (recommended)
5.	Photoperiod:	16 h light, 8 h dark (recommended)
6.	Test chamber size:	150 mL (recommended minimum)
7.	Test solution volume:	70 mL (recommended minimum)
8.	Renewal of test solutions:	Daily (required)
9.	Age of test organisms:	Less than 36-h old embryos (Maximum of 48-h if shipped) (required)
10.	No. embryos per test chamber:	15 (recommended) 10 (required minimum)
11.	No. replicate test chambers per concentration:	4 (recommended) 3 (required minimum)
12.	No. embryos per concentration:	60 (recommended) 30 (required minimum)
13.	Feeding regime:	Feeding not required
14.	Aeration:	None unless DO falls below 4.0 mg/L (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

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TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS,* EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1001.0) (CONTINUED)

15. Dilution water:	Uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or DMW (see Section 7, Dilution Water). The hardness of the test solutions should equal or exceed 25 mg/L ($CaCO_3$) to ensure hatching success (available options)
16. Test concentrations:	Effluents: 5 and a control (required minimum) Receiving waters: 100% receiving water (or minimum of 5) and a control (recommended)
17. Dilution factor:	Effluents: $ 0.5 (recommended) Receiving waters: None, or $ 0.5 (recommended)
18. Test duration:	7 days (required)
19. Endpoint:	Combined mortality (dead and deformed organisms) (required)
20. Test acceptability criteria:	80% or greater survival in controls (required)
21. Sampling requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
22. Sample volume required:	1.5 to 2.5 L/day depending on the volume of test solutions used (recommended)

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12.13 **DATA ANALYSIS**

12.13.1 GENERAL

12.13.1.1 Tabulate and summarize the data (Figure 3).

12.13.1.2 The endpoints of this toxicity test are based on total mortality, combined number of dead embryos, and dead and deformed larvae. The EC1 is calculated using Probit Analysis (Finney, 1971; see Appendix I). Separate analyses are performed for the estimation of LOEC and NOEC endpoints and for the estimation of the EC1 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the EC1 endpoint. See the Appendices for examples of the manual computations and examples of data input and output for the computer programs.

12.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

12.13.2 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY DATA

12.13.2.1 Formal statistical analysis of the total mortality data is outlined on the flowchart in Figure 4. The response used in the analysis is the total mortality proportion in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the EC endpoint. Concentrations at which there is 100% total mortality in all of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the EC1 endpoint.

12.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

12.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

12.13.2.4 Probit Analysis (Finney, 1971) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined.

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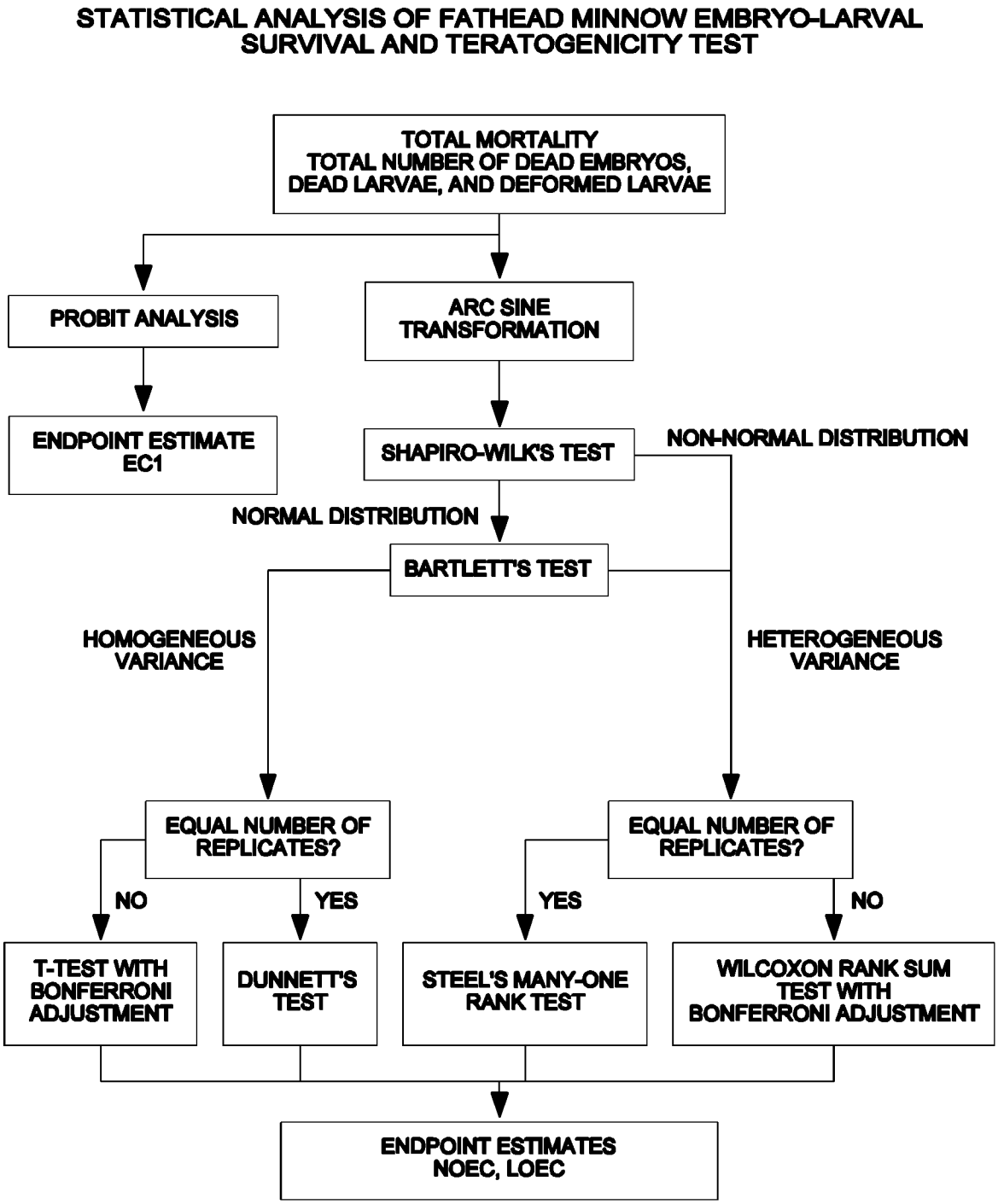

Figure 4. Flowchart for statistical analysis of fathead minnow, *Pimephales promelas*, embryo-larval data.

12.13.2.5 The data for this example are listed in Table 2. Total mortality, expressed as a proportion (combined total number of dead embryos, dead larvae and deformed larvae divided by the number of embryos at start of test), is the response of interest. The total mortality proportion in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 3. A plot of the data is provided in Figure 5. Since there is 100% total mortality in replicates for the 50.0% concentration, it is not included in this statistical analysis and is considered a qualitative mortality effect.

TABLE 2. DATA FROM FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL TOXICITY TEST WITH GROUND WATER EFFLUENT

Effluent Conc. (%)	No. Eggs at Start	Dead at Test Termination		Deformed at Test Termination		Dead + Deformed at Termination	
		No.	%	No.	%	No.	%
Control	10	0	0	0	0	0	0
	10	2	20	0	0	2	20
	10	0	0	0	0	0	0
	10	1	10	0	0	1	10
3.125	10	0	0	0	0	0	0
	10	0	0	1	10	1	10
	10	0	0	0	0	0	0
	10	1	10	0	0	1	10
6.25	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	0	0	1	10	1	10
12.5	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	0	0	0	0	0	0
	10	1	10	0	0	1	10
25.0	10	1	10	9	90	10	100
	10	2	20	8	80	10	100
	10	2	20	8	80	10	100
	10	1	10	4	40	5	50
50.0	10	4	40	6	60	10	100
	10	3	30	7	70	10	100
	10	5	50	5	50	10	100
	10	3	30	7	70	10	100

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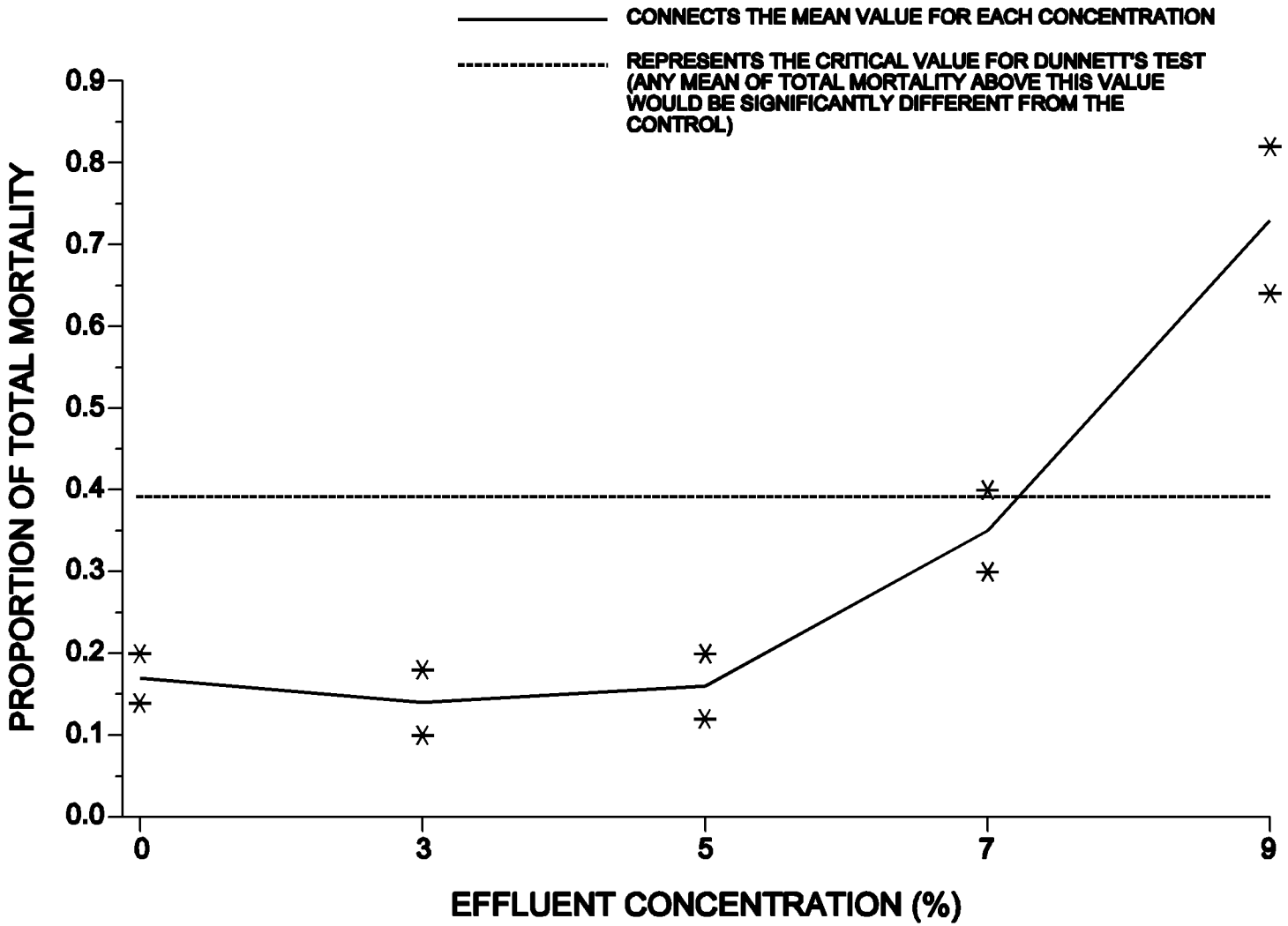

Figure 5. Plot of fathead minnow, *Pimephales promelas*, total mortality data from the embryo-larval test.

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TABLE 3. FATHEAD MINNOW, *PIMEPHALES PROMELAS,* EMBRYO-LARVAL TOTAL MORTALITY DATA

			Effluent Concentration (%)				
	Replicate	Control	3.125	6.25	12.5	25.0	50.0
RAW	A	0.00	0.00	0.00	0.00	1.00	1.00
	B	0.20	0.10	0.00	0.00	1.00	1.00
	C	0.00	0.00	0.00	0.00	1.00	1.00
	D	0.10	0.10	0.10	0.10	0.50	1.00
ARC SINE	A	0.159	0.159	0.159	0.159	1.412	-
TRANS-	B	0.464	0.322	0.159	0.159	1.412	-
FORMED	C	0.159	0.159	0.159	0.159	1.412	-
	D	0.322	0.322	0.322	0.322	0.785	-
Mean($\bar{Y}_i$)		0.276	0.241	0.200	0.200	1.255	
S_i^2		0.022	0.009	0.007	0.007	0.098	
i		1	2	3	4	5	

12.13.2.6 Test for Normality

12.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 4.

TABLE 4. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Effluent Concentration (%)				
Replicate	Control	3.125	6.25	12.5	25.0	50.0
A	-0.117	-0.082	-0.041	-0.041	0.157	-
B	0.188	0.081	-0.041	-0.041	0.157	-
C	-0.117	0.081	-0.041	-0.041	0.157	-
D	0.046	-0.082	0.122	0.122	-0.470	-

12.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

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n = the total number of centered observations

12.13.2.6.3 For this set of data, n = 20

$$\bar{X} \; ' \; \frac{1}{20}(\&0.003) \; ' \; 0.000$$

$$D = 0.4261$$

12.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 5.

TABLE 5. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.470	11	-0.041
2	-0.117	12	0.046
3	-0.117	13	0.081
4	-0.082	14	0.081
5	-0.082	15	0.122
6	-0.041	16	0.122
7	-0.041	17	0.157
8	-0.041	18	0.157
9	-0.041	19	0.157
10	-0.041	20	0.188

12.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 6.

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TABLE 6. COEFFICIENTS AND DIFFERENCES FOR THE SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.658	$X^{(20)} - X^{(1)}$
2	0.3211	0.274	$X^{(19)} - X^{(2)}$
3	0.2565	0.274	$X^{(18)} - X^{(3)}$
4	0.2085	0.239	$X^{(17)} - X^{(4)}$
5	0.1686	0.204	$X^{(16)} - X^{(5)}$
6	0.1334	0.163	$X^{(15)} - X^{(6)}$
7	0.1013	0.122	$X^{(14)} - X^{(7)}$
8	0.0711	0.122	$X^{(13)} - X^{(8)}$
9	0.0422	0.087	$X^{(12)} - X^{(9)}$
10	0.0140	0.000	$X^{(11)} - X^{(10)}$

12.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 6. For the data in this example,

$$W = \frac{1}{0.4261}(0.6004)^2$$
$$= 0.846$$

12.13.2.6.7 The decision rule for this test is to compare W as calculated in Section 13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 20 observations is 0.868. Since W = 0.846 is less than the critical value, conclude that the data are not normally distributed.

12.13.2.6.8 Since the data do not meet the assumption of normality, Steel's Many-one Rank Test will be used to analyze the total mortality data.

12.13.2.7 Steel's Many-one Rank Test

12.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ..., 8) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

12.13.2.7.2 An example of assigning ranks to the combined data for the control and 3.125% effluent concentration is given in Table 7. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 8. The control group ranks are next summed for each effluent concentration pairing, as shown in Table 9.

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TABLE 7. ASSIGNING RANKS TO THE CONTROL AND 3.125% EFFLUENT CONCENTRATION FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Mortality	Effluent Concentration (%)
2.5	0.159	Control
2.5	0.159	Control
2.5	0.159	3.125
2.5	0.159	3.125
6	0.322	Control
6	0.322	3.125
6	0.322	3.125
8	0.464	Control

TABLE 8. TABLE OF RANKS FOR STEEL'S MANY-ONE RANK TEST

			Effluent Concentration (%)							
Repl.	Control		3.125		6.25		12.5		25.0	
A	0.159	(2.5,3,3,1.5)	0.159	(2.5)	0.159	(3)	0.159	(3)	1.412	(7)
B	0.464	(8,8,8,4)	0.322	(6)	0.159	(3)	0.159	(3)	1.412	(7)
C	0.159	(2.5,3,3,1.5)	0.159	(2.5)	0.159	(3)	0.159	(3)	1.412	(7)
D	0.322	(6,6.5,6.5,3)	0.322	(6)	0.322	(3)	0.159	(3)	0.785	(5)

TABLE 9. RANK SUMS

Effluent Concentration (%)	Control Rank Sum
3.125	19
6.25	20.5
12.5	20.5
25.0	10

12.13.2.7.3 For this example, we want to determine if the total mortality in any of the effluent concentrations is significantly higher than the total mortality in the control. If this occurs, the rank sum of the control would be significantly less than the rank sum at that concentration. Thus we are only concerned with comparing the control rank sum for each pairing with the various effluent concentrations with some "minimum" or critical rank sum, at or below which the concentration total mortality would be considered significantly greater than the control. At a signficance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control) and four replicates per concentration is 10 (see Table 5, Appendix E).

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12.13.2.7.4 Since the control rank sum for the 25.0% effluent concentration pairing is equal to the critical value, the total proportion mortality in the 25.0% concentration is considered significantly greater than that in the control. Since no other rank sums are less than or equal to the critical value, no other concentrations have signficantly higher total proportion mortality than the control. Hence the NOEC is 12.5% and the LOEC is 25.0%.

12.13.2.8 Calculation of the LC50

12.13.2.8.1 The data used for the Probit Analysis is summarized in Table 10. To perform the Probit Analysis, run the USEPA Probit Analysis Program. An example of the program input and output is supplied in Appendix I.

12.13.2.8.2 For this example, the chi-square test for heterogeneity was not significant. Thus Probit Analysis appears appropriate for this data.

12.13.2.8.3 Figure 6 shows the output data for the Probit Analysis of the data from Table 10 using the USEPA Probit Program.

TABLE 10. DATA FOR PROBIT ANALYSIS

		Effluent Concentration (%)				
	Control	3.125	6.25	12.5	25.0	50.0
Number Dead	3	1	0	1	6	15
Number Exposed	40	40	40	40	40	40

12.14 **PRECISION AND ACCURACY**

12.14.1 PRECISION

12.14.1.1 Single-laboratory Precision

12.14.1.1.1 Data shown in Tables 11 and 12 indicate that the precision of the embryo-larval survival and teratogenicity test, expressed as the relative standard deviation (or coefficient of variation, CV) of the LC1 values, was 62% for cadmium (Table 11) and 41% for Diquat (Table 12).

12.14.1.1.2 Precision data are also available from four embryo-larval survival and teratogenicity tests on trickling filter pilot plant effluent (Table 13). Although the data could not be analyzed by Probit Analysis, the NOECs and LOECs obtained using Dunnett's Procedure were the same for all four tests, 7% and 11% effluent, respectively, indicating maximum precision in terms of the test design.

12.14.1.2 Multilaboratory Precision

12.14.1.2.1 Data on the multilaboratory precision of this test are not yet available.

12.14.2 ACCURACY

12.14.2.1 The accuracy of toxicity tests cannot be determined.

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USEPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Probit Analysis of Fathead Minnow Embryo-Larval Survival
and Teratogenicity Data

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	20	2	0.1000	0.0000
0.5000	20	2	0.1000	0.0174
1.0000	20	1	0.0500	-.0372
2.0000	20	4	0.2000	0.1265
4.0000	20	16	0.8000	0.7816
8.0000	20	20	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 0.441
Chi - Square for Heterogeneity (tabular value) = 7.815

Probit Analysis of Fathead Minnow Embryo-Larval Survival
and Teratogenicity Data

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
LC/EC 1.00	1.346	0.453	1.922
LC/EC 50.00	3.018	2.268	3.672

Figure 6. Output for USEPA Probit Program, Version 1.5.

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TABLE 11. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST, USING CADMIUM AS A REFERENCE TOXICANT[1,2]

Test	LC1[3] (mg/L)	95% Confidence Limits	NOEC[4] (mg/L)
1	0.014	0.009 - 0.018	0.012
2	0.006	0.003 - 0.010	0.012
3	0.005	0.003 - 0.009	0.013
4	0.003	0.002 - 0.004	0.011
5	0.006	0.003 - 0.009	0.012
N	5		5
Mean	0.0068		NA
SD	0.0042		
CV(%)	62		NA

1 Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Bioassessment and Ecotoxicology Branch, EMSL, USEPA, Cincinnati, OH.

2 Cadmium chloride was used as the reference toxicant. The nominal concentrations, expressed as cadmium (mg/L), were: 0.01, 0.032, 0.100, 0.320, and 1.000. The dilution water was reconstituted water with a hardness of 100 mg/L as calcium carbonate, and a pH of 7.8.

3 Determined by Probit Analysis.

4 Highest no-observed-effect concentration determined by independent statistical analysis (2X2 Chi-square Fisher's Exact Test). NOEC range of 0.011 - 0.013 represents a difference of one exposure concentration.

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TABLE12. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL, SURVIVAL AND TERATOGENICITY TOXICITY TEST, USING DIQUAT AS A REFERENCE TOXICANT[1,2]

Test	LC1[3] (mg/L)	95% Confidence Limits
1	0.58	0.32 - 0.86
2	2.31	--[4]
3	1.50	1.05 - 1.87
4	1.71	1.24 - 2.09
5	1.43	0.93 - 1.83
N	5	
Mean	1.51	
SD	0.62	
CV(%)	41.3	

1 Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Bioassessment and Ecotoxicology Branch, EMSL, USEPA, Cincinnati, OH.

2 The Diquat concentrations were determined by chemical analysis. The dilution water was reconstituted water with a hardness of 100 mg/L as calcium carbonate, and a pH of 7.8.

3 Determined by Probit Analysis.

4 Cannot be calculated.

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TABLE 13. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY STATIC-RENEWAL TEST CONDUCTED WITH TRICKLING FILTER EFFLUENT[1,2,3]

Test No.	NOEC (% Effluent)	LOEC (% Effluent)
1	7	11
2	7	11
3	7	11
4	7	11

[1] Data provided by Timothy Neiheisel, Bioassessment and Ecotoxiology Branch, EMSL, USEPA, Cincinnati, OH.
[2] Effluent concentrations used: 3, 5, 7, 11 and 16%
[3] Maximum precision achieved in terms of NOEC-LOEC interval. For a discusssion of the precision of data from chronic toxicity tests (see Section 4, Quality Assurance).

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SECTION 13

TEST METHOD

DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TEST METHOD 1002.0

13.1 SCOPE AND APPLICATION

13.1.1 This method measures the chronic toxicity of effluents and receiving water to the daphnid, *Ceriodaphnia dubia*, using less than 24 h old neonates during a three-brood (seven-day), static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

13.1.2 Daily observations on mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, and 96-h LC50s).

13.1.3 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

13.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly degradable or highly volatile toxicants in the source may not be detected in the test.

13.1.5 This test method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

13.2 SUMMARY OF METHOD

13.2.1 *Ceriodaphnia dubia* are exposed in a static renewal system to different concentrations of effluent, or to receiving water, until 60% or more of surviving control females have three broods of offspring. Test results are based on survival and reproduction. If the test is conducted as described, the surviving control organisms should produce 15 or more young in three broods. If these criteria are not met at the end of 8 days, the test must be repeated.

13.3 INTERFERENCES

13.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

13.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.3.3 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival and confound test results.

13.3.4 The amount and type of natural food in the effluent or dilution water may confound test results.

13.3.5 Food added during the test may sequester metals and other toxic substances and confound test results. Daily renewal of solutions, however, will reduce the probability of reduction of toxicity caused by feeding.

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13.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 13.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 13.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

13.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 13.3.6.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the initial pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

13.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ±0.2 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ±0.2 pH units in pH-controlled tests (USEPA, 1992).

13.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

13.3.6.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 13.3.6.1.1).

13.3.6.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 13.3.6.2) is applied routinely to subsequent testing of the effluent.

13.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992);

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or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, CO_2 is injected to maintain the test pH at the pH of the sample upon completion of collection. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

13.4 SAFETY

13.4.1 See Section 3, Health and Safety.

13.5 APPARATUS AND EQUIPMENT

13.5.1 *Ceriodaphnia* and algal culture units -- See *Ceriodaphnia* and algal culturing methods below and algal culturing methods in Section 14 and USEPA, 2002a.

13.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, capable of collecting a 24-h composite sample of 5 L or more.

13.5.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.5.4 Environmental chambers, incubators, or equivalent facilities with temperature control (25 ± 1EC).

13.5.5 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

13.5.6 Balance -- analytical, capable of accurately weighing 0.00001 g.

13.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the material to be weighed.

13.5.8 Test chambers -- 10 test chambers are required for each concentration and control. Test chambers such as 30-mL borosilicate glass beakers or disposable polystyrene cups are recommended because they will fit in the viewing field of most stereoscopic microscopes. The glass beakers and plastic cups are rinsed thoroughly with dilution water before use. To avoid potential contamination from the air and excessive evaporation of the test solutions during the test, the test vessels should be covered with safety glass plates or sheet plastic (6 mm thick).

13.5.9 Mechanical shaker or magnetic stir plates -- for algal cultures.

13.5.10 Light meter -- with a range of 0-200 $\mu E/m^2/s$ (0-1000 ft-c).

13.5.11 Fluorometer (optional) -- equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

13.5.12 UV-VIS spectrophotometer (optional) -- capable of accommodating 1-5 cm cuvettes.

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13.5.13 Cuvettes for spectrophotometer -- 1-5 cm light path.

13.5.14 Electronic particle counter (optional) -- Coulter Counter, ZBI, or equivalent, with mean cell (particle) volume determination.

13.5.15 Microscope with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condensor, and light source (inverted or conventional microscope) -- for determining sex and verifying identification.

13.5.16 Dissecting microscope, stereoscopic, with zoom objective, magnification to 50X -- for examining and counting the neonates in the test vessels.

13.5.17 Counting chamber -- Sedgwick-Rafter, Palmer-Maloney, or hemocytometer.

13.5.18 Centrifuge (optional) -- plankton, or with swing-out buckets having a capacity of 15-100 mL.

13.5.19 Centrifuge tubes -- 15-100 mL, screw-cap.

13.5.20 Filtering apparatus -- for membrane and/or glass fiber filters.

13.5.21 Racks (boards) -- to hold test chambers. It is convenient to use a piece of styrofoam insulation board, 50 cm x 30 cm x 2.5 cm (20 in x 12 in x 1 in), drilled to hold 60 test chambers, in six rows of 10 (see Figure 1).

13.5.22 Light box -- for illuminating organisms during examination.

13.5.23 Volumetric flasks and graduated cylinders -- class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL, for culture work and preparation of test solutions.

13.5.24 Pipettors, adjustable volume repeating dispensers -- for feeding. Pipettors such as the Gilson REPETMAN®, Eppendorf, Oxford, or equivalent, provide a rapid and accurate means of dispensing small volumes (0.1 mL) of food to large numbers of test chambers.

13.5.25 Volumetric pipets -- class A, 1-100 mL.

13.5.26 Serological pipets -- 1-10 mL, graduated.

13.5.27 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

13.5.28 Disposable polyethylene pipets, droppers, and glass tubing with fire-polished edges, $ 2mm ID -- for transferring organisms.

13.5.29 Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

13.5.30 Thermometer, glass or electronic, laboratory grade, -- for measuring water temperatures.

13.5.31 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

13.5.32 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA 1979b) -- to calibrate laboratory thermometers.

13.5.33 Meters, DO, pH, and specific conductivity -- for routine physical and chemical measurements.

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13.6 REAGENTS AND CONSUMABLE MATERIALS

13.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.6.2 Data sheets (one set per test) -- for recording the data.

13.6.3 Vials, marked -- for preserving specimens for verification (optional).

13.6.4 Tape, colored -- for labeling test vessels.

13.6.5 Markers, waterproof -- for marking containers.

13.6.6 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA, 1979b.

13.6.7 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for instrument calibration check (see USEPA Method 150.1, USEPA, 1979b).

13.6.8 Specific conductivity standards -- see USEPA Method 120.1, USEPA, 1979b.

13.6.9 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

13.6.10 Laboratory quality control samples and standards -- for calibration of the above methods.

13.6.11 Reference toxicant solutions -- see Section 4, Quality Assurance.

13.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

13.6.13 Effluent, surface water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

13.6.14 Trout chow, yeast, and CEROPHYL® food (or substitute food) -- for feeding the cultures and test organisms.

13.6.14.1 Digested trout chow, or substitute flake food (TETRAMIN®, BIORIL®, or equivalent), is prepared as follows:

1. Preparation of trout chow or substitute flake food requires one week. Use starter or No. 1 pellets prepared according to current U.S. Fish and Wildlife Service specifications.
2. Add 5.0 g of trout chow pellets or substitute flake food to 1 L of MILLI-Q® water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.
3. At the end of digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (i.e., NITEX® 110 mesh). Combine with equal volumes of supernatant from CEROPHYLL® and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the sediment.

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13.6.14.2 Yeast is prepared as follows:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S® Yeast, Lake State Kosher Certified Yeast, or equivalent, to 1 L of MILLI-Q® water.
2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.
3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and CEROPHYLL® preparations (below). Discard excess material.

13.6.14.3 CEROPHYLL® is prepared as follows:

1. Place 5.0 g of dried, powdered, cereal or alfalfa leaves, or rabbit pellets, in a blender. Cereal leaves, CEROPHYLL®, or equivalent are available from commercial sources. Dried, powdered, alfalfa leaves may be obtained from health food stores, and rabbit pellets are available at pet shops.
2. Add 1 L of MILLI-Q® water.
3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.
4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

13.6.14.4 Combined yeast-cerophyl-trout chow (YCT) is mixed as follows:

1. Thoroughly mix equal (approximately 300 mL) volumes of the three foods as described above.
2. Place aliquots of the mixture in small (50 mL to 100 mL) screw-cap plastic bottles and freeze until needed.
3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of two weeks. Do not store frozen over three months.
4. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1.7-1.9 g solids/L. Cultures or test solutions should contain 12-13 mg solids/L.

13.6.15 Algal food -- for feeding the cultures and test organisms.

13.6.15.1 Algal Culture Medium is prepared as follows:

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.
2. Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L, mix well. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.
3. Immediately filter the medium through a 0.45 µm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.
4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.
5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

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TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 mL MILLI-Q® WATER	
1. MACRONUTRIENTS			
A.	$MgCl_2 \cdot 6H_2O$	6.08	g
	$CaCl_2 \cdot 2H_2O$	2.20	g
	$NaNO_3$	12.75	g
B.	$MgSO_4 \cdot 7H_2O$	7.35	g
C.	K_2HPO_4	0.522	g
D.	$NaHCO_3$	7.50	g
2. MICRONUTRIENTS			
	H_3BO_3	92.8	mg
	$MnCl_2 \cdot 4H_2O$	208.0	mg
	$ZnCl_2$	1.64	mg[1]
	$FeCl_3 \cdot 6H_2O$	79.9	mg
	$CoCl_2 \cdot 6H_2O$	0.714	mg[2]
	$Na_2MoO_4 \cdot 2H_2O$	3.63	mg[3]
	$CuCl_2 \cdot 2H_2O$	0.006	mg[4]
	$Na_2EDTA \cdot 2H_2O$	150.0	mg
	Na_2SeO_4	1.196	mg[5]

[1] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[2] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[3] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[4] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock 2, micronutrients.

[5] Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

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TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRONUTRIENT	CONCENTRATION (mg/L)	ELEMENT	CONCENTRATION (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRONUTRIENT	CONCENTRATION (µg/L)	ELEMENT	CONCENTRATION (µg/L)
H_3BO_3	185.0	B	32.5
$MnCl_2 \cdot 4H_2O$	416.0	Mn	115.0
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160.0	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300.0	--	----
Na_2SeO_4	2.39	Se	0.91

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13.6.15.2 Algal Cultures

13.6.15.2.1 See Section 6, Test Organisms, for information on sources of "starter" cultures of *Selenastrum capricornutum, S. minutum*, and *Chlamydomonas reinhardti.*

13.6.15.2.2 Two types of algal cultures are maintained: "stock" cultures, and "food" cultures.

13.6.15.2.2.1 Establishing and Maintaining Stock Cultures of Algae:

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4EC.
2. The stock cultures are used as a source of algae to initiate "food" cultures for *Ceriodaphnia dubia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Ceriodaphnia dubia* cultures and tests. Stock culture volume may be rapidly "scaled up" to several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.
3. Culture temperature is not critical. Stock cultures may be maintained at 25EC in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
4. Cultures are mixed twice daily by hand.
5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5×10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.
6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4EC. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms (see Section 6, Test Organisms) every four to six months.

13.6.15.2.2.2 Establishing and Maintaining "Food" Cultures of Algae:

1. "Food" cultures are started seven days prior to use for *Ceriodaphnia dubia* cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5×10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and stored in the refrigerator.
2. Food cultures may be maintained at 25EC in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$ or 400 ft-c).
3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3EC.

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13.6.15.2.3 Preparing Algal Concentrate for Use as *Ceriodaphnia dubia* Food:

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for at least three weeks and siphoning off the supernatant.
2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer (see Section 14, Green Alga, *Selenastrum capricornutum* Growth Test), and used to determine the dilution (or further concentration) required to achieve a final cell count of 3.0 to 3.5 X 10^7/mL.
3. Assuming a cell density of approximately 1.5 X 10^6 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^9 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate (1500 feedings at 0.1 mL/feeding) for use as food. This would be enough algal food for four *Ceriodaphnia dubia* tests.
4. Algal concentrate may be stored in the refrigerator for one month.

13.6.15.3 Food Quality

13.6.15.3.1 USEPA recommends Fleishmann's® yeast, Cerophyll®, trout chow, and *Selenastrum capricornutum* as the preferred *Ceriodaphnia dubia* food combination. This recommendation is based on extensive data developed by many laboratories which indicated high *Ceriodaphnia dubia* survival and reproduction in culturing and testing. The use of substitute food(s) is acceptable only after side-by-side tests are conducted to determine that the quality of the substitute food(s) is equal to the USEPA recommended food combination based on survival and reproduction of *Ceriodaphnia dubia*.

13.6.15.3.2 The quality of food prepared with newly acquired supplies of yeast, trout chow, dried cereal leaves, algae, and/or any substitute food(s) should be determined in side-by-side comparisons of *Ceriodaphnia dubia* survival and reproduction, using the new food and food of known, acceptable quality, over a seven-day period in control medium.

13.6.16 TEST ORGANISMS, DAPHNIDS, *CERIODAPHNIA DUBIA*

13.6.16.1 Cultures of test organisms should be started at least three weeks before the brood animals are needed, to ensure an adequate supply of neonates for the test. Only a few individuals are needed to start a culture because of their prolific reproduction.

13.6.16.2 Neonates used for toxicity tests must be obtained from individually cultured organisms. Mass cultures may be maintained, however, to serve as a reserve source of organisms for use in initiating individual cultures and in case of loss of individual cultures.

13.6.16.3 Starter animals may be obtained from commercial sources and may be shipped in polyethylene bottles. Approximately 40 animals and 3 mL of food are placed in a l-L bottle filled full with culture water for shipment. Animals received from an outside source should be transferred to new culture media gradually over a period of 1-2 days to avoid mass mortality.

13.6.16.4 It is best to start the cultures with one animal, which is sacrificed after producing young, mounted on a microscope slide, and retained as a permanent slide mount to facilitate identification and permit future reference. The species identification of the stock culture should be verified by preparing slide mounts, regardless of the number of animals used to start the culture. The following procedure is recommended for making slide mounts of *Ceriodaphnia dubia* (modified from Beckett and Lewis, 1982):

1. Pipet the animal onto a watch glass.
2. Reduce the water volume by withdrawing excess water with the pipet.

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3. Add a few drops of carbonated water (club soda or seltzer water) or 70% ethanol to relax the specimen so that the post-abdomen is extended. (Optional: with practice, extension of the postabdomen may be accomplished by putting pressure on the cover slip).
4. Place a small amount (one to three drops) of mounting medium on a glass microscope slide. The recommended mounting medium is CMCP-9/10 Medium, prepared by mixing two parts of CMCP-9 with one part of CMCP-10 stained with enough acid fuchsin dye to color the mixture a light pink. For more viscosity and faster drying, CMC-10 stained with acid fuchsin may be used.
5. Using forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a 12 mm round cover slip and exert minimum pressure to remove any air bubbles trapped under the cover slip. Slightly more pressure will extend the postabdomen.
7. Allow mounting medium to dry.
8. Make slide permanent by placing varnish around the edges of the coverslip.
9. Identify to species (see Pennak, 1978; Pennak, 1989; and Berner, 1986).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

13.6.16.5 Mass Culture

13.6.16.5.1 Mass cultures are used only as a "backup" reservoir of organisms.

13.6.16.5.2 One-liter or 2-L glass beakers, crystallization dishes, "battery jars," or aquaria may be used as culture vessels. Vessels are commonly filled to three-fourths capacity. Cultures are fed daily. Four or more cultures are maintained in separate vessels and with overlapping ages to serve as back-up in case one culture is lost due to accident or other unanticipated problems, such as low DO concentrations or poor quality of food or laboratory water.

13.6.16.5.3 Mass cultures which will serve as a source of brood organisms for individual culture should be maintained in good condition by frequent renewal with new culture medium at least twice a week for two weeks. At each renewal, the adult survival is recorded, and the offspring and the old medium are discarded. After two weeks, the adults are also discarded, and the culture is re-started with neonates in fresh medium. Using this schedule, 1-L cultures will produce 500 to 1000 neonate *Ceriodaphnia dubia* each week.

13.6.16.6 Individual Culture

13.6.16.6.1 Individual cultures are used as the immediate source of neonates for toxicity tests.

13.6.16.6.2 Individual organisms are cultured in 15 mL of culture medium in 30-mL (1 oz) plastic cups or 30-mL glass beakers. One neonate is placed in each cup. It is convenient to place the cups in the same type of board used for toxicity tests (see Figure 1).

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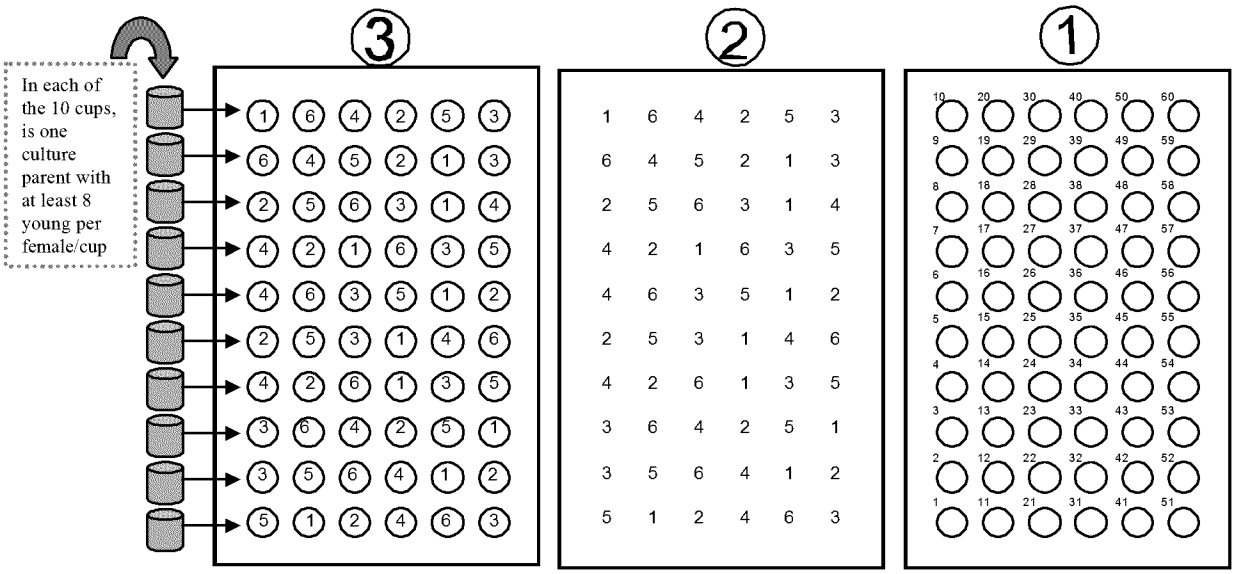

Figure 1. Examples of a test board and randomizing template: 1) test board with positions for six columns of ten replicate test chambers with each position numbered for recording results on data sheets, 2) cardboard randomizing template prepared by randomly drawing numbers (1-6) for each position in a row across the board, and 3) test board (1) placed on top of the randomizing template (2) for the purpose of assigning the position of test treatments (1-6) within each block (row on the test board). Following placement of test chambers, test organisms are allocated using blocking by known parentage. Test organisms from a single brood cup are distributed to each treatment within a given block (row on the test board).

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13.6.16.6.3 Organisms are fed daily (see Subsection 13.6.16.9) and are transferred to fresh medium a minimum of three times a week, typically on Monday, Wednesday, and Friday. On the transfer days, food is added to the new medium immediately before or after the organisms are transferred.

13.6.16.6.4 To provide cultures of overlapping ages, new boards are started weekly, using neonates from adults which produce at least eight young in their third or fourth brood. These adults can be used as sources of neonates until 14 days of age. A minimum of two boards are maintained concurrently to provide backup supplies of organisms in case of problems.

13.6.16.6.5 Cultures which are properly maintained should produce at least 20 young per adult in three broods (seven days or less). Typically, 60 adult females (one board) will produce more than the minimum number of neonates (120) required for two tests.

13.6.16.6.6 Records should be maintained on the survival of brood organisms and number of offspring at each renewal. Greater than 20% mortality of adults, or less than an average of 20 young per female would indicate problems, such as poor quality of culture media or food. Cultures that do not meet these criteria should not be used as a source of test organisms.

13.6.16.7 Culture Medium

13.6.16.7.1 Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW is recommended as a standard culture medium (see Section 7, Dilution Water).

13.6.16.8 Culture Conditions

13.6.16.8.1 The daphnid, *Ceriodaphnia dubia,* should be cultured at a temperature of 25 ± 1EC.

13.6.16.8.2 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A photoperiod of 16-h of light and 8-h of darkness is recommended. Light intensity should be 10-20 $\mu E/m^2/s$ or 50 to 100 ft-c.

13.6.16.8.3 Clear, double-strength safety glass or 6 mm plastic panels are placed on the culture vessels to exclude dust and dirt, and reduce evaporation.

13.6.16.8.4 The organisms are delicate and should be handled as carefully and as little as possible so that they are not unnecessarily stressed. They are transferred with a pipet of approximately 2-mm bore, taking care to release the animals under the surface of the water. Any organism that is injured during handling should be discarded.

13.6.16.9 Food and Feeding

13.6.16.9.1 Feeding the proper amount of the right food is extremely important in *Ceriodaphnia dubia* culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may reduce the toxicity of the test solutions, clog the animal's filtering apparatus, or greatly decrease the DO concentration and increase mortality. A combination of Yeast, CEROPHYLL®, and Trout chow (YCT), along with the unicellular green alga, *Selenastrum capricornutum*, will provide suitable nutrition if fed daily.

13.6.16.9.2 Other algal species (such as *S. minutum* or *Chlamydomonas reinhardti*), other substitute food combinations (such as Flake Fish Food), or different feeding rates may be acceptable as long as performance criteria are met and side-by-side comparison tests confirm acceptable quality (see Subsection 13.6.15.3).

13.6.16.9.3 Cultures should be fed daily to maintain the organisms in optimum condition so as to provide maximum reproduction. Stock cultures which are stressed because they are not adequately fed may produce low

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numbers of young, large numbers of males, and/or ephippial females. Also, their offspring may produce few young when used in toxicity tests.

13.6.16.9.4 Feed as follows:

1. If YCT is frozen, remove a bottle of food from the freezer 1h before feeding time, and allow to thaw.
2. YCT food mixture and algal concentrates should both be thoroughly mixed by shaking before dispensing.
3. Mass cultures are fed daily at the rate of 7 mL YCT and 7 mL algae concentrate/L culture.
4. Individual cultures are fed at the rate of 0.1 mL YCT and 0.1 mL algae concentrate per 15 mL culture.
5. Return unused YCT food mixture and algae concentrate to the refrigerator. Do not re-freeze YCT. Discard unused portion after two weeks.

13.6.16.10 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Daphnid, *Ceriodaphnia dubia,* neonates less than 24 h old, and all within 8 h of the same age are used to monitor the chronic toxicity of the reference toxicant to the *Ceriodaphnia dubia* produced by the culture unit (see Section 4, Quality Assurance).

13.6.16.11 Record Keeping

13.6.16.11.1 Records, kept in a bound notebook, include (1) source of organisms used to start the cultures, (2) type of food and feeding times, (3) dates culture were thinned and restarted, (4) rate of reproduction in individual cultures, (5) daily observations of the condition and behavior of the organisms in the cultures, and (6) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

13.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

13.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

13.8 CALIBRATION AND STANDARDIZATION

13.8.1 See Section 4, Quality Assurance.

13.9 QUALITY CONTROL

13.9.1 See Section 4, Quality Assurance.

13.10 TEST PROCEDURES

13.10.1 TEST SOLUTIONS

13.10.1.1 Receiving Waters

13.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared without dilution, against a control. For a test consisting of single receiving water and control, approximately 600 mL of sample would be required for each test, assuming 10 replicates of 15 mL, and sufficient additional sample for chemical analysis.

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13.10.1.2 Effluents

13.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5, and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the $ 0.5 dilution factor.**

13.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of effluent concentrations.

13.10.1.2.3 The volume of effluent required for daily renewal of 10 replicates per concentration, each containing 15 mL of test solution, with a dilution series of 0.5, is approximately 1 L/day. A volume of 15 mL of test solution is adequate for the organisms, and will provide a depth in which it is possible to count the animals under a stereomicroscope with a minimum of re-focusing. Ten test chambers are used for each effluent dilution and for the control. Sufficient test solution (approximately 550 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses at the high, medium, and low test concentrations.

13.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.10.1.2.5 Just prior to test initiation (approximately one h) the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature and maintained at that temperature during the preparation of the test solutions.

13.10.1.2.6 The DO of the test solutions should be checked prior to test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If any solution has a DO concentration below 4.0 mg/L, all the solutions and the control must be gently aerated.

13.10.1.3 Dilution Water

13.10.1.3.1 Dilution water may be uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other uncontaminated natural water (see Section 7, Dilution Water).

13.10.2 START OF THE TEST

13.10.2.1 Label the test chambers with a marking pen. Use of color-coded tape to identify each treatment and replicate is helpful. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) must have ten replicates.

13.10.2.2 The test chambers must be randomly assigned to a board using a template (Figure 1) or by using random numbers (see Appendix A). Randomizing the position of test chambers as described in Figure 1 (or equivalent) will assist in assigning test organisms using blocking by known parentage (Subsection 13.10.2.4). A number of different templates should be prepared, and the template used for each test should be identified on the data sheet. The same template must not be used for every test.

13.10.2.3 Neonates less than 24 h old, and all within 8 h of the same age, are required to begin the test. The neonates must be obtained from individual cultures using brood boards, as described above in Subsection 13.6.16.6,

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Individual Culture (also see Section 6, Test Organisms). Neonates must be taken only from adults in individual cultures that have eight or more young in their third or subsequent broods. These adults can be used as brood stock until they are 14 days old. If the neonates are held more than one or two hours before using in the test, they should be fed (0.1 mL YCT and 0.1 mL algal concentrate/15 mL of media). Record the age range of test organisms, source, and feeding of neonates on test data sheets.

13.10.2.4 Ten brood cups, each with 8 or more young, are randomly selected from a brood board for use in setting up a test. To start the test, neonates from these ten brood cups are distributed to each test chamber in the test board (one per test chamber). Test organisms must be assigned to test chambers using a block randomization procedure, such that offspring from a single female are distributed evenly among the treatments, appearing once in every test concentration. This arrangement is referred to as "blocking by known parentage". The technique used to achieve blocking by known parentage should be recorded in the test data report. One effective technique is to block randomize the test board as described in Figure 1 and transfer one neonate from the first brood cup to each of the six test chambers in the first row on the test board. One neonate from the second brood cup is then transferred to each of the six test chambers in the second row on the test board. This process is continued until each of the 60 test chambers contains one neonate. The set of six test chambers (one for each test treatment) containing organisms derived from a single female parent is referred to as a block. When using the technique described in Figure 1, each row of the test board will represent a block.

13.10.2.4.1 The brood cups and test chambers may be placed on a light table to facilitate counting the neonates. However, care must be taken to avoid temperature increase due to heat from the light table.

13.10.2.4.2 Following the allocation of test organisms to the test board, additional neonates might remain in the ten brood cups that were selected for test setup. These additional neonates may be discarded, used as future culture organisms if needed, or used to start additional tests (provided that at least 6 neonates remain and these neonates continue to meet test organism age requirements).

13.10.2.5 Blocking by known parentage allows the performance of each test organism to be tracked to its parent culture organism. This technique ensures that any brood effects (i.e., differences in test organism fecundity or sensitivity attributable to the source of parentage) are evenly distributed among the test treatments. Also, by knowing the parentage of each test organism, blocks consisting largely of males can be omitted from all test treatments at the end of the test (see Subsection 13.13.1.4), decreasing variability among replicates.

13.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

13.10.3.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 $\mu E/m^2/s$, or 50 to 100 ft-c, with a photoperiod of 16 h of light and 8 h of darkness.

13.10.3.2 It is critical that the test water temperature be maintained at 25 ± 1EC to obtain three broods in seven days.

13.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

13.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should be measured in the new solutions at the start of the test (Day 0) and before daily renewal of the test solutions on subsequent days. The DO concentration should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). Aeration is generally not practical during the daphnid, *Ceriodaphnia dubia*, test. If the DO in the effluent and/or dilution water is low, aerate gently before preparing the test solutions. The aeration rate should not exceed 100 bubbles/min using a pipet with an orifice of approximately 1.5 mm, such as a 1 ml KIMAX® serological pipet, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue physical stress to the organisms.

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13.10.5 FEEDING

13.10.5.1 The organisms are fed when the test is initiated, and daily thereafter. Food is added to the fresh medium immediately before or immediately after the adults are transferred. Each feeding consists of 0.1 mL YCT and 0.1 mL *Selenastrum capricornutum* concentrate/15 mL test solution (0.1 mL of algal concentrate containing 3.0-3.5 X 10^7 cells/mL will provide 2-2.3 X 10^5 cells/mL in the test chamber).

13.10.5.2 The YCT and algal suspension can be added accurately to the test chambers by using automatic pipettors, such as Gilson, Eppendorf, Oxford, or equivalent.

13.10.6 OBSERVATIONS DURING THE TEST

13.10.6.1 Routine Chemical and Physical Determinations

13.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period in at least one test chamber at each test concentration and in the control.

13.10.6.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test chamber at each test concentration and in the control. Temperature should be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in sufficient number of test vessels at least at the end of the test to determine the temperature variation in the environmental chamber.

13.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

13.10.6.1.4 Conductivity, alkalinity and hardness are measured in each new sample (100% effluent or receiving water) and in the control.

13.10.6.1.5 Record the data on data sheet (Figure 2).

13.10.6.2 Routine Biological Observations

13.10.6.2.1 Three or four broods are usually obtained in the controls in a 7-day test conducted at 25 ± 1EC. A brood is a group of offspring released from the female over a short period of time when the carapace is discarded during molting. In the controls, the first brood of two-to-five young is usually released on the third or fourth day of the test. Successive broods are released every 30 to 36 h thereafter. The second and third broods usually consist of eight to 20 young each. The total number of young produced by a healthy control organism in three broods often exceeds 30 per female. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test.

13.10.6.2.2 The release of a brood may be inadvertently interrupted during the daily transfer of organisms to fresh test solutions, resulting in a split in the brood count between two successive days. For example, four neonates of a brood of five might be released on Day 3, just prior to test solution renewal, and the fifth released just after renewal, and counted on Day 4. Partial broods, released over a two-day period, should be counted as one brood.

13.10.6.2.3 Each day, the live adults are transferred to fresh test solutions, and the numbers of live young are recorded (see data form, Figure 3). The young can be count ed with the aid of a stereomicroscope with substage lighting. Place the test chambers on a light box over a strip of black tape to aid in counting the neonates. The young are discarded after counting.

13.10.6.2.4 Some of the effects caused by toxic substances include, (1) a reduction in the number of young produced, (2) young may develop in the brood pouch of the adults, but may not be released during the exposure

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period, and (3) partially or fully developed young may be released, but are all dead at the end of the 24-h period. Such effects should be noted on the data sheets (Figure 3).

13.10.6.2.5 Protect the daphnids, *Ceriodaphnia dubia*, from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and transfer of females carefully. Make sure the females remain immersed during the performance of these operations.

13.10.7 DAILY PREPARATION OF TEST CHAMBERS

13.10.7.1 The test is started (Day 0) with new disposable polystyrene cups or precleaned 30-mL borosilicate glass beakers that are labeled and color-coded with tape. Each following day, a new set of plastic cups or precleaned glass beakers is prepared, labeled, and color-coded with tape similar to the original set. New solutions are placed in the new set of test chambers, and the test organisms are transferred from the original test chambers to the new ones with corresponding labels and color-codes. Each day, previously used glass beakers are recleaned (see Section 5, Facilities, Equipment, and Supplies) for the following day, and previously used plastic cups are discarded.

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Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 2. Data form for the daphnid, *Ceriodaphnia dubia,* survival and reproduction test. Routine chemical and physical determinations.

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Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 2. Data form for the daphnid, *Ceriodaphnia dubia,* survival and reproduction test. Routine chemical and physical determinations (CONTINUED).

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13.10.8 TEST SOLUTION RENEWAL

13.10.8.1 Freshly prepared solutions are used to renew the test daily. For on-site toxicity studies, fresh effluent or receiving water samples should be collected daily, and no more than 24 h should elapse between collection of the samples and their use in the tests (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples are collected, preferably on days one, three, and five. No more than 36 h should elapse between collection of the sample and the first use in the test. Maintain the samples in the refrigerator at 0-6EC until used.

13.10.8.2 New test solutions are prepared daily, and the test organisms are transferred to the freshly prepared solutions using a small-bore (2 mm) glass or polyethylene dropper or pipet. The animals are released under the surface of the water so that air is not trapped under the carapace. Organisms that are dropped or injured are discarded.

13.10.9 TERMINATION OF THE TEST

13.10.9.1 Tests should be terminated when 60% or more of the surviving control females have produced their third brood, or at the end of 8 days, whichever occurs first. Because of the rapid rate of development of *Ceriodaphnia dubia*, at test termination all observations on organism survival and numbers of offspring should be completed within two hours. An extension of more than a few hours in the test period would be a significant part of the brood production cycle of the animals, and could result in additional broods. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test.

13.10.9.2 Count the young, conduct required chemical measurements, and complete the data sheets (Figure 3).

13.10.9.3 Any animal not producing young should be examined to determine if it is a male (Berner, 1986). In most cases, the animal will need to be placed on a microscope slide before examining (see Subsection 13.6.16.4).

13.10.9.3.1 In general, the occurrence of males in healthy, well-maintained individual cultures is rare. In interlaboratory testing of the *Ceriodaphnia dubia* Survival and Reproduction Test, males were identified in only 7% (9 of 126 tests) of tests conducted (USEPA, 2001a). The number of males identified in these tests ranged from 1 to 12. In five tests containing a large number of males (4-12), laboratories conducting those tests also noted that organism cultures were experiencing or recovering from some stress. Since male production in cladoceran populations is generally associated with conditions of environmental stress (Pennak, 1989), culture conditions should be examined whenever males are identified in a test.

13.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

13.11.1 A summary of test conditions and test acceptability criteria is presented in Table 3.

13.12 ACCEPTABILITY OF TEST RESULTS

13.12.1 For the test results to be acceptable, at least 80% of all control organisms must survive, and 60% of surviving control females must produce at least three broods, with an average of 15 or more young per surviving female.

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Discharger:_____ Analyst:_____
Location: _____ Test Start-Date/Time: _____
Date Sample Collected: _____ Test Start-Date/time: _____

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

Figure 3. Data form for the daphnid, *Ceriodaphnia dubia*, survival and reproduction test. Daily summary of data.

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Discharger:_____ Analyst:_____
Location: _____ Test Start-Date/Time: _____
Date Sample Collected: _____ Test Start-Date/time: _____

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

Figure 3. Data form for the daphnid, *Ceriodaphnia dubia*, survival and reproduction test. Daily summary of data (CONTINUED).

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TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1002.0)[1]

1.	Test type:	Static renewal (required)
2.	Temperature (EC):	25 ± 1EC (recommended) Test temperatures should not deviate (i.e., maximum minus minimum temperature) by more than 3EC during the test (required)
3.	Light quality:	Ambient laboratory illumination (recommended)
4.	Light intensity:	10-20 $\mu E/m^2/s$, or 50-100 ft-c (ambient laboratory levels) (recommended)
5.	Photoperiod:	16 h light, 8 h dark (recommended)
6.	Test chamber size:	30 mL (recommended minimum)
7.	Test solution volume:	15 mL (recommended minimum)
8.	Renewal of test solutions:	Daily (required)
9.	Age of test organisms:	Less than 24 h; and all released within a 8-h period (required)
10.	No. neonates per test chamber:	1 Assigned using blocking by known parentage (Subsection 13.10.2.4) (required)
11.	No. replicate test chambers per concentration:	10 (required minimum)
12.	No. neonates per test concentration:	10 (required minimum)
13.	Feeding regime:	Feed 0.1 mL each of YCT and algal suspension per test chamber daily (recommended)
14.	Cleaning:	Use freshly cleaned glass beakers or new plastic cups daily (recommended)
15.	Aeration:	None (recommended)
16.	Dilution water:	Uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or DMW (see Section 7, Dilution Water) (available options)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

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TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1002.0) (CONTINUED)

17.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended)
18.	Dilution factor:	Effluents: $ 0.5 (recommended) Receiving Waters: None or $ 0.5 (recommended)
19.	Test duration:	Until 60% or more of surviving control females have three broods (maximum test duration 8 days) (required)
20.	Endpoints:	Survival and reproduction (required)
21.	Test acceptability criteria:	80% or greater survival of all control organisms and an average of 15 or more young per surviving female in the control solutions. 60% of surviving control females must produce three broods (required)
22.	Sampling requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
23.	Sample volume required:	1 L/day (recommended)

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13.13 **DATA ANALYSIS**

13.13.1 GENERAL

13.13.1.1 Tabulate and summarize the data. A sample set of survival and reproduction data is listed in Table 4.

TABLE 4. SUMMARY OF SURVIVAL AND REPRODUCTION DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, EXPOSED TO AN EFFLUENT FOR SEVEN DAYS

Effluent Concentration (%)	No. of Young per Adult Replicate										No. Live Adults
	1	2	3	4	5	6	7	8	9	10	
Control	27	30	29	31	16	15	18	17	14	27	10
1.56	32	35	32	26	18	29	27	16	35	13	10
3.12	39	30	33	33	36	33	33	27	38	44	10
6.25	27	34	36	34	31	27	33	31	33	31	10
12.5	10	13	7	7	7	10	10	16	12	2	10
25.0	0	0	0	0	0	0	0	0	0	0	2

13.13.1.2 The endpoints of toxicity tests using the daphnid, *Ceriodaphnia dubia*, are based on the adverse effects on survival and reproduction. The LC50, the IC25, the IC50 and the EC50 are calculated using point estimation techniques, and LOEC and NOEC values for survival and reproduction are obtained using a hypothesis test approach such as Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962), Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50, IC25, IC50 and EC50. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for reproduction, but included in the estimation of the LC50, IC25, IC50, and EC50. See the Appendices for examples of the manual computations, program listings, and examples of data input and program output.

13.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

13.13.1.4 At the end of the test, if 50% or more of the surviving organisms in a block are identified as males, the entire block must be excluded from data analysis for the reproduction endpoint (i.e., calculation of the reproduction NOEC and IC25 as described in Subsection 13.13.3), but may be used in the analysis of the survival endpoint (i.e., calculation of the survival NOEC and LC50 as described in Subsection 13.13.2). For blocks having fewer than 50% of surviving organisms identified as males, the males (not the entire block) must be excluded from the analysis of reproduction (i.e., calculation of the reproduction NOEC and IC25 as described in Subsection 13.13.3), but may be used in the analysis of survival (i.e., calculation of the survival NOEC and LC50 as described in Subsection 13.13.2). Note that the exclusion of males from the analysis of reproduction may create unequal sample sizes among the concentrations, influencing the statistical methods chosen for analysis of reproduction (Figure 6). Determinations regarding test acceptability criteria for survival and reproduction (Subsection 13.12) must be made prior to exclusion of any blocks. In addition to these test acceptability criteria, if fewer than eight replicates in the

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control remain after excluding males and blocks with 50% or more of surviving organisms identified as males, the test is invalid and must be repeated with a newly collected sample.

13.13.2 EXAMPLE OF ANALYSIS OF THE DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL DATA

13.13.2.1 Formal statistical analysis of the survival data is outlined on the flowchart in Figure 4. The response used in the analysis is the number of animals surviving at each test concentration. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the EC50, LC50, IC25, or IC50 endpoints. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the LC, EC, and IC endpoints.

13.13.2.2 Fisher's Exact Test is used to determine the NOEC and LOEC endpoints. It provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a Bernoulli (binomial) population. Additional information on Fisher's Exact Test is provided in Appendix G.

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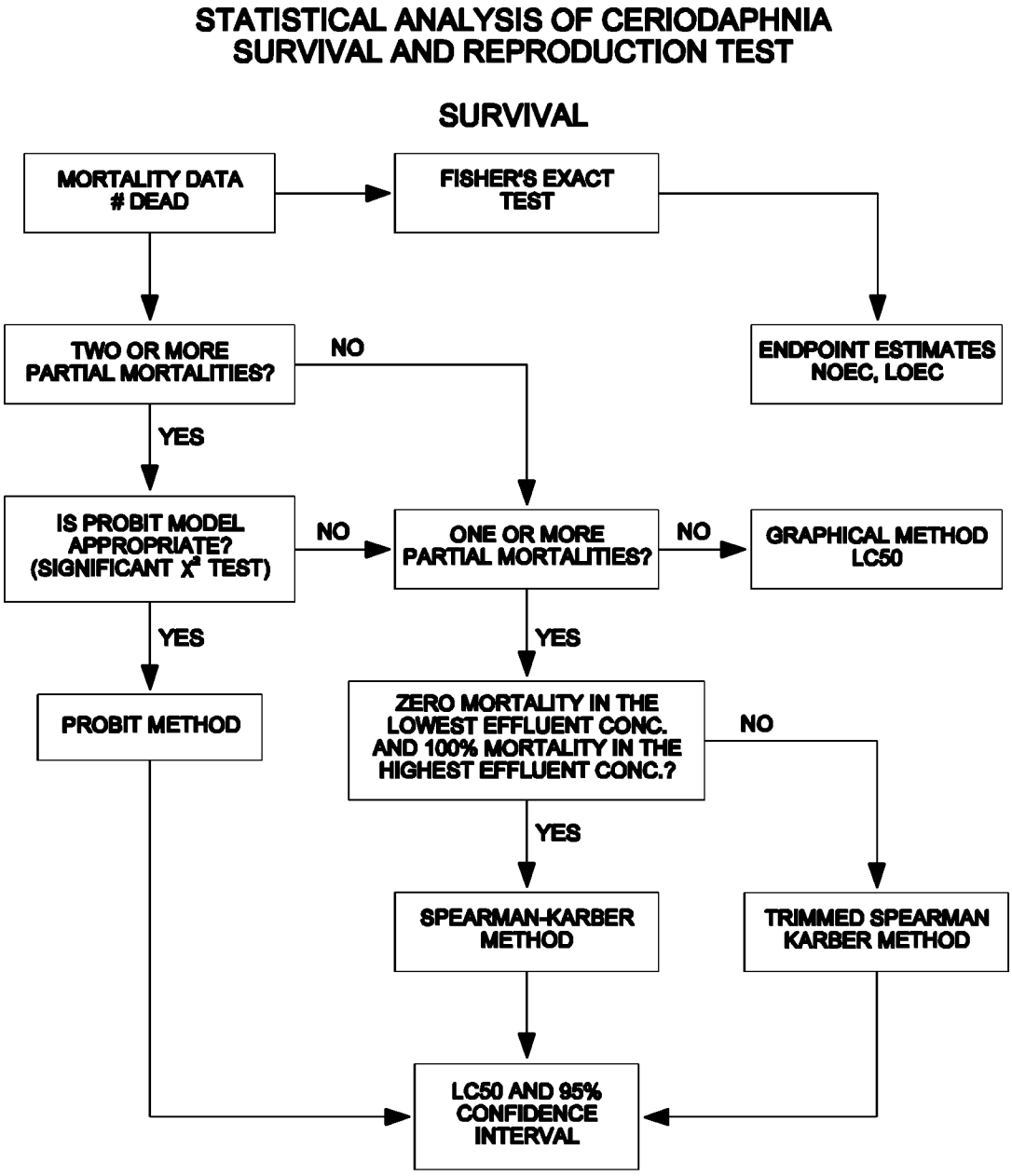

Figure 4. Flowchart for statistical analysis of the daphnid, *Ceriodaphnia dubia*, survival data.

13.13.2.3 Probit Analysis (Finney, 1971; Appendix I) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total number dead at a given concentration is the response.

13.13.2.4 Example of Analysis of Survival Data

13.13.2.4.1 The data in Table 4 will be used to illustrate the analysis of survival data from the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test. As can be seen from the data in Table 4, there were no deaths in the 1.56%, 3.12%, 6.25%, and 12.5% concentra tions. These concentrations are obviously not different from the control in terms of survival. This leaves only the 25% effluent concentration to be tested statistically for a difference in survival from the control.

13.13.2.5 Fisher's Exact Test

13.13.2.5.1 The basis for Fisher's Exact Test is a 2x2 contingency table. From the 2x2 table prepared by comparing the control and the effluent concentration, determine statistical significance by looking up a value in the table provided in Appendix G (Table G.5). However, to use this table the contingency table must be arranged in the format illustrated in Table 5.

TABLE 5. FORMAT OF THE 2x2 CONTINGENCY TABLE

	Number of		Number of
	Successes	Failures	Observations
Condition 1	a	A - a	A
Condition 2	b	B - b	B
Total	a + b	[(A+B) - a - b]	A + B

13.13.2.5.2 Arrange the table so that the total number of observations for row one is greater than or equal to the total for row two (A $ B). Categorize a success such that the proportion of successes for row one is greater than or equal to the proportion of successes for row two (a/A $ b/B). For these data, a success may be 'alive' or 'dead' whichever causes a/A $ b/B. The test is then conducted by looking up a value in the table of significance levels of b and comparing it to the b value given in the contingency table. The table of significance levels of b is included in Appendix G, Table G.5. Enter Table G.5 in the section for A, subsection for B, and the line for a. If the b value of the contingency table is equal to or less than the integer in the column headed 0.05 in Table G.5, then the survival proportion for the effluent concentration is significantly different from that of the control. A dash or absence of entry in Table G.5 indicates that no contingency table in that class is significant.

13.13.2.5.3 To compare the control and the effluent concentration of 25%, the appropriate contingency table for the test is given in Table 6.

13.13.2.5.4 Since 10/10 $ 3/10, the category 'alive' is regarded as a success. For A = 10, B = 10 and, a = 10, under the column headed 0.05, the value from Table G.5 is b = 6. Since the value of b (b = 3) from the contingency table (Table 6), is less than the value of b (b = 6) from Table G.5 in Appendix G, the test concludes that the proportion

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surviving in the 25% effluent concentration is significantly different from the control. Thus the NOEC for survival is 12.5% and the LOEC is 25%.

TABLE 6. 2x2 CONTIGENCY TABLE FOR CONTROL AND 25% EFFLUENT

	Number of		
	Alive	Dead	Number of Observations
Condition 1	10	0	10
Condition 2	3	7	10
Total	13	7	20

13.13.2.6 Calculation of the LC50

13.13.2.6.1 The data used for the Trimmed Spearman-Karber Method are summarized in Table 7. To perform the Trimmed Spearman-Karber Method, run the USEPA Trimmed Spearman-Karber Program. An example of the program input and output is supplied in Appendix J.

TABLE 7. DATA FOR TRIMMED SPEARMAN-KARBER ANALYSIS

	Effluent Concentration (%)					
	Control	1.56	3.12	6.25	12.5	25.0
Number Dead	0	0	0	0	0	8
Number Exposed	10	10	10	10	10	10

13.13.2.6.2 For this example, with only one partial mortality, Trimmed Spearman-Karber analysis appears appropriate for this data.

13.13.2.6.3 Figure 5 shows the output for the Trimmed Spearman-Karber Analysis of the data in Table 7 using the USEPA Program.

13.13.3 EXAMPLE OF ANALYSIS OF THE DAPHNID, *CERIODAPHNIA DUBIA,* REPRODUCTION DATA

13.13.3.1 Formal statistical analysis of the reproduction data is outlined on the flowchart in Figure 6. The response used in the statistical analysis is the number of young produced per adult female, which is determined by taking the total number of young produced until either the time of death of the adult or the end of the experiment, whichever comes first. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test. An animal that dies before

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producing young, if it has not been identified as a male, would be included in the analysis with zero entered as the number of young produced. The subsequent calculation of the mean number of live young produced per adult female for each toxicant concentration provides a combined measure of the toxicant's effect on both mortality and reproduction. An IC estimate can be calculated for the reproduction data using a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC for reproduction. Concentrations above the NOEC for survival are excluded from the hypothesis test for reproduction effects.

13.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested using the Shapiro Wilk's Test for normality, and Bartlett's Test for homogeneity of variance. If either of these tests fails, a nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

13.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

13.13.3.4 The data, mean, and variance of the observations at each concentration including the control are listed in Table 8. A plot of the number of young per adult female for each concentration is provided in Figure 7. Since there is significant mortality in the 25% effluent concentration, its effect on reproduction is not considered.

TABLE 8. THE DAPHNID, *CERIODAPHNIA DUBIA,* REPRODUCTION DATA

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	27	32	39	27	10
2	30	35	30	34	13
3	29	32	33	36	7
4	31	26	33	34	7
5	16	18	36	31	7
6	15	29	33	27	10
7	18	27	33	33	10
8	17	16	27	31	16
9	14	35	38	33	12
10	27	13	44	31	2
Mean $\bar{Y}_I$	22.4	26.3	34.6	31.7	9.4
S_i^2	48.0	64.0	23.4	8.7	15.1
i	1	2	3	4	5

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TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1 TEST NUMBER: 2 DURATION: 7 Days
TOXICANT: effluent
SPECIES: *Ceriodaphnia dubia*

RAW DATA:

Concentration (%)	Number Exposed	Mortalities
.00	10	0
1.25	10	0
3.12	10	0
6.25	10	0
12.5	10	0
25.0	10	8

SPEARMAN-KARBER TRIM: 20.00 %

SPEARMAN-KARBER ESTIMATES: LC50: 19.28
95% CONFIDENCE LIMITS
ARE NOT RELIABLE.

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure 5. Output for USEPA Trimmed Spearman-Karber program.

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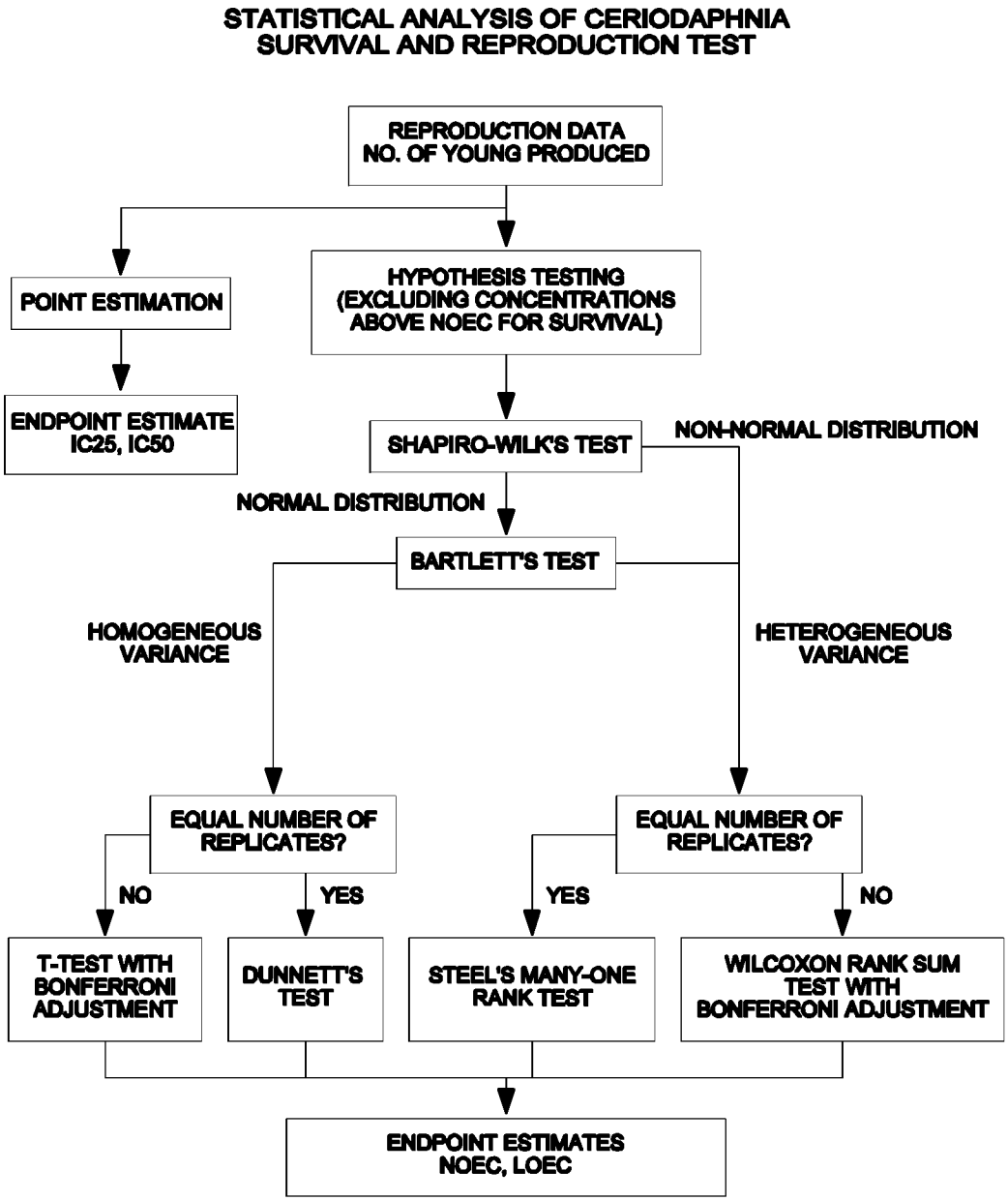

Figure 6. Flowchart for the statistical analysis of the daphnid, *Ceriodaphnia dubia*, reproduction data.

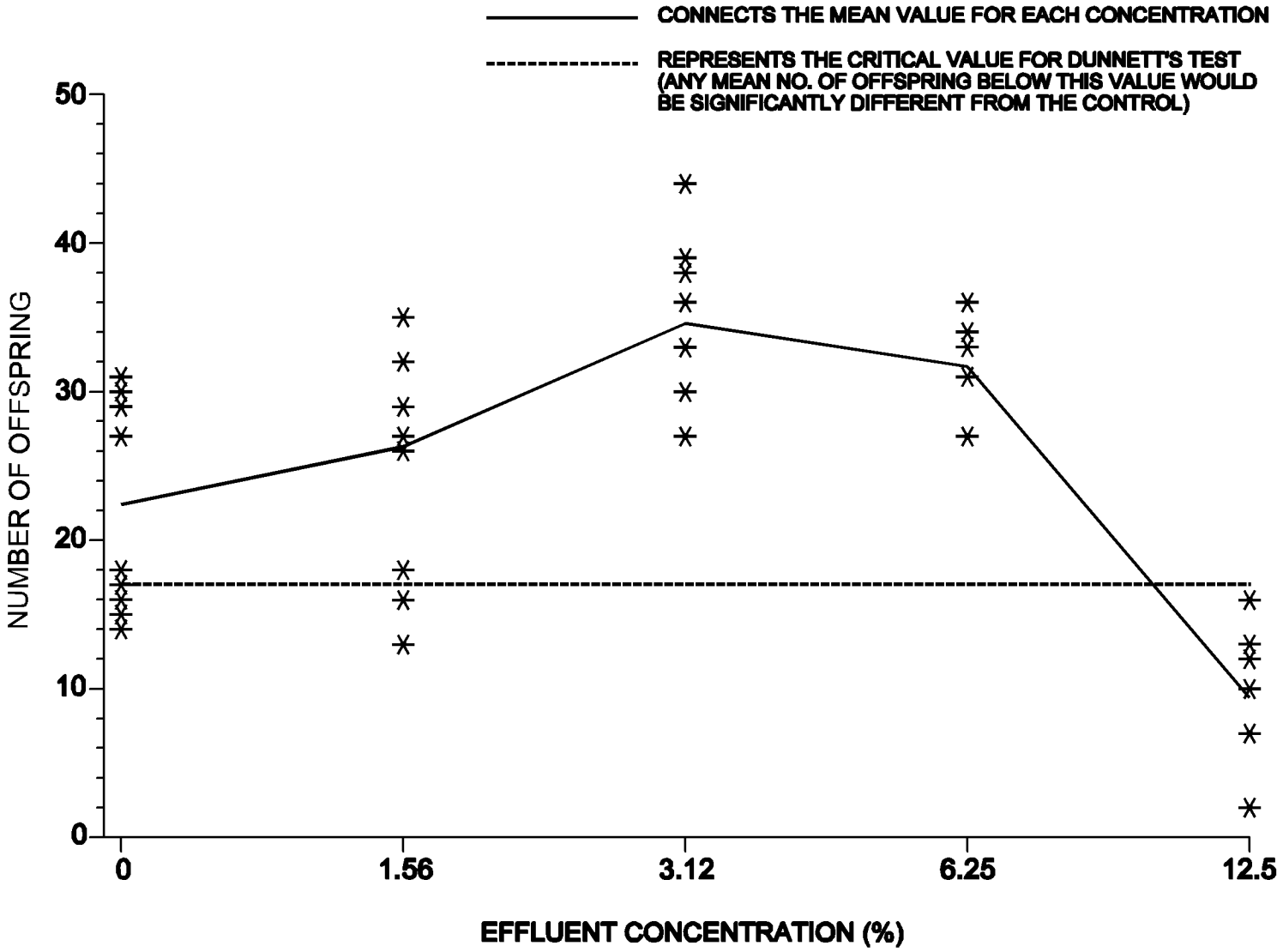

Figure 7. Plot of number of young per adult female from a daphnid, *Ceriodaphnia dubia*, survival and reproduction test.

13.13.3.5 Test for Normality

13.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 9.

TABLE 9. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	4.6	5.7	4.4	-4.7	0.6
2	7.6	8.7	-4.6	2.3	3.6
3	6.6	5.7	-1.6	4.3	-2.4
4	8.6	-0.3	-1.6	2.3	-2.4
5	-6.4	-8.3	1.4	-0.7	-2.4
6	-7.4	2.7	-1.6	-4.7	0.6
7	-4.4	0.7	-1.6	1.3	0.6
8	-5.4	-10.3	-7.6	-0.7	6.6
9	-8.4	8.7	3.4	1.3	2.6
10	4.6	-13.3	9.4	-0.7	-7.4

13.13.3.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the <u>overall</u> mean of the centered observations

n = the total number of centered observations.

For this set of data,

$$n = 50$$

$$\bar{X} = \frac{1}{50}(0.0) = 0.0$$

$$D = 1433.4$$

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13.13.3.5.3 Order the centered observations from smallest to largest

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 10.

13.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 50, k = 25. The a_i values are listed in Table 11.

13.13.3.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 11.

For this set of data:

$$W = \frac{1}{1433.4}(37.3)^2 = 0.97$$

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TABLE 10. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-13.3	26	0.6
2	-10.3	27	0.6
3	-8.4	28	0.7
4	-8.3	29	1.3
5	-7.6	30	1.3
6	-7.4	31	1.4
7	-7.4	32	2.3
8	-6.4	33	2.3
9	-5.4	34	2.6
10	-4.7	35	2.7
11	-4.7	36	3.4
12	-4.6	37	3.6
13	-4.4	38	4.3
14	-2.4	39	4.4
15	-2.4	40	4.6
16	-2.4	41	4.6
17	-1.6	42	5.7
18	-1.6	43	5.7
19	-1.6	44	6.6
20	-1.6	45	6.6
21	-0.7	46	7.6
22	-0.7	47	8.6
23	-0.7	48	8.7
24	-0.3	49	8.7
25	0.6	50	9.4

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TABLE 11. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3751	22.7	$X^{(50)} - X^{(1)}$
2	0.2574	19.0	$X^{(49)} - X^{(2)}$
3	0.2260	17.1	$X^{(48)} - X^{(3)}$
4	0.2032	16.9	$X^{(47)} - X^{(4)}$
5	0.1847	15.2	$X^{(46)} - X^{(5)}$
6	0.1691	14.0	$X^{(45)} - X^{(6)}$
7	0.1554	14.0	$X^{(44)} - X^{(7)}$
8	0.1430	12.1	$X^{(43)} - X^{(8)}$
9	0.1317	11.1	$X^{(42)} - X^{(9)}$
10	0.1212	9.3	$X^{(41)} - X^{(10)}$
11	0.1113	9.3	$X^{(40)} - X^{(11)}$
12	0.1020	9.0	$X^{(39)} - X^{(12)}$
13	0.0932	8.7	$X^{(38)} - X^{(13)}$
14	0.0846	6.0	$X^{(37)} - X^{(14)}$
15	0.0764	5.8	$X^{(36)} - X^{(15)}$
16	0.0685	5.1	$X^{(35)} - X^{(16)}$
17	0.0608	4.2	$X^{(34)} - X^{(17)}$
18	0.0532	3.9	$X^{(33)} - X^{(18)}$
19	0.0459	3.9	$X^{(32)} - X^{(19)}$
20	0.0386	3.0	$X^{(31)} - X^{(20)}$
21	0.0314	2.0	$X^{(30)} - X^{(21)}$
22	0.0244	2.0	$X^{(29)} - X^{(22)}$
23	0.0174	1.4	$X^{(28)} - X^{(23)}$
24	0.0104	0.9	$X^{(27)} - X^{(24)}$
25	0.0035	0.0	$X^{(26)} - X^{(25)}$

13.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 50 observations (n) is 0.930. Since W = 0.97 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

13.13.3.6 Test for Homogeneity of Variance

13.13.3.6.1 The test used to examine whether the variation in number of young produced is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i)\ln\bar{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, $V_i = (n_i - 1)$

p = number of levels of effluent concentration and control

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n_i = the number of replicates for concentration i

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

$$\bar{S}^2 = \frac{\left(\sum_{i=1}^{p} V_i S_i^2\right)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + (3(p-1))^{-1}\left[\sum_{i=1}^{p} \frac{1}{V_i} - \left(\sum_{i=1}^{p} V_i\right)^{-1}\right]$$

13.13.3.6.2 For the data in this example (see Table 8), all effluent concentrations including the control have the same number of replicates (n_i = 10 for all i). Thus, V_i = 9 for all i.

13.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(45)\ln(31.8) - 9\sum_{i=1}^{p} \ln(S_i^2)]/1.04$$

$$= [45(3.46) - 9(16.061)]/1.04$$

$$= 11.15/1.04$$

$$= 10.72$$

13.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with four degrees of freedom, is 13.3. Since B = 10.7 is less than the critical value of 13.3, conclude that the variances are not different.

13.13.3.7 Dunnett's Procedure

13.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 12.

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TABLE 12. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	= SSB/(p-1)
Within	N - p	SSW	= SSW/(N-p)
Total	N - 1	SST	

Where: p = number effluent concentrations including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the number of young produced by female j in effluent concentration i)

13.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 10$

N = 50

$T_1 = Y_{11} + Y_{12} + \ldots + Y_{110} = 224$
$T_2 = Y_{21} + Y_{22} + \ldots + Y_{210} = 263$

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$T_3 = Y_{31} + Y_{32} + \ldots + Y_{310} = 346$
$T_4 = Y_{41} + Y_{42} + \ldots + Y_{410} = 317$
$T_5 = Y_{51} + Y_{52} + \ldots + Y_{510} = 94$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 1244$

$$SSB = \sum_{i=1}^{p} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$

$$t_2 = \frac{(22.4-26.3)}{[5.64\sqrt{(\frac{1}{10})+(\frac{1}{10})}]} = -1.55$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$

$$= 36{,}272 - \frac{(1244)^2}{50} = 5321.28$$

$SSW = SST - SSB = 5321.28 - 3887.88 = 1433.40$

$S_B^2 = SSB/(p-1) = 3887.88/(5-1) = 971.97$

$S_W^2 = SSW/(N-p) = 1433.40/(50-5) = 31.85$

13.13.3.7.3 Summarize these calculations in an ANOVA table (Table 13).

TABLE 13. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	3887.88	971.97
Within	45	1433.40	31.85
Total	49	5321.28	

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13.13.3.7.4 To perform the individual comparisons, calculate the t statistic for each concentration and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w \sqrt{\left(\frac{1}{n_1}\right) + \left(\frac{1}{n_i}\right)}}$$

Where: $\bar{Y}_i$ = mean number of young produced for effluent concentration i

$\bar{Y}_1$ = mean number of young produced for the control

S_W = square root of within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

Since we are looking for a decrease in reproduction from the control, the mean for concentration i is subtracted from the control mean in the t statistic above. However, if we were looking for an increased response over the control, the control mean would be subtracted from the mean at a concentration.

13.13.3.7.5 Table 14 includes the calculated t values for each concentration and control combination. In this example, comparing the 1.56% concentration with the control the calculation is as follows:

$$t_2 = \frac{(22.4 - 26.3)}{[5.64 \sqrt{\left(\frac{1}{10}\right) + \left(\frac{1}{10}\right)}]} = -1.55$$

TABLE 14. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
1.56	2	-1.55
3.12	3	-4.84
6.25	4	-3.69
12.5	5	5.16

13.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean reproduction, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. Since an entry for 45 degrees of freedom for error is not provided in the table, the entry for 40 degrees of freedom for error, an alpha level of 0.05 and four concentrations (excluding the control) will be used, 2.23. The mean reproduction for concentration "i" is considered significantly less than the mean reproduction for the control if t_i is greater than the critical value.

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Since t_5 is greater than 2.23, the 12.5% concentration has significantly lower reproduction than the control. Hence the NOEC and the LOEC for reproduction are 6.25% and 12.5%, respectively.

13.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d S_w \sqrt{\left(\frac{1}{n_1}\right) + \left(\frac{1}{n}\right)}$$

Where: d = the critical value for the Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

13.13.3.7.8 In this example:

$$MSD = 2.23(5.64)\sqrt{\left(\frac{1}{10}\right) + \left(\frac{1}{10}\right)}$$

$$= 2.23\ (5.64)\ (0.447)$$

$$= 5.62$$

13.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 5.62.

13.13.3.7.10 This represents a 25% decrease in mean reproduction from the control.

13.13.3.8 Calculation of the IC

13.13.3.8.1 The reproduction data in Table 4 are utilized in this example. As can be seen from Figure 8, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

13.13.3.8.2 Starting with the observed control mean, $\bar{Y}_1$= 22.4, and the observed mean for the lowest effluent concentration, $\bar{Y}_2$= 26.3, we see that $\bar{Y}_1$ is less than $\bar{Y}_2$.

13.13.3.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (\bar{Y}_1+\bar{Y}_2)/2 = 24.35$$

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13.13.3.8.4 Since Y_3= 34.6 is larger than M_2, average Y_3 with the previous concentrations:

$$M_1 = M_2 = M_3 = (M_1 + M_2 + Y_3)/3 = 27.7.$$

13.13.3.8.5 Additionally, Y_4 = 31.7 is larger than M_3, and is pooled with the first three means. Thus:

$$(M_1 + M_2 + M_3 + Y_4)/4 = 28.7 = M_1 = M_2 = M_3 = M_4$$

13.13.3.8.6 Since $M_4 > Y_5$= 9.4, set M_5 = 9.4. Likewise, $M_5 > Y_6$ = 0, and M_6 becomes 0. Table 15 contains the smoothed means and Figure 8 gives a plot of the smoothed means and the interpolated response curve.

TABLE 15. DAPHNID, *CERIODAPHNIA DUBIA*, REPRODUCTION MEAN RESPONSE AFTER SMOOTHING

Response Effluent Conc. (%)	i	Smoothed Means, Y_i (young/female)	Means, M_i (young/female)
Control	1	22.4	28.75
1.56	2	26.3	28.75
3.12	3	34.6	28.75
6.25	4	31.7	28.75
12.5	5	9.4	9.40
25.0	6	0.0	0.00

13.13.3.8.7 Estimates of the IC25 and IC50 can be calculated using the Linear Interpolation Method. A 25% reduction in reproduction, compared to the controls, would result in a mean reproduction of 21.56 young per adult, where $M_1(1 - p/100) = 28.75(1 - 25/100)$. A 50% reduction in reproduction, compared to the controls, would result in a mean reproduction of 14.38 young per adult, where $M_1(1 - p/100) = 28.75(1 - 50/100)$. Examining the smoothed means and their associated concentrations (Table 15), the two effluent concentrations bracketing 21.56 young per adult are C_4 = 6.25% effluent and C_5 = 12.5% effluent. The two effluent concentrations bracketing a response of 14.38 young per adult are also C_4 = 6.25% and C_5 = 12.5%.

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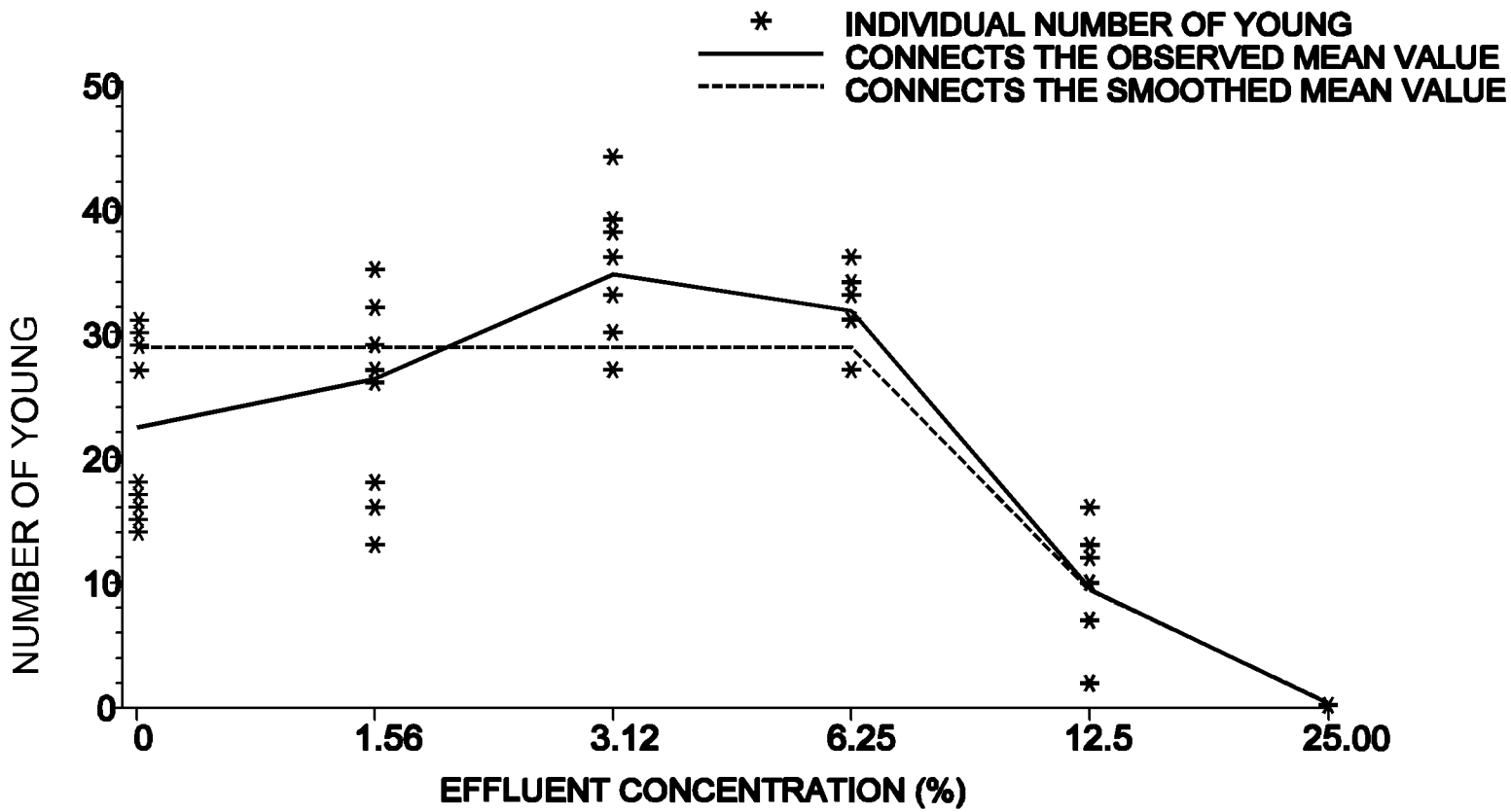

Figure 8. Plot of raw data, observed means, and smoothed means for the daphnid, *Ceriodaphnia dubia*, reproductive data.

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13.13.3.8.8 Using equation from Section 4.2 in Appendix M, the estimate of the IC25 is as follows:

$$ICp = C_j + [M_1(1 - \frac{p}{100}) - M_j]\frac{(C_{(p+1)} - C_j)}{(M_{(p+1)} - M_j)}$$

$$IC25 = 6.25 + [28.75(1 - \frac{25}{100}) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

= 8.57% effluent

13.13.3.8.9 The estimate of the IC50 is as follows:

$$ICp = C_j + [M_1(1 - \frac{p}{100}) - M_j]\frac{(C_{(p+1)} - C_j)}{(M_{(p+1)} - M_j)}$$

$$IC50 = 6.25 + [28.75(1 - \frac{50}{100}) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

= 10.89% effluent

13.13.3.8.10 When the ICPIN program was used to analyze this data set for the IC25, requesting 80 resamples, the estimate of the IC25 was 8.5715% effluent. The empirical 95% confidence interval for the true mean was 8.3112% and 9.0418% effluent. The computer output for this data set is provided in Figure 9.

13.13.3.8.11 When the ICPIN program was used to analyze this data set for the IC50, requesting 80 resamples, the estimate of the IC50 was 10.8931% effluent. The empirical 95% confidence interval for the true mean was 10.4373% and 11.6269% effluent. The computer output for this data set is provided in Figure 10.

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Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Example Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration: 7-d
DATA FILE: cdmanual.icp
OUTPUT FILE: cdmanual.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 8.5715 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 8.5891 Standard Deviation: 0.1831
Original Confidence Limits: Lower: 8.3112 Upper: 9.0418
Resampling time in Seconds: 2.53 Random Seed: -641671986

Figure 9. Example of ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Example Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration: 7-d
DATA FILE: cdmanual.icp
OUTPUT FILE: cdmanual.i50

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 10.8931 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 10.9316 Standard Deviation: 0.3357
Original Confidence Limits: Lower: 10.4373 Upper: 11.6269
Resampling time in Seconds: 2.58 Random Seed: 172869646

Figure 10. Example of ICPIN program output for the IC50.

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13.14 **PRECISION AND ACCURACY**

13.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 13.14.1.1 and 13.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

13.14.1.1 Single-Laboratory Precision

13.14.1.1.1 Information on the single-laboratory precision of the daphnid, *Ceriodaphnia dubia*, survival and reproduction test is based on the NOEC and LOEC values from nine tests with the reference toxicant sodium pentachlorophenate (NaPCP) is provided in Table 16. The NOECs and LOECs of all tests fell in the same concentration range, indicating maximum possible precision. Table 17 gives precision data for the IC25 and IC50 values for seven tests with the reference toxicant NaPCP. Coefficient of variation was 41% for the IC25 and 28% for the IC50.

13.14.1.1.2 Ten sets of data from six laboratories met the acceptability criteria, and were statistically analyzed using nonparametric procedures to determine NOECs and LOECs.

13.14.1.1.3 EPA evaluated within-laboratory precision of the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test using a database of routine reference toxicant test results from 33 laboratories (USEPA, 2000b). The database consisted of 393 reference toxicant tests conducted in 33 laboratories using a variety of reference toxicants including: cadmium, copper, potassium chloride, sodium chloride, and sodium pentachlorophenate. Among the 33 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 27% for the IC25 reproduction endpoint. In 25% of laboratories, the within-laboratory CV was less than 17%; and in 75% of laboratories, the within-laboratory CV was less than 45%.

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TABLE 16: SINGLE LABORATORY PRECISION OF THE DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TEST, USING NAPCP AS A REFERENCE TOXICANT[1,2]

Test	NOEC (mg/L)	LOEC (mg/L)	Chronic Value (mg/L)
1[3]	0.25	0.50	0.35
2[4]	0.20	0.60	0.35
3	0.20	0.60	0.35
4[5]	0.30	0.60	0.42
5	0.30	0.60	0.42
6	0.30	0.60	0.42
7	0.30	0.60	0.42
8	0.30	0.60	0.42
9	0.30	0.60	0.42

1 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

2 Data from Tests performed by Philip Lewis, Aquatic Biology Branch, EMSL-Cincinnati, OH. Tests were conduted in reconstituted hard water (hardness = 180 mg $CaC0_3$/L; pH - 8.1).

3 Concentrations used in Test 1 were: 0.03, 0.06, 0.12, 0.25, 0.50, 1.0 mg NaPCP/L.

4 Concentrations used in Tests 2 and 3 were: 0.007, 0.022, 0.067, 0.020, 0.60 mg NaPCP/L.

5 Concentrations used in Tests 4 through 9 were: 0.0375, 0.075, 0.150, 0.30, 0.60 mg NaPCP/L.

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TABLE 17. THE DAPHNID, *CERIODAPHNIA DUBIA,* SEVEN-DAY SURVIVAL AND REPRODUCTION TEST PRECISION FOR A SINGLE LABORATORY USING NAPCP AS THE REFERENCE TOXICANT (USEPA, 1991a)

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)
19	0.30	0.3754	0.4508
46A	0.20	0.0938	0.2608
46B	0.20	0.2213	0.2879
49	0.20	0.2303	0.2912
55	0.20	0.2306	0.3177
56	0.10	0.2241	0.2827
n	7	7	7
Mean	NA	0.2157	0.2953
CV(%)	NA	41.1	27.9

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13.14.1.2 Multilaboratory Precision

13.14.1.2.1 A multilaboratory study was performed by the Aquatic Biology Branch, EMSL-Cincinnati in 1985e, involving a total of 11 analysts in 10 different laboratories (Neiheisel et. al., 1988; USEPA, 1988e). Each analyst performed one-to-three seven-day tests using aliquots of a copper-spiked effluent sample, for a total of 25 tests. The tests were performed on the same day in all participating laboratories, using a pre-publication draft of Method 1002.0. The NOECs and LOECs for these tests were within one concentration interval which, with a dilution factor of 0.5, is equivalent to a two-fold range in concentration (Table 18).

13.14.1.2.2 A second multilaboratory study of Method 1002.0 (using the first edition of this manual; USEPA, 1985c), was coordinated by Battelle, Columbus Division, and involved 11 participating laboratories (Table 19) (DeGraeve et al., 1989). All participants used 10% DMW (10% PERRIER® Water) as the culture and dilution water, and used their own formulation of food for culturing and testing the *Ceriodaphnia dubia*. Each laboratory was to conduct at least one test with each of eight blind samples. Each test consisted of 10 replicates of one organism each for five toxicant concentrations and a control. Of the 116 tests planned, 91 were successfully initiated, and 70 (77%) met the survival and reproduction criteria for acceptability of the results (80% survival and nine young per initial female). If the reproduction criteria of 15 young/female, used in this edition of the method, had been applied to the results of the interlaboratory study, 22 additional tests would have been unacceptable. The overall precision (CV) of the test was 27% for the survival data (7-day LC50s) and 37.5% and 39.0% for the reproduction data (IC50s and IC25s, respectively).

13.14.1.2.3 In 2000, EPA conducted an interlaboratory variability study of the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 34 participant laboratories tested 3 or 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Of the 122 *Ceriodaphnia dubia* Survival and Reproduction tests conducted in this study, 82.0% were successfully completed and met the required test acceptability criteria. Of 27 tests that were conducted on blank samples, none showed false positive results for survival endpoints, and only one resulted in false positive results for the growth endpoint, yielding a false positive rate of 3.70%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 20 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 35.0% for IC25 results. Table 21 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned three concentrations for the reference toxicant and effluent sample types and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 97.2%, 91.3%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned five concentrations for the reference toxicant sample type, three concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 83.3%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

13.14.2 ACCURACY

13.14.2.1 The accuracy of toxicity tests cannot be determined.

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TABLE 18. INTERLABORATORY PRECISION FOR THE DAPHNID, *CERIODAPHNIA DUBIA,* SURVIVAL AND REPRODUCTION TEST WITH COPPER SPIKED EFFLUENT (USEPA, 1988e)

		Endpoints (% Effluent)			
		Reproduction		Survival	
Analyst	Test	NOEC	LOEC	NOEC	LOEC
3	1	12	25	25	50
4	1	6	12	12	25
4	2	6	12	25	50
5	1	6	12	12	25
5	2	12	25	12	25
6	1	12	25	25	50
6	2	6	12	25	50
10	1	6	12	12	25
10	2	6	12	12	25
11	1	12	25	25	50

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TABLE 19. INTERLABORATORY PRECISION DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, SUMMARIZED FOR EIGHT REFERENCE TOXICANTS AND EFFLUENTS (USEPA, 1991a)

Test Material	Mean IC50	CV%	Mean IC25	CV%
Sodium chloride	1.34	29.9	1.00	34.3
Industrial	3.6	83.3	3.2	78.1
Sodium chloride	0.96	57.4	0.09	44.4
Pulp and Paper	60.0	28.3	47.3	27.0
Potassium dichromate	35.8	30.8	23.4	32.7
Pulp and Paper	70.2	7.5	55.7	12.2
Potassium dichromate	53.2	25.9	29.3	46.8
Industrial	69.8	37.0	67.3	36.7
n		8		8
Mean		37.5		39.0
Standard Deviation		23.0		19.1

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TABLE 20. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	-	-	-
	Effluent	17.4	27.6	32.6
	Receiving water	-	-	37.4
	Average	17.4	27.6	35.0

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the reference toxicant sample type a majority of the results were outside of the test concentration range, so precision estimates were not calculated. For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

3 The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

4 The between-laboratory component of variability for duplicate samples tested at different laboratories.

5 The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

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TABLE 21. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results $2[3]
Survival NOEC	Reference toxicant	100%	97.2	0.00	2.78
	Effluent	25%	65.2	26.1	8.70
	Receiving water	25%	90.0	10.0	0.00
Growth NOEC	Reference toxicant	100%	72.2	11.1	16.7
	Effluent	12.5%	70.8	29.2	0.00
	Receiving water	25%	70.0	30.0	0.00

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
2 Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
3 Percent of values two or more concentration intervals above or below the median.

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SECTION 14

TEST METHOD

GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH TEST METHOD 1003.0

14.1 SCOPE AND APPLICATION

14.1.1 This method measures the chronic toxicity of effluents and receiving water to the freshwater green alga, *Selenastrum capricornutum*, in a four-day static test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

14.1.2 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

14.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly degradable or highly volatile toxicants present in the source may not be detected in the test.

14.1.4 This test method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

14.1.5 This test is very versatile because it can also be used to identify wastewaters which are biostimulatory and may cause nuisance growths of algae, aquatic weeds, and other organisms at higher trophic levels.

14.2 SUMMARY OF METHOD

14.2.1 A green alga, *Selenastrum capricornutum,* population is exposed in a static system to a series of concentrations of effluent, or to receiving water, for 96 h. The response of the population is measured in terms of changes in cell density (cell counts per mL), biomass, chlorophyll content, or absorbance.

14.3 INTERFERENCES

14.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

14.3.2 Adverse effects of high concentrations of suspended and/or dissolved solids, color, and extremes of pH may mask the presence of toxic substances.

14.3.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.3.4 Pathogenic organisms and/or planktivores in the dilution water and effluent may affect test organism survival and growth, and confound test results.

14.3.5 Nutrients in the effluent or dilution water may confound test results.

14.4 SAFETY

14.4.1 See Section 3, Safety and Health.

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14.5 APPARATUS AND EQUIPMENT

14.5.1 Laboratory *Selenastrum capricornutum* culture unit -- see culturing methods below and USEPA, 2002a. To test effluent toxicity, sufficient numbers of log-phase-growth organisms must be available.

14.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L or more.

14.5.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.5.4 Environmental chamber, incubator, or equivalent facility -- with "cool-white" fluorescent illumination (86 ± 8.6 $\mu E/m^2/s$, 400 ± 40 ft-c, or 4306 lux) and temperature control (25 ± 1EC).

14.5.5 Mechanical shaker -- capable of providing orbital motion at the rate of 100 cycles per minute (cpm).

14.5.6 Light meter -- with a range of 0-200 $\mu E/m^2/s$ (0-1000 ft-c).

14.5.7 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

14.5.8 Balance -- analytical, capable of accurately weighing 0.00001 g.

14.5.9 Reference weights, class S -- for checking performance of balance.

14.5.10 Volumetric flasks and graduated cylinders -- class A, 10-1000 mL, borosilicate glass, for culture work and preparation of test solutions.

14.5.11 Volumetric pipets -- class A, 1-100 mL.

14.5.12 Serological pipets -- 1-10 mL, graduated.

14.5.13 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

14.5.14 Wash bottles -- for rinsing small glassware, instrument electrodes, and probes.

14.5.15 Test chambers -- four 125 or 250 mL borosilicate, Erlenmeyer flasks, with foam plugs or stainless steel or Shumadzu closures. For special glassware cleaning requirements (see Section 5, Facilities, Equipment, and Supplies).

14.5.16 Culture chambers -- 1-4 L borosilicate, Erlenmeyer flasks.

14.5.17 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

14.5.18 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

14.5.19 Thermometer, National Bureau of Standards Certified, (see USEPA Method 170.1, USEPA, 1979b) -- to calibrate laboratory thermometers.

14.5.20 Meters, pH and specific conductivity -- for routine physical and chemical measurements.

14.5.21 Tissue grinder -- for chlorophyll extraction.

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14.5.22 Fluorometer (Optional) -- equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

14.5.23 UV-VIS spectrophotometer -- capable of accommodating 1-5 cm cuvettes.

14.5.24 Cuvettes for spectrophotometer -- 1-5 cm light path.

14.5.25 Electronic particle counter (Optional) -- Coulter Counter, Model ZBI, or equivalent, with mean cell (particle) volume determination.

14.5.26 Microscope -- with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condenser, and light source (inverted or conventional microscope).

14.5.27 Counting chamber -- Sedgwick-Rafter, Palmer-Maloney, or hemocytometer.

14.5.28 Centrifuge -- with swing-out buckets having a capacity of 15-100 mL.

14.5.29 Centrifuge tubes -- 15-100 mL, screw-cap.

14.5.30 Filtering apparatus -- for membrane and/or glass fiber filters.

14.6 REAGENTS AND CONSUMABLE MATERIALS

14.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.6.2 Data sheets (one set per test) -- for recording data.

14.6.3 Tape, colored -- for labeling test chambers.

14.6.4 Markers, waterproof -- for marking containers, etc.

14.6.5 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA, 1979b.

14.6.6 Buffers pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) for instrument calibration (see USEPA Method 150.1, USEPA, 1979b).

14.6.7 Specific conductivity standards (see USEPA Method 120.1, USEPA, 1979b).

14.6.8 Standard particles -- such as chicken or turkey fibroblasts or polymer microspheres, 5.0 ± 0.03 µm diameter, 65.4 μm^3 volume, for calibration of electronic particle counters.

14.6.9 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

14.6.10 Laboratory quality control samples and standards -- for calibration of the above methods.

14.6.11 Reference toxicant solutions -- see Section 4, Quality Assurance.

14.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

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14.6.13 Effluent or receiving water and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Testing.

14.6.14 Acetone -- pesticide-grade or equivalent.

14.6.15 Dilute (10%) hydrochloric acid -- carefully add 10 mL of concentrated HCl to 90 mL of MILLI-Q® water.

14.6.16 TEST ORGANISMS, GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*

14.6.16.1 *Selenastrum capricornutum*, a unicellular coccoid green alga, is the test organism. The genus and species name of this organism was formally changed to *Pseudokirchneriella subcapitata* (Hindak, 1990), however, the method manual will continue to refer to *Selenastrum capricornutum* to maintain consistency with previous versions of the method.

14.6.16.2 Algal Culture Medium is prepared as follows:

14.6.16.2.1 Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.

14.6.16.2.2 Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L, mix well, and adjust the pH to 7.5 ± 0.1, using 0.1N NaOH or HCl, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.

14.6.16.2.3 Immediately filter the pH-adjusted medium through a 0.45 µm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.

14.6.16.2.4 If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels. If a 0.22 µg filter is used no sterilization is needed.

14.6.16.2.5 Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

14.6.16.2.6 When prepared according to Table 1, the micronutrient stock solution contains ethylenediaminetetraacetic acid (EDTA). EPA requires the addition of EDTA to nutrient stock solutions when conducting the *Selenastrum capricornutum* Growth Test and submitting data under NPDES permits. The use of EDTA improves test method performance by reducing the incidence of false positives and increasing test method precision. In interlaboratory testing of split samples analyzed with and without the addition of EDTA, false positive rates were 0.00% with EDTA and 33.3% without EDTA (USEPA, 2001a). Interlaboratory variability, expressed as the CV for IC25 values, was 34.3% with EDTA and 58.5% without EDTA (USEPA, 2001a). While the addition of EDTA improves test performance, EPA also cautions that the addition of EDTA may cause the *Selenastrum capricornutum* Growth Test to underestimate the toxicity of metals. Regulatory authorities should consider this possibility when selecting test methods for monitoring effluents that are suspected to contain metals. As recommended in EPA's Technical Support Document for Water Quality-Based Toxics Control (USEPA, 1991a), the most sensitive of at least three test species from different phyla should be used for monitoring the toxicity of effluents.

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TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 mL MILLI-Q® WATER	
1. MACRONUTRIENTS			
A.	$MgCl_2 \cdot 6H_2O$	6.08	g
	$CaCl_2 \cdot 2H_2O$	2.20	g
	$NaNO_3$	12.75	g
B.	$MgSO_4 \cdot 7H_2O$	7.35	g
C	K_2HPO_4	0.522	g
D.	$NaHCO_3$	7.50	g
2. MICRONUTRIENTS			
	H_3BO_3	92.8	mg
	$MnCl_2 \cdot 4H_2O$	208.0	mg
	$ZnCl_2$	1.64	mg[1]
	$FeCl_3 \cdot 6H_2O$	79.9	mg
	$CoCl_2 \cdot 6H_2O$	0.714	mg[2]
	$Na_2MoO_4 \cdot 2H_2O$	3.63	mg[3]
	$CuCl_2 \cdot 2H_2O$	0.006	mg[4]
	$Na_2EDTA \cdot 2H_2O$	150.0	mg
	Na_2SeO_4	1.196	mg[5]

[1] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[2] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[3] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[4] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock 2, micronutrients.

[5] Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

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TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRONUTRIENT	CONCENTRATION (mg/L)	ELEMENT	CONCENTRATION (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRONUTRIENT	CONCENTRATION (µg/L)	ELEMENT	CONCENTRATION (µg/L)
H_3BO_3	185.0	B	32.5
$MnCl_2 \cdot 4H_2O$	416.0	Mn	115.0
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160.0	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300.0	--	----
Na_2SeO_4	2.39	Se	0.91

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14.6.16.3 Stock Algal Cultures

14.6.16.3.1 See Section 6, Test Organisms, for information on sources of "starter" cultures of the green alga, *Selenastrum capricornutum*.

14.6.16.3.2 Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring 1 mL to a culture flask containing control algal culture medium (prepared as described above). The volume of stock culture medium initially prepared will depend upon the number of test flasks to be inoculated later from the stock, or other planned uses, and may range from 25 mL in a 125 mL flask to 2 L in a 4-L flask. The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4EC.

14.6.16.3.3 Maintain the stock cultures at 25 ± 1EC, under continuous "Cool-White" fluorescent lighting of 86 ± 8.6 $\mu E/m^2/s$ (400 ± 40 ft-c). Shake continuously at 100 cpm or twice daily by hand.

14.6.16.3.4 Transfer 1 to 2 mL of stock culture weekly to 50 - 100 mL of new culture medium to maintain a continuous supply of "healthy" cells for tests. Aseptic techniques should be used in maintaining the algal cultures, and extreme care should be exercised to avoid contamination. Examine the stock cultures with a microscope for contaminating microorganisms at each transfer.

14.6.16.3.5 Viable unialgal culture material may be maintained for long periods of time if placed in a refrigerator at 4EC.

14.6.16.4 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Algal cells four to seven days old are used to monitor the chronic toxicity (growth) of the reference toxicant to the algal stock produced by the culture unit (see Section 4, Quality Assurance, Subsection 4.17).

14.6.16.5 Record Keeping

14.6.16.5.1 Records, kept in a bound notebook, include (1) dates culture media was prepared, (2) source of "starter" cultures, (3) date stock cultures were started, (4) cell density in stock cultures, and (5) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

14.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

14.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

14.8 CALIBRATION AND STANDARDIZATION

14.8.1 See Section 4, Quality Assurance.

14.9 QUALITY CONTROL

14.9.1 See Section 4, Quality Assurance.

14.10 TEST PROCEDURES

14.10.1 TEST SOLUTIONS

14.10.1.1 Receiving Waters

14.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared

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without dilution against a control. Using four replicate chambers per test, each containing 100 mL and 400 mL for chemical analyses, would require approximately 1 L or more of sample for the test.

14.10.1.2 Effluents

14.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5 and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends using a $ 0.5 dilution factor**.

14.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of the effluent concentrations.

14.10.1.2.3 The volume of effluent required for the test is 1 to 2 L. Sufficient test solution (approximately 900 or 1500 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses at the high, medium, and low test concentrations. There is no daily renewal of test solution.

14.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be usedfor the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.10.1.2.5 Just prior to test initiation (approximately 1 h) the temperature of sufficient quantity of the sample to make test solutions should be adjusted to the test temperature and maintained at that temperature during the addition of dilution water.

14.10.1.2.6 The DO of the test solutions should be checked prior to test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If or any solution has a DO concentration below 4.0 mg/L, all of the solutions and the control must be gently aerated.

14.10.1.2.7 Effluents may be toxic and/or nutrient poor. "Poor" growth in an algal toxicity test, therefore, may be due to toxicity or nutrient limitation, or both. To eliminate false negative results due to low nutrient concentrations, 1 mL of each stock nutrient solution is added per liter of effluent prior to use in preparing the test dilutions. Thus, all test treatments and controls will contain at a minimum the concentration of nutrients in the stock culture medium.

14.10.1.2.8 If samples contain volatile substances, the test sample should be added below the surface of the dilution water towards the bottom of the test container through an appropriate delivery tube.

14.10.1.3 Dilution Water

14.10.1.3.1 Dilution water may be stock culture medium, any uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other natural water (see Section 7, Dilution Water). However, if water other than the stock culture medium is used for dilution water, 1 mL of each stock nutrient solution should be added per liter of dilution water. Natural waters used as dilution water must be filtered through a prewashed filter, such as a GF/A, GF/C, or equivalent filter, that provides 0.45 µm particle size retention.

14.10.1.3.2 If the growth of the algae in the test solutions is to be measured with an electronic particle counter, the effluent and dilution water must be filtered through a GF/A or GF/C filter, or other filter providing 0.45 µm particle

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size retention, and checked for "background" particle count before it is used in the test. Glass-fiber filters generally provide more rapid filtering rates and greater filtrate volume before plugging.

14.10.1.4 Preparation of Inoculum

14.10.1.4.1 The inoculum is prepared no more than 2 to 3 h prior to the beginning of the test, using *Selenastrum capricornutum* harvested from a four- to-seven-day stock culture. Each milliliter of inoculum must contain enough cells to provide an initial cell density of approximately 10,000 cells/mL (± 10%) in the test flasks. Assuming the use of 250 mL flasks, each containing 100 mL of test solution, the inoculum must contain 1,000,000 cells/mL.

14.10.1.4.2 Estimate the volume of stock culture required to prepare the inoculum. As an example, if the four-to-seven-day-old stock culture used as the source of the inoculum has a cell density of 2,000,000 cells/mL, a test employing 24 flasks, each containing 100 mL of test medium and inoculated with a total of 1,000,000 cells, would require 24,000,000 cells or 15 mL of stock solution (24,000,000/2,000,000) to provide sufficient inoculum. It is advisable to prepare a volume 20% to 50% in excess of the minimum volume required, to cover accidental loss in transfer and handling.

14.10.1.4.3 Prepare the inoculum as follows:

1. Centrifuge 15 mL of stock culture at 1000 x g for 5 min. This volume will provide a 50% excess in the number of cells.
2. Decant the supernatant and resuspend the cells in 10 mL of control medium.
3. Repeat the centrifugation and decantation step, and resuspend the cells in 10 mL control medium.
4. Mix well and determine the cell density in the algal concentrate. Some cells will be lost in the concentration process.
5. Determine the density of cells (cells/mL) in the stock culture (for this example, assume 2,000,000 per mL).
6. Calculate the required volume of stock culture as follows:

$$\text{Volume (mL) of Stock Culture Required} = \frac{\text{Number test flasks to be used} \times \text{Volume of test Solutions/flask} \times \text{10,000 cells/mL}}{\text{Cell density (cells/mL) in the stock culture}}$$

$$= \frac{24 \text{ flasks} \times 100 \text{ mL/flask} \times 10{,}000 \text{ cells/mL}}{2{,}000{,}000 \text{ cells/mL}}$$

$$= 12.0 \text{ mL Stock Culture}$$

7. Dilute the cell concentrate as needed to obtain a cell density of 1,000,000 cells/mL, and check the cell density in the final inoculum.
8. The volume of the algal inoculum should be considered in calculating the dilution of toxicant in the test flasks.

14.10.2 START OF THE TEST

14.10.2.1 Label the test chambers with a marking pen and use the color-coded tape to identify each treatment and replicate. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) should have a minimum of four replicates.

14.10.2.2 Randomize the position of the test flasks at the beginning of the test (see Appendix A). Preparation of a position chart may be helpful.

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14.10.2.3 The test begins when the algae are added to the test flasks. Mix the inoculum well, and add 1 mL to the test solution in each randomly arranged flask. Make a final check of the cell density in three of the test solutions at time "zero" (within 2 h of the inoculation).

14.10.2.3.1 Alkalinity, hardness, and conductivity are measured at the beginning of the test in the high, medium, and low effluent concentrations and control before they are dispensed to the test chambers and the data recorded on the data sheet (Figure 1).

Discharger:_____ Test Dates: _____
Location:_____ Analyst: _____

Effluent Concentration

Parameter	Control							Remarks
Temperature								
pH								
Alkalinity								
Hardness								
Conductivity								
Chlorine								

Figure 1. Data form for the green alga, *Selenastrum capricornutum*, growth test. Routine chemical and physical determinations.

14.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

14.10.3.1 Test flasks are incubated under continuous illumination at 86 ± 8.6 $\mu E/m^2/s$ (400 ± 40 ft-c), at 25 ± 1EC, and should be shaken continuously at 100 cpm on a mechanical shaker or twice daily by hand. Flask positions in the incubator should be randomly rotated each day to minimize possible spatial differences in illumination and temperature on growth rate. If it can be verified that test specifications are met at all positions, this need not be done.

14.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

14.10.4.1 Because of the continuous illumination of the test flasks, DO concentration should never be a problem during the test and no aeration will be required.

14.10.5 OBSERVATIONS DURING THE TEST

14.10.5.1 Routine Chemical and Physical Determinations

14.10.5.1.1 Temperature should be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be checked in a sufficient number of test vessels at least at the end of the test to determine variability in the environmental chamber.

14.10.5.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test flask at each concentration and in the control.

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14.10.5.1.3 Record all the measurements on the data sheet (Figure 1).

14.10.5.2 Biological Observations

14.10.5.2.1 Toxic substances in the test solutions may degrade or volatilize rapidly, and the inhibition in algal growth may be detectable only during the first one or two days in the test. It may be desirable, therefore, to determine the algal growth response daily. Otherwise, biological observations are not required until the test is terminated and the test solutions are not renewed during the test period.

14.10.6 TERMINATION OF THE TEST

14.10.6.1 The test is terminated 96 h after initiation. The algal growth in each flask is measured by one of the following methods: (a) cell counts, (b) chlorophyll content, or (c) turbidity (light absorbance).

14.10.6.2 Cell counts

14.10.6.2.1 Automatic Particle Counters

14.10.6.2.1.1 Several types of automatic electronic and optical particle counters are available for use in the rapid determination of cell density (cells/mL) and mean cell volume (MCV) in μm^3/cell. The Coulter Counter is widely used and is discussed in detail in USEPA (1978b).

14.10.6.2.1.2 If biomass data are desired for algal growth potential measurements, a Model ZM Coulter Counter is used. However, the instrument must be calibrated with a reference sample of particles of known volume.

14.10.6.2.1.3 When the Coulter Counter is used, an aliquot (usually 1 mL) of the test culture is diluted 10X to 20X with a 1% sodium chloride electrolyte solution, such as ISOTON®, to facilitate counting. The resulting dilution is counted using an aperture tube with a 100-µm diameter aperture. Each cell (particle) passing through the aperture causes a voltage drop proportional to its volume. Depending on the model, the instrument stores the information on the number of particles and the volume of each, and calculates the mean cell volume. The following procedure is used:

1. Mix the algal culture in the flask thoroughly by swirling the contents of the flask approximately six times in a clockwise direction, and then six times in the reverse direction; repeat the two-step process at least once.
2. At the end of the mixing process, stop the motion of the liquid in the flask with a strong brief reverse mixing action, and quickly remove 1 mL of cell culture from the flask with a sterile pipet.
3. Place the aliquot in a counting beaker, and add 9 mL (or 19 mL) of electrolyte solution (such as Coulter ISOTON®).
4. Determine the cell density (and MCV, if desired).

14.10.6.2.2 Manual microscope counting method

14.10.6.2.2.1 Cell counts may be determined using a Sedgwick-Rafter, Palmer-Maloney, hemocytometer, inverted microscope, or similar methods. For details on microscope counting methods, see APHA (1992) and USEPA (1973). Whenever feasible, 400 cells per replicate are counted to obtain ± 10% precision at the 95% confidence level. This method has the advantage of allowing for the direct examination of the condition of the cells.

14.10.6.3 Chlorophyll Content

14.10.6.3.1 Chlorophyll may be estimated in-vivo fluorometrically, or in-vitro either fluorometrically or spectrophotometrically. In-vivo fluorometric measurements are recommended because of the simplicity and sensitivity of the technique and rapidity with which the measurements can be made (Rehnberg et al., 1982).

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14.10.6.3.2 The in-vivo chlorophyll measurements are made as follows:

1. Adjust the "blank" reading of the fluorometer using the filtrate from an equivalent dilution of effluent filtered through a 0.45 µm particle retention filter.
2. Mix the contents of the test culture flask by swirling successively in opposite directions (at least three times), and remove 1 mL of culture from the flask with a sterile pipet.
3. Place the aliquot in a small disposable vial and record the fluorescence as soon as the reading stabilizes. (Do not allow the sample to stand in the instrument more than 1 min).
4. Discard the sample.

14.10.6.3.3 For additional information on chlorophyll measurement methods, (see APHA, 1992).

14.10.6.4 Turbidity (Absorbance)

14.10.6.4.1 A second rapid technique for growth measurement involves the use of a spectrophotometer to determine the turbidity, or absorbance, of the cultures at a wavelength of 750 nm. Because absorbance is a complex function of the volume, size, and pigmentation of the algae, it would be useful to construct a calibration curve to establish the relationship between absorbance and cell density.

14.10.6.4.2 The algal growth measurements are made as follows:

1. A blank is prepared as described for the fluorometric analysis.
2. The culture is thoroughly mixed as described above.
3. Sufficient sample is withdrawn from the test flask with a sterile pipet and transferred to a 1- to 5-cm cuvette.
4. The absorbance is read at 750 nm and divided by the light path length of the cuvette, to obtain an "absorbance-per-centimeter" value.
5. The 1-cm absorbance values are used in the same manner as the cell counts.

14.10.6.5 Record the data as indicated in Figure 2.

14.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

14.11.1 A summary of test conditions and test acceptability criteria is presented in Table 3.

14.12 ACCEPTABILITY OF TEST RESULTS

14.12.1 For the test results to be acceptable, the mean algal cell density in the control flasks must exceed 1×10^6 cells/mL at the end of the test, and the coefficient of variation (CV, calculated as standard deviation X 100 / mean) for algal cell density among the control replicates must not exceed 20%.

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Discharger: _____ Test Dates: _____
Location: _____ Analyst: _____

Concentration	Cell Density Measurement Replicate 1	2	3	4	Treatment Mean	Comments
Control						
Conc:						
Conc:						
Conc:						
Conc:						
Conc:						

Comments:

Figure 2. Data form for the green alga, *Selenastrum capricornutum*, growth test, cell density determinations.

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TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GREEN ALGA, *SELENASTRUM CAPRICORNUTUM,* GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1003.0)[1]

1.	Test type:	Static non-renewal (required)
2.	Temperature:	25 ± 1EC (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
3.	Light quality:	"Cool white" fluorescent lighting (recommended)
4.	Light intensity:	86 ± 8.6 $\mu E/m^2/s$ (400 ± 40 ft-c or 4306 lux) (recommended)
5.	Photoperiod:	Continuous illumination (required)
6.	Test chamber size:	125 mL or 250 mL (recommended)
7.	Test solution volume:	50 mL or 100 mL[2] (recommended)
8.	Renewal of test solutions:	None (required)
9.	Age of test organisms:	4 to 7 days (required)
10.	Initial cell density in test chambers:	10,000 cells/mL (recommended)
11.	No. replicate chambers per concentration:	4 (required minimum)
12.	Shaking rate:	100 cpm continuous, or twice daily by hand (recommended)
13.	Aeration:	None (recommended)
14.	Dilution water:	Algal stock culture medium, enriched uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals, or DMW (see Section 7, Dilution Water) (available options)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

[2] For tests not continuously shaken use 25 mL in 125 mL flasks and 50 mL in 250 mL flasks.

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TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GREEN ALGA, *SELENASTRUM CAPRICORNUTUM,* GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1003.0) (CONTINUED)

15.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended)
16.	Test dilution factor:	Effluents: $ 0.5 (recommended) Receiving Waters: None or $ 0.5 (recommended)
17.	Test duration:	96 h (required)
18.	Endpoint:	Growth (cell counts, chlorophyll fluorescence, absorbance, or biomass) (required)
19.	Test acceptability criteria:[3]	Mean cell density of at least 1 X 10^6 cells/mL in the controls; and variability (CV%) among control replicates less than or equal to 20% (required)
20.	Sampling requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
21.	Sample volume required:	1 or 2 L depending on test volume (recommended)

[3] If the test is conducted under non-NPDES applications (i.e., data are not submitted under NPDES permits) and used without EDTA in the nutrient stock solution, the test acceptability criteria are a mean cell density of at least 2 X 10^5 cells/mL in the controls, and variability (CV%) among control replicates less than or equal to 20%.

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14.13 **DATA ANALYSIS**

14.13.1 GENERAL

14.13.1.1 Tabulate and summarize the data. A sample set of algal growth response data is shown in Table 4.

TABLE 4. GREEN ALGA, *SELENASTRUM CAPRICORNUTUM,* GROWTH RESPONSE DATA

			Toxicant Concentration (µg Cd/L)				
	Replicate	Control	5	10	20	40	80
	A	1209	1212	826	493	127	49.3
	B	1180	1186	628	416	147	40.0
	C	1340	1204	816	413	147	44.0
Log_{10}	A	3.082	3.084	2.917	2.693	2.104	1.693
Trans-	B	3.072	3.074	2.798	2.619	2.167	1.602
formed	C	3.127	3.081	2.912	2.616	2.167	1.643
	Mean($\bar{Y}_i$)	3.094	3.080	2.876	2.643	2.146	1.646

14.13.1.2 The endpoints of toxicity tests using the green alga, *Selenastrum capricornutum,* are based on the adverse effects on cell growth (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). The EC50, the IC25, and the IC50 are calculated using the point estimation techniques, and LOEC and NOEC values for growth are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the EC50, IC25, and IC50. See the Appendices for examples of the manual computations, and examples of data input and program output.

14.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

14.13.2 EXAMPLE OF ANALYSIS OF ALGAL GROWTH DATA

14.13.2.1 Formal statistical analysis of the growth data is outlined on the flowchart in Figure 3. The response used in the statistical analysis is the number of cells per milliliter per replicate. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 and IC50 endpoints.

14.13.2.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Tests, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

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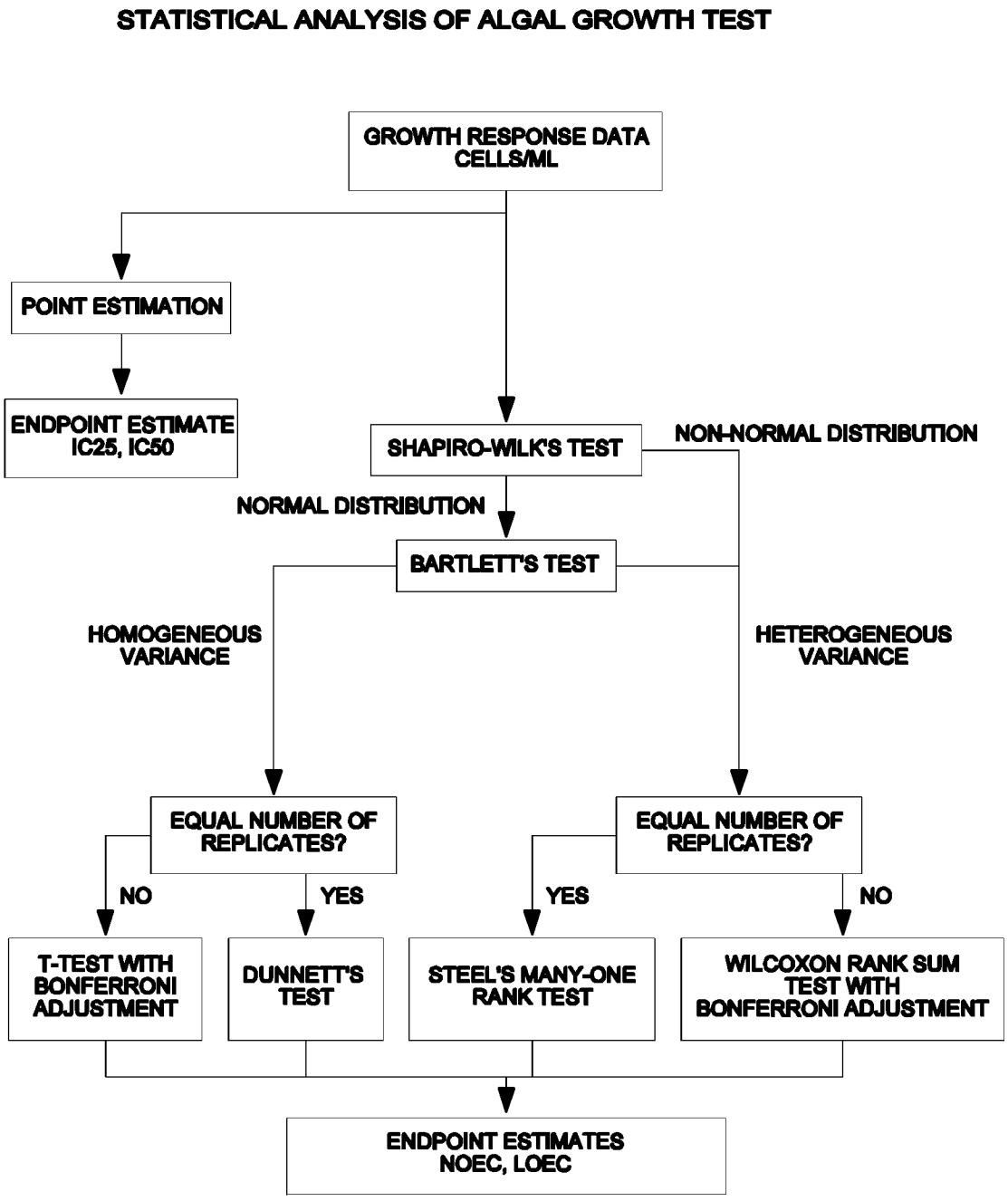

Figure 3. Flowchart for statistical analysis of the green alga, *Selenastrum capricornutum*, growth response data.

14.13.2.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

14.13.2.4 Data from an algal growth test with cadmium chloride will be used to illustrate the statistical analysis. The cell counts were $\log_{10}$ transformed in an effort to stabilize the variance for the ANOVA analysis. The raw data, $\log_{10}$ transformed data, mean and standard deviation of the observations at each concentration including the control are listed in Table 4. A plot of the $\log_{10}$ transformed cell counts for each treatment is provided in Figure 4.

14.13.2.5 Test for Normality

14.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 5.

TABLE 5. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Toxicant Concentration (µg Cd/L)				
Replicate	Control	5	10	20	40	80
A	-0.012	0.004	0.041	0.050	-0.042	0.047
B	-0.022	-0.006	-0.078	-0.024	0.021	-0.044
C	0.033	0.001	0.036	-0.027	0.021	-0.003

14.13.2.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations.

For this set of data, n = 18

$$\bar{X} = \frac{1}{18}(0.000) = 0.000$$

D = 0.0214

14.13.2.5.3 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 6.

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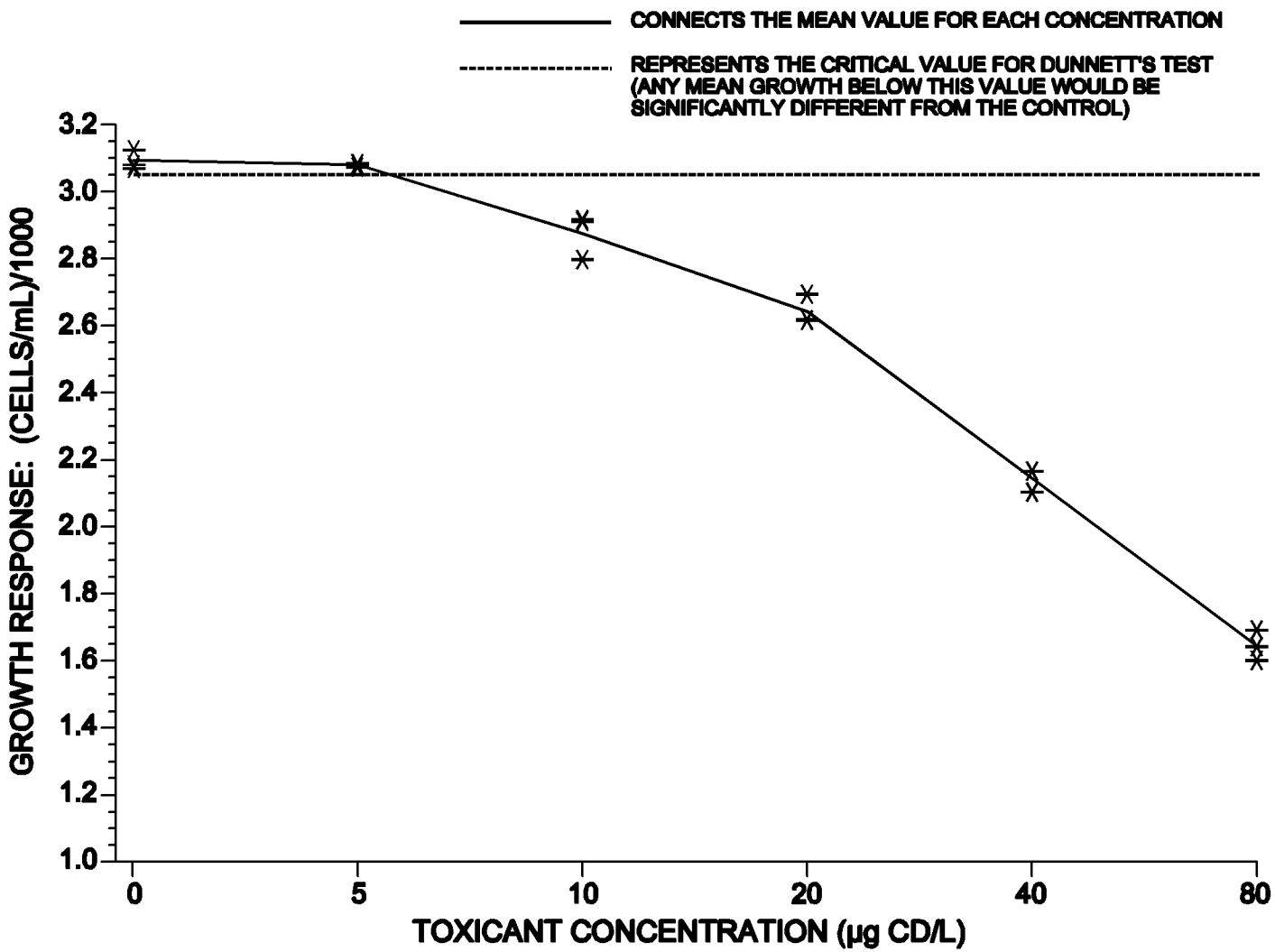

Figure 4. Plot of the $\log_{10}$ transformed cell count data from the green alga, *Selenastrum capricornutum*, growth response test in Table 4.

TABLE 6. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.078	10	0.001
2	-0.044	11	0.004
3	-0.042	12	0.021
4	-0.027	13	0.021
5	-0.024	14	0.033
6	-0.022	15	0.036
7	-0.012	16	0.041
8	-0.006	17	0.047
9	-0.003	18	0.050

14.13.2.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ..., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 18 , k = 9. The a_i values are listed in Table 7.

14.13.2.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 7.

TABLE 7. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4886	0.128	$X^{(18)} - X^{(1)}$
2	0.3253	0.091	$X^{(17)} - X^{(2)}$
3	0.2553	0.083	$X^{(16)} - X^{(3)}$
4	0.2027	0.063	$X^{(15)} - X^{(4)}$
5	0.1587	0.057	$X^{(14)} - X^{(5)}$
6	0.1197	0.043	$X^{(13)} - X^{(6)}$
7	0.0837	0.033	$X^{(12)} - X^{(7)}$
8	0.0496	0.010	$X^{(11)} - X^{(8)}$
9	0.0163	0.004	$X^{(10)} - X^{(9)}$

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For this set of data:

$$W = \frac{1}{0.0214}(0.1436)^2 = 0.964$$

14.13.2.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 18 observations (n) is 0.858. Since W = 0.964 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

14.13.2.6 Test for Homogeneity of Variance

14.13.2.6.1 The test used to examine whether the variation in mean cell count is the same across all toxicant concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i)\ln \bar{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each toxicant concentration and control, $V_i = (n_i - 1)$

p = number of levels of toxicant concentration including the control

n_i = the number of replicates for concentration i

ln = $\log_e$

i = 1, 2, ..., p, where p is the number of concentrations including the control

$$\bar{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + (3(p-1))^{-1}[\sum_{i=1}^{p} \frac{1}{V_i} - (\sum_{i=1}^{p} V_i)^{-1}]$$

14.13.2.6.2 For the data in this example, (see Table 4) all toxicant concentrations including the control have the same number of replicates (n_i = 3 for all i). Thus, V_i = 2 for all i.

14.13.2.6.3 Bartlett's statistic is therefore:

$$B = \frac{[(12)\ln(0.0018) - 2\sum_{i=1}^{p} \ln(S_i^2)]}{1.194}$$

= [12(-6.3200) - 2(-41.9082)]/1.194

= 7.9764/1.194

= 6.6804

14.13.2.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with five degrees of freedom, is 15.09. Since B = 6.6804 is less than the critical value of 15.09, conclude that the variances are not different.

14.13.2.7 Dunnett's Procedure

14.13.2.7.1 To obtain an estimate of the pooled variance for Dunnett's Procedure, construct an ANOVA table as described in Table 8.

TABLE 8. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of toxicant concentrations including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the cell count for toxicant concentration i in test chamber j)

14.13.2.7.2 For the data in this example:

$$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 3$$

$$N = 18$$

$$T_1 = Y_{11} + Y_{12} + Y_{13} = 9.281$$
$$T_2 = Y_{21} + Y_{22} + Y_{23} = 9.239$$
$$T_3 = Y_{31} + Y_{32} + Y_{33} = 8.627$$
$$T_4 = Y_{41} + Y_{42} + Y_{43} = 7.928$$
$$T_5 = Y_{51} + Y_{52} + Y_{53} = 6.438$$
$$T_6 = Y_{61} + Y_{62} + Y_{63} = 4.938$$

$$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 46.451$$

$$SSB = \sum_{i=1}^{P} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$

$$= \frac{1}{3}(374.606) - \frac{(46.451)^2}{18} = 4.997$$

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$

$$= 124.890 - \frac{(46.451)^2}{18} = 5.018$$

$$SSW = SST - SSB = 5.018 - 4.997 = 0.0210$$

$$S_B^2 = SSB/(p-1) = 4.996/(6-1) = 0.9990$$

$$S_W^2 = SSW/(N-p) = 0.021/(18-6) = 0.0018$$

14.13.2.7.3 Summarize these calculations in the ANOVA table (Table 9).

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TABLE 9. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	5	4.997	0.999
Within	12	0.021	0.0018
Total	17	5.017	

14.13.2.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(\frac{1}{n_1}) + (\frac{1}{n_i})}}$$

Where: $\bar{Y}_i$ = mean cell count for toxicant concentration i

$\bar{Y}_1$ = mean cell count for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

14.13.2.7.5 Table 10 includes the calculated t values for each concentration and control combination. In this example, comparing the 5 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(3.094 - 3.080)}{[0.0424\sqrt{(1/3) + (1/3)}]} = 0.405$$

TABLE 10. CALCULATED T VALUES

Toxicant Concentration (µg Cd/L)	i	t_i
5	2	0.405
10	3	6.300
20	4	13.035
40	5	27.399
80	6	41.850

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14.13.2.7.6 Since the purpose of this test is to detect a significant reduction in mean cell count, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 12 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.50. The mean count for concentration i is considered significantly less than the mean count for the control if t_i is greater than the critical value. Since t_3, t_4, t_5 and t_6 are greater than 2.50, the 10, 20, 40 and 80 µg/L concentrations have significantly lower mean cell counts than the control. Hence the NOEC and the LOEC for the test are 5 µg/L and 10 µg/L, respectively.

14.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated.

$$MSD = d\,S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

14.13.2.7.8 In this example:

$$MSD = 2.50(0.0424)\sqrt{(1/3)+(1/3)}$$

$$= 2.50\ (0.0424)(0.8165)$$

$$= 0.086$$

14.13.2.7.9 The MSD (0.086) is in transformed units. An approximate MSD in terms of cell count per 100 mL may be calculated via the following conversion.

1. Subtract the MSD from the transformed control mean.

   3.094 - 0.086 = 3.008

2. Obtain the untransformed values for the control mean and the difference calculated in 1.

   $10^{(3.094)} = 1241.6$

   $10^{(3.008)} = 1018.6$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from 2.

   $MSU_u = 1241.6 - 1018.6 = 223$

14.13.2.7.10 Therefore, for this set of data, the minimum difference in mean cell count between the control and any toxicant concentration that can be detected as statistically significant is 223.

14.13.2.7.11 This represents a decrease in growth of 18% from the control.

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14.13.2.8 Calculation of the ICp

14.13.2.8.1 The growth data in Table 4 are utilized in this example. Table 11 contains the means for each toxicant concentration. As can be seen, the observed means are monotonically non-increasing with respect to concentration. Therefore, it is not necessary to smooth the means prior to calculating the ICp. See Figure 5 for a plot of the response curve.

TABLE 11. ALGAL MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Concentration (µg Cd/L)	i	Response means, $\bar{Y}_i$ (cells/mL)	Smoothed mean, M_i (cells/mL)
Control	1	1243	1243
5	2	1201	1201
10	3	757	757
20	4	441	441
40	5	140	140
80	6	44	44

14.13.2.8.2 An IC25 and IC50 can be estimated using the Linear Interpolation Method (Appendix M). A 25% reduction in cell count, compared to the controls, would result in a mean count of 932 cells, where $M_1(1-p/100)$ = 1243(1-25/100). A 50% reduction in cell count, compared to the controls, would result in a mean count of 622 cells. Examining the means and their associated concentrations (Table 11), the response, 932 cells, is bracketed by C_2 = 5 µg Cd/L and C_3 = 10 µg Cd/L. The response, 622 cells, is bracketed by C_3 = 10 µg Cd/L and C_4 = 20 µg Cd/L.

14.13.2.8.3 Using the equation from section 4.2 of Appendix M, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1-p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 5 + [1243(1-25/100) - 1201]\frac{(10-5)}{(757-1201)}$$

= 8µg Cd/L.

14.13.2.8.4 The IC50 estimate is 14 µg Cd/L:

$$IC25 = 6.25 + [28.75(1-25/100) - 28.75]\frac{(12.5-6.25)}{(9.40-28.75)}$$

$$IC50 = 10 + [1243(1-50/100) - 757]\frac{(20-10)}{(441-757)}$$

= 14 µg Cd/L.

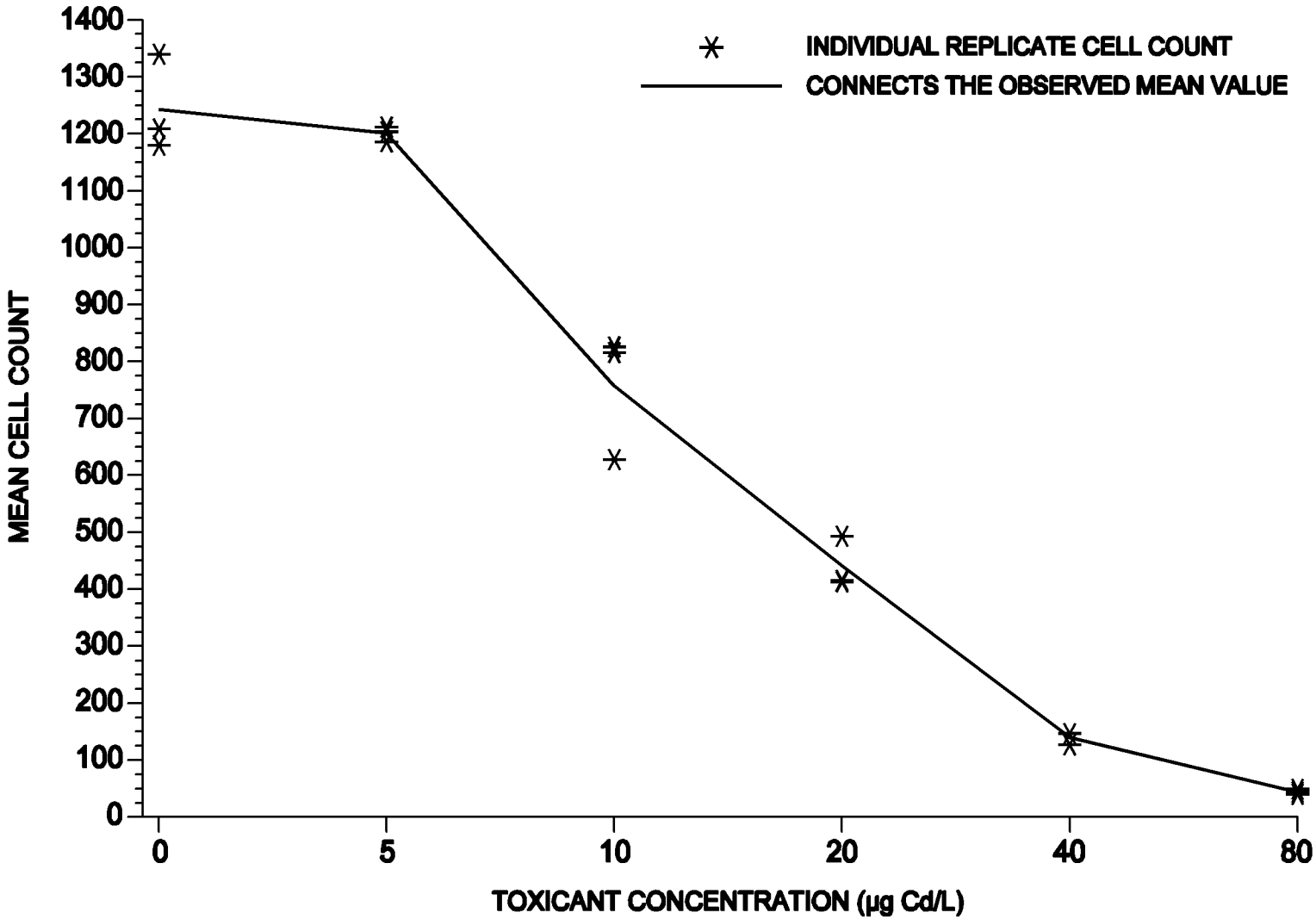

Figure 5. Plot of raw data and observed means for the green alga, *Selenastrum capricornutum*, growth data.

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14.13.2.8.5 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 8.0227µg Cd/L. The empirical 95% confidence interval for the true mean was 6.4087 µg Cd/L and 10.0313 µg Cd/L. The ICPIN computer program output for the IC25 for this data set is shown in Figure 6.

14.13.2.8.6 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 14.2774 µg Cd/L. The empirical 95% confidence interval for the true mean was 9.7456 µg Cd/L and 18.5413 µg Cd/L. The computer program output for the IC50 for this data set is shown in Figure 7.

14.13.3 BIOSTIMULATION

14.13.3.1 Where the growth response in effluent (or surface water) exceeds growth in the control flasks, the percent stimulation, S(%), is calculated as shown below. Values which are significantly greater than the control indicate a possible degrading enrichment effect on the receiving water (Walsh et al., 1980):

$$S\ (\%)\ '\ \frac{T\&C}{C}\ x\ 100$$

Where: T = Mean effluent or surface water response

C = Mean control response

14.14 PRECISION AND ACCURACY

14.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 14.14.1.1 and 14.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

14.14.1.1 Single-Laboratory Precison

14.14.1.1.1 Data from repetitive 96-h toxicity tests conducted with cadmium chloride as the reference toxicant, using medium containing EDTA, are shown in Table 12. The precision (CV) of the 10 EC50s was 10.2%.

14.14.1.1.2 EPA evaluated within-laboratory precision of the green alga, *Selenastrum capricornutum*, Growth Test using a database of routine reference toxicant test results from nine laboratories (USEPA, 2000b). The database consisted of 85 reference toxicant tests conducted in 9 laboratories using a variety of reference toxicants including: copper, sodium chloride, and zinc. Among the 9 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 26% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 25%; and in 75% of laboratories, the within-laboratory CV was less than 39%.

14.14.1.2 Multilaboratory Precision

14.14.1.2.1 In 2000, EPA conducted an interlaboratory variability study of the green alga, *Selenastrum capricornutum*, Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 11 participant laboratories tested 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a

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municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Each sample was tested with and without the addition of EDTA. Of the 44 *Selenastrum capricornutum* Growth tests conducted with EDTA, 63.6% were successfully completed and met the required test acceptability criteria. Of the 44 tests conducted without EDTA, 65.9% were successfully completed and met the required test acceptability criteria. Of five tests that were conducted on blank samples with the addition of EDTA, none showed false positive results for the growth endpoint. Of 6 tests that were conducted on blank samples without the addition of EDTA, 2 showed false positive results for the growth endpoint, yielding a false positive rate of 33.3%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 13 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 34.3% and 58.5% for IC25 results in tests with EDTA and without EDTA, respectively. Table 14 shows the precision of growth NOEC endpoints for each sample type. NOEC values for tests with EDTA spanned three concentrations for the effluent sample type and four concentrations for the reference toxicant and receiving water sample types. NOEC values for tests without EDTA, spanned six concentrations for the reference toxicant sample type, four concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median for tests conducted with EDTA was 85.7%, 100%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively. The percentage of values within one concentration of the median for tests conducted without EDTA was 40.0%, 50.0%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

14.14.2 ACCURACY

14.14.2.1 The accuracy of toxicity tests cannot be determined.

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Conc. ID		1	2	3	4	5	6
Conc. Tested		0	5	10	20	40	80
Response	1	1209	1212	826	493	127	49.3
Response	2	1180	1186	628	416	147	40.0
Response	3	1340	1204	816	413	147	44.0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Cadmium
Test Start Date: Example Test Ending Date:
Test Species: Selenastrum capricornutum
Test Duration: 96 h
DATA FILE: scmanual.icp
OUTPUT FILE: scmanual.i25

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	1243.000	85.247	1243.000
2	3	5.000	1200.667	13.317	1200.667
3	3	10.000	756.667	111.541	756.667
4	3	20.000	440.667	45.347	440.667
5	3	40.000	140.333	11.547	140.333
6	3	80.000	44.433	4.665	44.433

The Linear Interpolation Estimate: 8.0227 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 8.1627 Standard Deviation: 0.4733
Original Confidence Limits: Lower: 7.2541 Upper: 8.9792
Expanded Confidence Limits: Lower: 6.4087 Upper: 10.0313
Resampling time in Seconds: 1.65 Random Seed: -1575623987

Figure 6. ICPIN program output for the IC25.

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Conc. ID	1	2	3	4	5	6
Conc. Tested	0	5	10	20	40	80
Response 1	1209	1212	826	493	127	49.3
Response 2	1180	1186	628	416	147	40.0
Response 3	1340	1204	816	413	147	44.0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Cadmium
Test Start Date: Example Test Ending Date:
Test Species: Selenastrum capricornutum
Test Duration: 96 h
DATA FILE: scmanual.icp
OUTPUT FILE: scmanual.i50

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	1243.000	85.247	1243.000
2	3	5.000	1200.667	13.317	1200.667
3	3	10.000	756.667	111.541	756.667
4	3	20.000	440.667	45.347	440.667
5	3	40.000	140.333	11.547	140.333
6	3	80.000	44.433	4.665	44.433

The Linear Interpolation Estimate: 14.2774 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 14.2057 Standard Deviation: 1.1926
Original Confidence Limits: Lower: 12.1194 Upper: 16.3078
Expanded Confidence Limits: Lower: 9.7456 Upper: 18.5413
Resampling time in Seconds: 1.65 Random Seed: -1751550803

Figure 7. ICPIN program output for the IC50.

TABLE 12. SINGLE LABORATORY PRECISION OF THE GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, 96-H TOXICITY TESTS, USING THE REFERENCE TOXICANT CADMIUM CHLORIDE (USEPA, 1991a)

Test Number	EC_{50} (mg/L)
1	2.3
2	2.4
3	2.3
4	2.8
5	2.6
6	2.1
7	2.1
8	2.1
9	2.6
10	2.4
n	10.0
Mean	2.37
CV (%)	10.2

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TABLE 13. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	10.9	20.8	23.5
(with EDTA)	Effluent	39.5	8.48	40.4
	Receiving water	-	-	38.9
	Average	25.2	14.6	34.3
IC25	Reference toxicant	25.6	83.6	87.5
(without EDTA)	Effluent	21.0	60.3	63.9
	Receiving water	-	-	24.1
	Average	23.3	72.0	58.5

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

3 The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

4 The between-laboratory component of variability for duplicate samples tested at different laboratories.

5 The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

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TABLE 14. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results $2[3]
Growth NOEC	Reference toxicant	25%	57.1	28.6	14.3
(with EDTA)	Effluent	6.25%	42.9	57.1	0.00
	Receiving water	12.5%	28.6	57.1	14.3
Growth NOEC	Reference toxicant	18.8%	-[4]	40.0	60.0
(without EDTA)	Effluent	18.8%	-[4]	50.0	50.0
	Receiving water	6.25%	75.0	25.0	0.00

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
2 Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
3 Percent of values two or more concentration intervals above or below the median.
4 The median NOEC fell between test concentrations, so no test results fell precisely on the median.

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APPENDIX A

INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

1. STATISTICAL INDEPENDENCE

1.1 Dunnett's Procedure and the t test with Bonferroni's adjustment are parametric procedures based on the assumptions that (1) the observations within treatments are independent and normally distributed, and (2) that the variance of the observations is homogeneous across all toxicant concentrations and the control. Of the three possible departures from the assumptions, non-normality, heterogeneity of variance, and lack of independence, those caused by lack of independence are the most difficult to resolve (see Scheffe, 1959). For toxicity data, statistical independence means that given knowledge of the true mean for a given concentration or control, knowledge of the error in any one actual observation would provide no information about the error in any other observation. Lack of independence is difficult to assess and difficult to test for statistically. It may also have serious effects on the true alpha or beta level. Therefore, it is of utmost importance to be aware of the need for statistical independence between observations and to be constantly vigilant in avoiding any patterned experimental procedure that might compromise independence. One of the best ways to help ensure independence is to follow proper randomization procedures throughout the test.

2. RANDOMIZATION

2.1 Randomization of the distribution of test organisms among test chambers and the arrangement of treatments and replicate chambers is an important part of conducting a valid test. The purpose of randomization is to avoid situations where test organisms are placed serially into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.

2.2 An example of randomization of the distribution of test organisms among test chambers, and an example of randomization of arrangement of treatments and replicate chambers are described using the Fathead Minnow Larval Survival and Growth test. For the purpose of the example, the test design is as follows: five effluent concentrations are tested in addition to the control. The effluent concentrations are as follows: 6.25%, 12.5%, 25.0%, 50.0%, and 100.0%. There are four replicate chambers per treatment. Each replicate chamber contains ten fish.

2.3 RANDOMIZATION OF FISH TO REPLICATE CHAMBERS EXAMPLE

2.3.1 Consider first the random assignment of the fish to the replicate chambers. The first step is to label each of the replicate chambers with the control or effluent concentration and the replicate number. The next step is to assign each replicate chamber four double-digit numbers. An example of this assignment is provided in Table A.1. Note that the double digits 00 and 97 through 99 were not used.

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TABLE A.1. RANDOM ASSIGNMENT OF FISH TO REPLICATE CHAMBERS EXAMPLE ASSIGNED NUMBERS FOR EACH REPLICATE CHAMBER

Assigned Numbers	Replicate Chamber	
01, 25, 49, 73	Control,	replicate chamber 1
02, 26, 50, 74	Control,	replicate chamber 2
03, 27, 51, 75	Control,	replicate chamber 3
04, 28, 52, 76	Control,	replicate chamber 4
05, 29, 53, 77	6.25% effluent,	replicate chamber 1
06, 30, 54, 78	6.25% effluent,	replicate chamber 2
07, 31, 55, 79	6.25% effluent,	replicate chamber 3
08, 32, 56, 80	6.25% effluent,	replicate chamber 4
09, 33, 57, 81	12.5% effluent,	replicate chamber 1
10, 34, 58, 82	12.5% effluent,	replicate chamber 2
11, 35, 59, 83	12.5% effluent,	replicate chamber 3
12, 36, 60, 84	12.5% effluent,	replicate chamber 4
13, 37, 61, 85	25.0% effluent,	replicate chamber 1
14, 38, 62, 86	25.0% effluent,	replicate chamber 2
15, 39, 63, 87	25.0% effluent,	replicate chamber 3
16, 40, 64, 88	25.0% effluent,	replicate chamber 4
17, 41, 65, 89	50.0% effluent,	replicate chamber 1
18, 42, 66, 90	50.0% effluent,	replicate chamber 2
19, 43, 67, 91	50.0% effluent,	replicate chamber 3
20, 44, 68, 92	50.0% effluent,	replicate chamber 4
21, 45, 69, 93	100.0% effluent,	replicate chamber 1
22, 46, 70, 94	100.0% effluent,	replicate chamber 2
23, 47, 71, 95	100.0% effluent,	replicate chamber 3
24, 48, 72, 96	100.0% effluent,	replicate chamber 4

2.3.2 The random numbers used to carry out the random assignment of fish to replicate chambers are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double digit number. The first number read identifies the replicate chamber for the first fish taken from the tank. For the example, the first entry in row 2 was chosen as the starting position. The first number in this row is 37. According to Table A.1, this number corresponds to replicate chamber 1 of the 25.0% effluent concentration. Thus, the first fish taken from the tank is to be placed in replicate chamber 1 of the 25.0% effluent concentration.

2.3.3 The next step is to read the double digit number to the right of the first one. The second number identifies the replicate chamber for the second fish taken from the tank. Continuing the example, the second number read in row 2 of Table A.2 is 54. According to Table A.1, this number corresponds to replicate chamber 2 of the 6.25% effluent concentration. Thus, the second fish taken from the tank is to be placed in replicate chamber 2 of the 6.25% effluent concentration.

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TABLE A.2. TABLE OF RANDOM NUMBERS (Dixon and Massey, 1983)

10	09	73	25	33	76	52	01	35	86	34	67	35	43	76	80	95	90	91	17	39	29	27	49	45
37	54	20	48	05	64	89	47	42	96	24	80	52	40	37	20	63	61	04	02	00	82	29	16	65
08	42	26	89	53	19	64	50	93	03	23	20	90	25	60	15	95	33	47	64	35	08	03	36	06
99	01	90	25	29	09	37	67	07	15	38	31	13	11	65	88	67	67	43	97	04	43	62	76	59
12	80	79	99	70	80	15	73	61	47	64	03	23	66	53	98	95	11	68	77	12	27	17	68	33
66	06	57	47	17	34	07	27	68	50	36	69	73	61	70	65	81	33	98	85	11	19	92	91	70
31	06	01	08	05	45	57	18	24	06	35	30	34	26	14	86	79	90	74	39	23	40	30	97	32
85	26	97	76	02	02	05	16	56	92	68	66	57	48	18	73	05	38	52	47	18	62	38	85	79
63	57	33	21	35	05	32	54	70	48	90	55	35	75	48	28	46	82	87	09	83	49	12	56	24
73	79	64	57	53	03	52	96	47	78	35	80	83	42	82	60	93	52	03	44	35	27	38	84	35
98	52	01	77	67	14	90	56	86	07	22	10	94	05	58	60	97	09	34	33	50	50	07	39	98
11	80	50	54	31	39	80	82	77	32	50	72	56	82	48	29	40	52	42	01	52	77	56	78	51
83	45	29	96	34	06	28	89	80	83	13	74	67	00	78	18	47	54	06	10	68	71	17	78	17
88	68	54	02	00	86	50	75	84	01	36	76	66	79	51	90	36	47	64	93	29	60	91	10	62
99	59	46	73	48	87	51	76	49	69	91	82	60	89	28	93	78	56	13	68	23	47	83	41	13
65	48	11	76	74	17	46	85	09	50	58	04	77	69	74	73	03	95	71	86	40	21	81	65	44
80	12	43	56	35	17	72	70	80	15	45	31	82	23	74	21	11	57	82	53	14	38	55	37	63
74	35	09	98	17	77	40	27	72	14	43	23	60	02	10	45	52	16	42	37	96	28	60	26	55
69	91	62	68	03	66	25	22	91	48	36	93	68	72	03	76	62	11	39	90	94	40	05	64	18
09	89	32	05	05	14	22	56	85	14	46	42	75	67	88	96	29	77	88	22	54	38	21	45	98
91	49	91	45	23	68	47	92	76	86	46	16	28	35	54	94	75	08	99	23	37	08	92	00	48
80	33	69	45	98	26	94	03	68	58	70	29	73	41	35	53	14	03	33	40	42	05	08	23	41
44	10	48	19	49	85	15	74	79	54	32	97	92	65	75	57	60	04	08	81	22	22	20	64	13
12	55	07	37	42	11	10	00	20	40	12	86	07	46	97	96	64	48	94	39	28	70	72	58	15
63	60	64	93	29	16	50	53	44	84	40	21	95	25	63	43	65	17	70	82	07	20	73	17	90
61	19	69	04	46	26	45	74	77	74	51	92	43	37	29	65	39	45	95	93	42	58	26	05	27
15	47	44	52	66	95	27	07	99	53	59	36	78	38	48	82	39	61	01	18	33	21	15	94	66
94	55	72	85	73	67	89	75	43	87	54	62	24	44	31	91	19	04	25	92	92	92	74	59	73
42	48	11	62	13	97	34	40	87	21	16	86	84	87	67	03	07	11	20	59	25	70	14	66	70
23	52	37	83	17	73	20	88	98	37	68	93	59	14	16	26	25	22	96	63	05	52	28	25	62
04	49	35	24	94	75	24	63	38	24	45	86	25	10	25	61	96	27	93	35	65	33	71	24	72
00	54	99	76	54	64	05	18	81	59	96	11	96	38	96	54	69	28	23	91	23	28	72	95	29
35	96	31	53	07	26	89	80	93	45	33	35	13	54	62	77	97	45	00	24	90	10	33	93	33
59	80	80	83	91	45	42	72	68	42	83	60	94	97	00	13	02	12	48	92	78	56	52	01	06
46	05	88	52	36	01	39	09	22	86	77	28	14	40	77	93	91	08	36	47	70	61	74	29	41
32	17	90	05	97	87	37	92	52	41	05	56	70	70	07	86	74	31	71	57	85	39	41	18	38
69	23	46	14	06	20	11	74	52	04	15	95	66	00	00	18	74	39	24	23	97	11	89	63	38
19	56	54	14	30	01	75	87	53	79	40	41	92	15	85	66	67	43	68	06	84	96	28	52	07
45	15	51	49	38	19	47	60	72	46	43	66	79	45	43	59	04	79	00	33	20	82	66	95	41
94	86	43	19	94	36	16	81	08	51	34	88	88	15	53	01	54	03	54	56	05	01	45	11	76
98	08	62	48	26	45	24	02	84	04	44	99	90	88	96	39	09	47	34	07	35	44	13	18	80
33	18	51	62	32	41	94	15	09	49	89	43	54	85	81	88	69	54	19	94	37	54	87	30	43
80	95	10	04	06	96	38	27	07	74	20	15	12	33	87	25	01	62	52	98	94	62	46	11	71
79	75	24	91	40	71	96	12	82	96	69	86	10	25	91	74	85	22	05	39	00	38	75	95	79
18	63	33	25	37	98	14	50	65	71	31	01	02	46	74	05	45	56	14	27	77	93	89	19	36
74	02	94	39	02	77	55	73	22	70	97	79	01	71	19	52	52	75	80	21	80	81	45	17	48
54	17	84	56	11	80	99	33	71	43	05	33	51	29	69	56	12	71	92	55	36	04	09	03	24
11	66	44	98	83	52	07	98	48	27	59	38	17	15	39	09	97	33	34	40	88	46	12	33	56
48	32	47	79	28	31	24	96	47	10	02	29	53	68	70	32	30	75	75	46	15	02	00	99	94
69	07	49	41	38	87	63	79	19	76	35	58	40	44	01	10	51	82	16	15	01	84	87	69	38

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2.3.4 Continue in this fashion until all the fish have been randomly assigned to a replicate chamber. In order to fill each replicate chamber with ten fish, the assigned numbers will be used more than once. If a number is read from the table that was not assigned to a replicate chamber, then ignore it and continue to the next number. If a replicate chamber becomes filled and a number is read from the table that corresponds to it, then ignore that value and continue to the next number. The first ten random assignments of fish to replicate chambers for the example are summarized in Table A.3.

TABLE A.3. EXAMPLE OF RANDOM ASSIGNMENT OF FIRST TEN FISH TO REPLICATE CHAMBERS

Fish		Assignment	
First	fish taken from tank	25.0% effluent,	replicate chamber 1
Second	fish taken from tank	6.25% effluent,	replicate chamber 2
Third	fish taken from tank	50.0% effluent,	replicate chamber 4
Fourth	fish taken from tank	100.0% effluent,	replicate chamber 4
Fifth	fish taken from tank	6.25% effluent,	replicate chamber 1
Sixth	fish taken from tank	25.0% effluent,	replicate chamber 4
Seventh	fish taken from tank	50.0% effluent,	replicate chamber 1
Eighth	fish taken from tank	100.0% effluent,	replicate chamber 3
Ninth	fish taken from tank	50.0% effluent,	replicate chamber 2
Tenth	fish taken from tank	100.0% effluent,	replicate chamber 4

2.3.5 Four double-digit numbers were assigned to each replicate chamber (instead of one, two, or three double-digit numbers) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each replicate chamber: the first column of assigned numbers in Table A.1. Whenever the numbers 00 and 25 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

2.4 RANDOMIZATION OF REPLICATE CHAMBERS TO POSITIONS EXAMPLE

2.4.1 Next consider the random assignment of the 24 replicate chambers to positions within the water bath (or equivalent). Assume that the replicate chambers are to be positioned in a four row by six column rectangular array. The first step is to label the positions in the water bath. Table A.4 provides an example layout.

TABLE A.4 RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE LABELING THE POSITIONS WITHIN THE WATER BATH

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24

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2.4.2 The second step is to assign each of the 24 positions four double-digit numbers. An example of this assignment is provided in Table A.5. Note that the double digits 00 and 97 through 99 were not used.

TABLE A.5. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNED NUMBERS FOR EACH POSITION

Assigned Numbers	Position
01, 25, 49, 73	1
02, 26, 50, 74	2
03, 27, 51, 75	3
04, 28, 52, 76	4
05, 29, 53, 77	5
06, 30, 54, 78	6
07, 31, 55, 79	7
08, 32, 56, 80	8
09, 33, 57, 81	9
10, 34, 58, 82	10
11, 35, 59, 83	11
12, 36, 60, 84	12
13, 37, 61, 85	13
14, 38, 62, 86	14
15, 39, 63, 87	15
16, 40, 64, 88	16
17, 41, 65, 89	17
18, 42, 66, 90	18
19, 43, 67, 91	19
20, 44, 68, 92	20
21, 45, 69, 93	21
22, 46, 70, 94	22
23, 47, 71, 95	23
24, 48, 72, 96	24

2.4.3 The random numbers used to carry out the random assignment of replicate chambers to positions are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double-digit number. The first number read identifies the position for the first replicate chamber of the control. For the example, the first entry in row 10 of Table A.2 was chosen as the starting position. The first number in this row was 73. According to Table A.5, this number corresponds to position 1. Thus, the first replicate chamber for the control will be placed in position 1.

2.4.4 The next step is to read the double-digit number to the right of the first one. The second number identifies the position for the second replicate chamber of the control. Continuing the example, the second number read in row 10 of Table A.2 is 79. According to Table A.5, this number corresponds to position 7. Thus, the second replicate chamber for the control will be placed in position 7.

2.4.5 Continue in this fashion until all the replicate chambers have been assigned to a position. The first four numbers read will identify the positions for the control replicate chambers, the second four numbers read will identify the positions for the lowest effluent concentration replicate chambers, and so on. If a number is read from the table that was not assigned to a position, then ignore that value and continue to the next number. If a number is repeated in Table A.2, then ignore the repeats and continue to the next number. The complete randomization of

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replicate chambers to positions for the example is displayed in Table A.6.

TABLE A.6. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNMENT OF ALL 24 POSITIONS

Control	100.0%	6.25%	6.25%	6.25%	12.5%
Control	12.5%	Control	25.0%	12.5%	25.0%
100.0%	50.0%	100.0%	Control	100.0%	25.0%
50.0%	50.0%	25.0%	50.0%	12.5%	6.25%

2.4.6 Four double-digit numbers were assigned to each position (instead of one, two, or three) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each position: the first column of assigned numbers in Table A.5. Whenever the numbers 00 and 25 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

3. **OUTLIERS**

3.1 An outlier is an inconsistent or questionable data point that appears unrepresentative of the general trend exhibited by the majority of the data. Outliers may be detected by tabulation of the data, plotting, and by an analysis of the residuals. An explanation should be sought for any questionable data points. Without an explanation, data points should be discarded only with extreme caution. If there is no explanation, the analysis should be performed both with and without the outlier, and the results of both analyses should be reported.

3.2 Gentleman-Wilk's A statistic gives a test for the condition that the extreme observation may be considered an outlier. For a discussion of this, and other techniques for evaluating outliers, see Draper and John (1981).

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APPENDIX B

VALIDATING NORMALITY AND HOMOGENEITY OF VARIANCE ASSUMPTIONS

1. INTRODUCTION

1.1 Dunnett's Procedure and the t test with Bonferroni's adjustment are parametric procedures based on the assumptions that the observations within treatments are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using these tests, to determine if they have been met. Tests for validating the assumptions are provided in the following discussion. If the tests fail (if the data do not meet the assumptions), a nonparametric procedure such as Steel's Many-one Rank Test may be more appropriate. However, the decision on whether to use parametric or nonparametric tests may be a judgment call, and a statistician should be consulted in selecting the analysis.

2. TEST FOR NORMAL DISTRIBUTION OF DATA

2.1 SHAPIRO-WILK'S TEST

2.1.1 One formal test for normality is the Shapiro-Wilk's Test (Conover, 1980). The test statistic is obtained by dividing the square of an appropriate linear combination of the sample order statistics by the usual symmetric estimate of variance. The calculated W must be greater than zero and less than or equal to one. This test is recommended for a sample size of 50 or less. If the sample size is greater than 50, the Kolmogorov "D" statistic (Stephens, 1974) is recommended. An example of the Shapiro-Wilk's test is provided below.

2.2 The example uses growth data from the Fathead Minnow Larval Survival and Growth Test. The same data are used in the discussion of the homogeneity of variance determination in Paragraph 3 and Dunnett's Procedure in Appendix C. The data, the mean and variance of the observations at each concentration, including the control, are listed in Table B.1.

TABLE B.1. FATHEAD LARVAL, *PIMEPHALES PROMELAS*, LARVAL GROWTH DATA (WEIGHT IN MG) FOR THE SHAPIRO-WILK'S TEST

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.711	0.646	0.669	0.629	0.650
B	0.662	0.626	0.669	0.680	0.558
C	0.718	0.723	0.694	0.513	0.606
D	0.767	0.700	0.676	0.672	0.508
Mean($\bar{Y}_i$)	0.714	0.674	0.677	0.624	0.580
S_i^2	0.0018	0.0020	0.0001	0.0059	0.0037
i	1	2	3	4	5

2.3 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are listed in Table B.2.

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TABLE B.2. EXAMPLE OF SHAPIRO-WILK'S TEST: CENTERED OBSERVATIONS

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	-0.003	-0.028	-0.008	0.005	0.070
B	-0.052	-0.048	-0.008	0.056	-0.022
C	0.004	0.049	0.017	-0.111	0.026
D	0.053	0.026	-0.001	0.048	-0.072

2.4 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the centered observations and $\bar{X}$ is the overall mean of the centered observations. For this set of data, $\bar{X}$ = 0, and D = 0.0412.

2.5 Order the centered observations from smallest to largest.

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations are listed in Table B.3.

TABLE B.3. EXAMPLE OF THE SHAPIRO-WILK'S TEST: ORDERED OBSERVATIONS

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.111	11	0.004
2	-0.072	12	0.005
3	-0.052	13	0.017
4	-0.048	14	0.026
5	-0.028	15	0.026
6	-0.022	16	0.048
7	-0.008	17	0.049
8	-0.008	18	0.053
9	-0.003	19	0.056
10	-0.001	20	0.070

2.6 From Table B.4, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$, where k is n/2 if n is even, and (n-1)/2 if n is odd. For the data in this example, n = 20, k = 10. The a_i values are listed in Table B.5.

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TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (Conover, 1980)

	Number of Observations								
$i \pm^n$	2	3	4	5	6	7	8	9	10
1	0.7071	0.7071	0.6872	0.6646	0.6431	0.6233	0.6052	0.5888	0.5739
2	-	0.0000	0.1667	0.2413	0.2806	0.3031	0.3164	0.3244	0.3291
3	-	-	-	0.0000	0.0875	0.1401	0.1743	0.1976	0.2141
4	-	-	-	-	-	0.0000	0.0561	0.0947	0.1224
5	-	-	-	-	-	-	-	0.0000	0.0399

	Number of Observations									
$i \pm^n$	11	12	13	14	15	16	17	18	19	20
1	0.5601	0.5475	0.5359	0.5251	0.5150	0.5056	0.4968	0.4886	0.4808	0.4734
2	0.3315	0.3325	0.3325	0.3318	0.3306	0.3209	0.3273	0.3253	0.3232	0.3211
3	0.2260	0.2347	0.2412	0.2460	0.2495	0.2521	0.2540	0.2553	0.2561	0.2565
4	0.1429	0.1586	0.1707	0.1802	0.1878	0.1939	0.1988	0.2027	0.2059	0.2085
5	0.0695	0.0922	0.1099	0.1240	0.1353	0.1447	0.1524	0.1587	0.1641	0.1686
6	0.0000	0.0303	0.0539	0.0727	0.0880	0.1005	0.1109	0.1197	0.1271	0.1334
7	-	-	0.0000	0.0240	0.0433	0.0593	0.0725	0.0837	0.0932	0.1013
8	-	-	-	-	0.0000	0.0196	0.0359	0.0496	0.0612	0.0711
9	-	-	-	-	-	-	0.0000	0.0163	0.0303	0.0422
10	-	-	-	-	-	-	-	-	0.0000	0.0140

	Number of Observations									
$i \pm^n$	21	22	23	24	25	26	27	28	29	30
1	0.4643	0.4590	0.4542	0.4493	0.4450	0.4407	0.4366	0.4328	0.4291	0.4254
2	0.3185	0.3156	0.3126	0.3098	0.3069	0.3043	0.3018	0.2992	0.2968	0.2944
3	0.2578	0.2571	0.2563	0.2554	0.2543	0.2533	0.2522	0.2510	0.2499	0.2487
4	0.2119	0.2131	0.2139	0.2145	0.2148	0.2151	0.2152	0.2151	0.2150	0.2148
5	0.1736	0.1764	0.1787	0.1807	0.1822	0.1836	0.1848	0.1857	0.1864	0.1870
6	0.1399	0.1443	0.1480	0.1512	0.1539	0.1563	0.1584	0.1601	0.1616	0.1630
7	0.1092	0.1150	0.1201	0.1245	0.1283	0.1316	0.1346	0.1372	0.1395	0.1415
8	0.0804	0.0878	0.0941	0.0997	0.1046	0.1089	0.1128	0.1162	0.1192	0.1219
9	0.0530	0.0618	0.0696	0.0764	0.0923	0.0876	0.0923	0.0965	0.1002	0.1036
10	0.0263	0.0368	0.0459	0.0539	0.0610	0.0672	0.0728	0.0778	0.0822	0.0862
11	0.0000	0.0122	0.0228	0.0321	0.0403	0.0476	0.0540	0.0598	0.0650	0.0697
12	-	-	0.0000	0.0107	0.0200	0.0284	0.0358	0.0424	0.0483	0.0537
13	-	-	-	-	0.0000	0.0094	0.0178	0.0253	0.0320	0.0381
14	-	-	-	-	-	-	0.0000	0.0084	0.0159	0.0227
15	-	-	-	-	-	-	-	-	0.0000	0.0076

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TABLE B.4. COEFFICIENTS FOR THE SHAPIRO WILK'S TEST (CONTINUED)

	Number of Observations									
i±[n]	31	32	33	34	35	36	37	38	39	40
1	0.4220	0.4188	0.4156	0.4127	0.4096	0.4068	0.4040	0.4015	0.3989	0.3964
2	0.2921	0.2898	0.2876	0.2854	0.2834	0.2813	0.2794	0.2774	0.2755	0.2737
3	0.2475	0.2462	0.2451	0.2439	0.2427	0.2415	0.2403	0.2391	0.2380	0.2368
4	0.2145	0.2141	0.2137	0.2132	0.2127	0.2121	0.2116	0.2110	0.2104	0.2098
5	0.1874	0.1878	0.1880	0.1882	0.1883	0.1883	0.1883	0.1881	0.1880	0.1878
6	0.1641	0.1651	0.1660	0.1667	0.1673	0.1678	0.1663	0.1686	0.1689	0.1691
7	0.1433	0.1449	0.1463	0.1475	0.1487	0.1496	0.1505	0.1513	0.1520	0.1526
8	0.1243	0.1265	0.1284	0.1301	0.1317	0.1331	0.1344	0.1356	0.1366	0.1376
9	0.1066	0.1093	0.1118	0.1140	0.1160	0.1179	0.1196	0.1211	0.1225	0.1237
10	0.0899	0.0931	0.0961	0.0988	0.1013	0.1036	0.1056	0.1075	0.1092	0.1108
11	0.0739	0.0777	0.0812	0.0844	0.0873	0.0900	0.0924	0.0947	0.0967	0.0986
12	0.0585	0.0629	0.0669	0.0706	0.0739	0.0770	0.0798	0.0824	0.0848	0.0870
13	0.0435	0.0485	0.0530	0.0572	0.0610	0.0645	0.0677	0.0706	0.0733	0.0759
14	0.0289	0.0344	0.0395	0.0441	0.0484	0.0523	0.0559	0.0592	0.0622	0.0651
15	0.0144	0.0206	0.0262	0.0314	0.0361	0.0404	0.0444	0.0481	0.0515	0.0546
16	0.0000	0.0068	0.0131	0.0187	0.0239	0.0287	0.0331	0.0372	0.0409	0.0444
17	-	-	0.0000	0.0062	0.0119	0.0172	0.0220	0.0264	0.0305	0.0343
18	-	-	-	-	0.0000	0.0057	0.0110	0.0158	0.0203	0.0244
19	-	-	-	-	-	-	0.0000	0.0053	0.0101	0.0146
20	-	-	-	-	-	-	-	-	0.0000	0.0049

	Number of Observations									
i±[n]	41	42	43	44	45	46	47	48	49	50
1	0.3940	0.3917	0.3894	0.3872	0.3850	0.3830	0.3808	0.3789	0.3770	0.3751
2	0.2719	0.2701	0.2684	0.2667	0.2651	0.2635	0.2620	0.2604	0.2589	0.2574
3	0.2357	0.2345	0.2334	0.2323	0.2313	0.2302	0.2291	0.2281	0.2271	0.2260
4	0.2091	0.2085	0.2078	0.2072	0.2065	0.2058	0.2052	0.2045	0.2038	0.2032
5	0.1876	0.1874	0.1871	0.1868	0.1865	0.1862	0.1859	0.1855	0.1851	0.1847
6	0.1693	0.1694	0.1695	0.1695	0.1695	0.1695	0.1695	0.1693	0.1692	0.1691
7	0.1531	0.1535	0.1539	0.1542	0.1545	0.1548	0.1550	0.1551	0.1553	0.1554
8	0.1384	0.1392	0.1398	0.1405	0.1410	0.1415	0.1420	0.1423	0.1427	0.1430
9	0.1249	0.1259	0.1269	9,1278	0.1286	0.1293	0.1300	0.1306	0.1312	0.1317
10	0.1123	0.1136	0.1149	0.1160	9.1170	0.1180	0.1189	0.1197	0.1205	0.1212
11	0.1004	0.1020	0.1035	0.1049	0.1062	0.1073	0.1085	0.1095	0.1105	0.1113
12	0.0891	0.0909	0.0927	0.0943	0.0959	0.0972	0.0986	0.0998	0.1010	0.1020
13	0.0782	0.0804	0.0824	0.0842	0.0860	0.0876	0.0892	0.0906	0.0919	0.0932
14	0.0677	0.0701	0.0724	0.0745	0.0765	0.0783	0.0801	0.0817	0.0832	0.0846
15	0.0575	0.0602	0.0628	0.0651	0.0673	0.0694	0.0713	0.0731	0.0748	0.0764
16	0.0476	0.0506	0.0534	0.0560	0.0584	0.0607	0.0628	0.0648	0.0667	0.0685
17	0.0379	0.0411	0.0442	0.0471	0.0497	0.0522	0.0546	0.0568	0.0588	0.0608
18	0.0283	0.0318	0.0352	0.0383	0.0412	0.0439	0.0465	0.0489	0.0511	0.0532
19	0.0188	0.0227	0.0263	0.0296	0.0328	0.0357	0.0385	0.0411	0.0436	0.0459
20	0.0094	0.0136	0.0175	0.0211	0.0245	0.0277	0.0307	0.0335	0.0361	0.0386
21	0.0000	0.0045	0.0087	0.0126	0.0163	0.0197	0.0229	0.0259	0.0288	0.0314
22	-	-	0.0000	0.0042	0.0081	0.0118	0.0153	0.0185	0.0215	0.0244
23	-	-	-	-	0.0000	0.0039	0.0076	0.0111	0.0143	0.0174
24	-	-	-	-	-	-	0.0000	0.0037	0.0071	0.0104
25	-	-	-	-	-	-	-	-	0.0000	0.0035

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2.7 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i \left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table B.5.

2.8 The decision rule for this test is to compare the critical value from Table B.6 to the computed W. If the computed value is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 20 observations (n) is 0.868. The calculated value, 0.959, is not less than the critical value. Therefore, conclude that the data are normally distributed.

TABLE B.5. EXAMPLE OF THE SHAPIRO-WILK'S TEST: TABLE OF COEFFICIENTS AND DIFFERENCES

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.181	$X^{(20)} - X^{(1)}$
2	0.3211	0.128	$X^{(19)} - X^{(2)}$
3	0.2565	0.105	$X^{(18)} - X^{(3)}$
4	0.2085	0.097	$X^{(17)} - X^{(4)}$
5	0.1686	0.076	$X^{(16)} - X^{(5)}$
6	0.1334	0.048	$X^{(15)} - X^{(6)}$
7	0.1013	0.034	$X^{(14)} - X^{(7)}$
8	0.0711	0.025	$X^{(13)} - X^{(8)}$
9	0.0422	0.008	$X^{(12)} - X^{(9)}$
10	0.0140	0.005	$X^{(11)} - X^{(10)}$

2.9 In general, if the data fail the test for normality, a transformation such as to log values may normalize the data. After transforming the data, repeat the Shapiro-Wilk's Test for normality.

2.10 KOLMOGOROV "D" TEST

2.10.1 A formal two-sided test for normality is the Kolmogorov "D" Test. The test statistic is calculated by obtaining the difference between the cumulative distribution function estimated from the data and the standard normal cumulative distribution function for each standardized observation. This test is recommended for a sample size greater than 50. If the sample size is less than or equal to 50, then the Shapiro Wilk's Test is recommended. An example of the Kolmogorov "D" test is provided below.

2.10.2 The example uses reproduction data from the daphnid, *Ceriodaphnia dubia,* Survival and Reproduction Test. The observed data and the mean of the observations at each concentration, including the control, are listed in Table B.7.

2.10.3 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations for the example are listed in Table B.8.

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TABLE B.6. QUANTILES OF THE SHAPIRO-WILK'S TEST STATISTIC (Conover, 1980)

n	0.01	0.02	0.05	0.10	0.50	0.90	0.95	0.98	0.99
3	0.753	0.756	0.767	0.789	0.959	0.998	0.999	1.000	1.000
4	0.687	0.707	0.748	0.792	0.935	0.987	0.992	0.996	0.997
5	0.686	0.715	0.762	0.806	0.927	0.979	0.986	0.991	0.993
6	0.713	0.743	0.788	0.826	0.927	0.974	0.981	0.986	0.989
7	0.730	0.760	0.803	0.838	0.928	0.972	0.979	0.985	0.988
8	0.749	0.778	0.818	0.851	0.932	0.972	0.978	0.984	0.987
9	0.764	0.791	0.829	0.859	0.935	0.972	0.978	0.984	0.986
10	0.781	0.806	0.842	0.869	0.938	0.972	0.978	0.983	0.986
11	0.792	0.817	0.850	0.876	0.940	0.973	0.979	0.984	0.986
12	0.805	0.828	0.859	0.883	0.943	0.973	0.979	0.984	0.986
13	0.814	0.837	0.866	0.889	0.945	0.974	0.979	0.984	0.986
14	0.825	0.846	0.874	0.895	0.947	0.975	0.980	0.984	0.986
15	0.835	0.855	0.881	0.901	0.950	0.975	0.980	0.984	0.987
16	0.844	0.863	0.887	0.906	0.952	0.976	0.981	0.985	0.987
17	0.851	0.869	0.892	0.910	0.954	0.977	0.981	0.985	0.987
18	0.858	0.874	0.897	0.914	0.956	0.978	0.982	0.986	0.988
19	0.863	0.879	0.901	0.917	0.957	0.978	0.982	0.986	0.988
20	0.868	0.884	0.905	0.920	0.959	0.979	0.983	0.986	0.988
21	0.873	0.888	0.908	0.923	0.960	0.980	0.983	0.987	0.989
22	0.878	0.892	0.911	0.926	0.961	0.980	0.984	0.987	0.989
23	0.881	0.895	0.914	0.928	0.962	0.981	0.984	0.987	0.989
24	0.884	0.898	0.916	0.930	0.963	0.981	0.984	0.987	0.989
25	0.888	0.901	0.918	0.931	0.964	0.981	0.985	0.988	0.989
26	0.891	0.904	0.920	0.933	0.965	0.982	0.985	0.988	0.989
27	0.894	0.906	0.923	0.935	0.965	0.982	0.985	0.988	0.990
28	0.896	0.908	0.924	0.936	0.966	0.982	0.985	0.988	0.990
29	0.898	0.910	0.926	0.937	0.966	0.982	0.985	0.988	0.990
30	0.900	0.912	0.927	0.939	0.967	0.983	0.985	0.988	0.990
31	0.902	0.914	0.929	0.940	0.967	0.983	0.986	0.988	0.990
32	0.904	0.915	0.930	0.941	0.968	0.983	0.986	0.988	0.990
33	0.906	0.917	0.931	0.942	0.968	0.983	0.986	0.989	0.990
34	0.908	0.919	0.933	0.943	0.969	0.983	0.986	0.989	0.990
35	0.910	0.920	0.934	0.944	0.969	0.984	0.986	0.989	0.990
36	0.912	0.922	0.935	0.945	0.970	0.984	0.986	0.989	0.990
37	0.914	0.924	0.936	0.946	0.970	0.984	0.987	0.989	0.990
38	0.916	0.925	0.938	0.947	0.971	0.984	0.987	0.989	0.990
39	0.917	0.927	0.939	0.948	0.971	0.984	0.987	0.989	0.991
40	0.919	0.928	0.940	0.949	0.972	0.985	0.987	0.989	0.991
41	0.920	0.929	0.941	0.950	0.972	0.985	0.987	0.989	0.991
42	0.922	0.930	0.942	0.951	0.972	0.985	0.987	0.989	0.991
43	0.923	0.932	0.943	0.951	0.973	0.985	0.987	0.990	0.991
44	0.924	0.933	0.944	0.952	0.973	0.985	0.987	0.990	0.991
45	0.926	0.934	0.945	0.953	0.973	0.985	0.988	0.990	0.991
46	0.927	0.935	0.945	0.953	0.974	0.985	0.988	0.990	0.991
47	0.928	0.936	0.946	0.954	0.974	0.985	0.988	0.990	0.991
48	0.929	0.937	0.947	0.954	0.974	0.985	0.988	0.990	0.991
49	0.929	0.937	0.947	0.955	0.974	0.985	0.988	0.990	0.991
50	0.930	0.938	0.947	0.955	0.974	0.985	0.988	0.990	0.991

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TABLE B.7. *CERIODAPHNIA DUBIA* REPRODUCTION DATA FOR THE KOLMOGOROV "D" TEST

	Effluent Concentration (%)					
Replicate	Control	1.56	3.12	6.25	12.5	25.0
1	27	32	39	27	19	10
2	30	35	30	34	25	13
3	29	32	33	36	26	7
4	31	26	33	34	17	7
5	16	18	36	31	16	7
6	15	29	33	27	21	10
7	18	27	33	33	23	10
8	17	16	27	31	15	16
9	14	35	38	33	18	12
10	27	13	44	31	10	2
Mean	22.4	26.3	34.6	31.7	19.0	9.4

TABLE B.8. CENTERED OBSERVATIONS FOR KOLMOGOROV “D” EXAMPLE

	Effluent Concentration (%)					
Replicate	Control	1.56	3.12	6.25	12.5	25.0
1	4.6	5.7	4.4	-4.7	0.0	0.6
2	7.6	8.7	-4.6	2.3	6.0	3.6
3	6.6	5.7	-1.6	4.3	7.0	-2.4
4	8.6	-0.3	-1.6	2.3	-2.0	-2.4
5	-6.4	-8.3	1.4	-0.7	-3.0	-2.4
6	-7.4	2.7	-1.6	-4.7	2.0	0.6
7	-4.4	0.7	-1.6	1.3	4.0	0.6
8	-5.4	-10.3	-7.6	-0.7	-4.0	6.6
9	-8.4	8.7	3.4	1.3	-1.0	2.6
10	4.6	-13.3	9.4	-0.7	-9.0	-7.4

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2.10.4 Order the centered observations from smallest to largest:

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation, and n denotes the total number of centered observations. The ordered observations for the example are listed in Table B.9.

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TABLE B.9. EXAMPLE CALCULATION OF THE KOLMOGOROV "D" STATISTIC

i	$X^{(i)}$	z_i	p_i	D_i+	D_i-
1	-13.3	-2.51	0.0060	0.0107	0.0060
2	-10.3	-1.94	0.0262	0.0071	0.0095
3	-9.0	-1.70	0.0446	0.0054	0.0113
4	-8.4	-1.58	0.0571	0.0096	0.0071
5	-8.3	-1.57	0.0582	0.0251	-0.0085
6	-7.6	-1.43	0.0764	0.0236	-0.0069
7	-7.4	-1.40	0.0808	0.0359	-0.0192
8	-7.4	-1.40	0.0808	0.0525	-0.0359
9	-6.4	-1.21	0.1131	0.0369	-0.0202
10	-5.4	-1.02	0.1539	0.0128	0.0039
11	-4.7	-0.89	0.1867	-0.0034	0.0200
12	-4.7	-0.89	0.1867	0.0133	0.0034
13	-4.6	-0.87	0.1922	0.0245	-0.0078
14	-4.4	-0.83	0.2033	0.0300	-0.0134
15	-4.0	-0.75	0.2266	0.0234	-0.0067
16	-3.0	-0.57	0.2843	-0.0176	0.0343
17	-2.4	-0.45	0.3264	-0.0431	0.0597
18	-2.4	-0.45	0.3264	-0.0264	0.0431
19	-2.4	-0.45	0.3264	-0.0097	0.0264
20	-2.0	-0.38	0.3520	-0.0187	0.0353
21	-1.6	-0.30	0.3821	-0.0321	0.0488
22	-1.6	-0.30	0.3821	-0.0154	0.0321
23	-1.6	-0.30	0.3821	0.0012	0.0154
24	-1.6	-0.30	0.3821	0.0179	-0.0012
25	-1.0	-0.19	0.4247	-0.0080	0.0247
26	-0.7	-0.13	0.4483	-0.0150	0.0316
27	-0.7	-0.13	0.4483	0.0017	0.0150
28	-0.7	-0.13	0.4483	0.0184	-0.0017
29	-0.3	-0.06	0.4761	0.0072	0.0094
30	0.0	0.00	0.5000	0.0000	0.0167
31	0.6	0.11	0.5438	-0.0271	0.0438
32	0.6	0.11	0.5438	-0.0105	0.0271
33	0.6	0.11	0.5438	0.0062	0.0105
34	0.7	0.13	0.5517	0.0150	0.0017
35	1.3	0.25	0.5987	-0.0154	0.0320
36	1.3	0.25	0.5987	0.0013	0.0154
37	1.4	0.26	0.6026	0.0141	0.0026
38	2.0	0.38	0.6480	-0.0147	0.0313
39	2.3	0.43	0.6664	-0.0164	0.0331
40	2.3	0.43	0.6664	0.0003	0.0164
41	2.6	0.49	0.6879	-0.0046	0.0212
42	2.7	0.51	0.6950	0.0050	0.0117
43	3.4	0.64	0.7389	-0.0222	0.0389
44	3.6	0.68	0.7517	-0.0184	0.0350
45	4.0	0.75	0.7734	-0.0234	0.0401
46	4.3	0.81	0.7910	-0.0243	0.0410
47	4.4	0.83	0.7967	-0.0134	0.0300

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TABLE B.9. EXAMPLE CALCULATION OF THE KOLMOGOROV "D" STATISTIC (CONTINUED)

i	$X^{(i)}$	z_i	p_i	D_i+	D_i-
48	4.6	0.87	0.8078	-0.0078	0.0245
49	4.6	0.87	0.8078	0.0089	0.0078
50	5.7	1.08	0.8599	-0.0266	0.0432
51	5.7	1.08	0.8599	-0.0099	0.0266
52	6.0	1.13	0.8708	-0.0041	0.0208
53	6.6	1.25	0.8944	-0.0111	0.0277
54	6.6	1.25	0.8944	0.0056	0.0111
55	7.0	1.32	0.9066	0.0101	0.0066
56	7.6	1.43	0.9236	0.0097	0.0069
57	8.6	1.62	0.9474	0.0026	0.0141
58	8.7	1.64	0.9495	0.0172	-0.0005
59	8.7	1.64	0.9495	0.0338	-0.0172
60	9.4	1.77	0.9616	0.0384	-0.0217

2.10.5 The next step is to standardize the ordered observations. Let z_i denote the standardized value of the ith ordered observation. Then,

$$z_i = \frac{X^{(i)}}{s} \quad and \quad s^2 = \frac{\sum [X^{(i)}]^2}{(n-1)}$$

For the example, s = 5.3, and the standardized observations are listed in Table B.9.

2.10.6 From Table B.10, obtain the value of the standard normal cumulative distribution function (standard normal CDF) at z_i. Denote this value as p_i. Note that negative z are not listed in Table B.10. The value of the standard normal CDF at a negative number is one minus the value of the standard normal CDF at the absolute value of that number. For example, since the value of the standard normal CDF at 3.21 is 0.9993, the value of the standard normal CDF at -3.21 is 1 - 0.9993 = 0.0007. The p_i values for the example data are listed in Table B.9.

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TABLE B.10. P IS THE VALUE OF THE STANDARD NORMAL CUMULATIVE DISTRIBUTION AT Z

z	p	z	p	z	p	z	p
0.00	0.5000	0.41	0.6591	0.82	0.7939	1.23	0.8907
0.01	0.5040	0.42	0.6628	0.83	0.7967	1.24	0.8925
0.02	0.5080	0.43	0.6664	0.84	0.7995	1.25	0.8944
0.03	0.5120	0.44	0.6700	0.85	0.8023	1.26	0.8962
0.04	0.5160	0.45	0.6736	0.86	0.8051	1.27	0.8980
0.05	0.5199	0.46	0.6772	0.87	0.8078	1.28	0.8997
0.06	0.5239	0.47	0.6808	0.88	0.8106	1.29	0.9015
0.07	0.5279	0.48	0.6844	0.89	0.8133	1.30	0.9032
0.08	0.5319	0.49	0.6879	0.90	0.8159	1.31	0.9049
0.09	0.5359	0.50	0.6915	0.91	0.8186	1.32	0.9066
0.10	0.5398	0.51	0.6950	0.92	0.8212	1.33	0.9082
0.11	0.5438	0.52	0.6985	0.93	0.8238	1.34	0.9099
0.12	0.5478	0.53	0.7019	0.94	0.8264	1.35	0.9115
0.13	0.5517	0.54	0.7054	0.95	0.8289	1.36	0.9131
0.14	0.5557	0.55	0.7088	0.96	0.8315	1.37	0.9147
0.15	0.5596	0.56	0.7123	0.97	0.8340	1.38	0.9162
0.16	0.5636	0.57	0.7157	0.98	0.8365	1.39	0.9177
0.17	0.5675	0.58	0.7190	0.99	0.8389	1.40	0.9192
0.18	0.5714	0.59	0.7224	1.00	0.8413	1.41	0.9207
0.19	0.5753	0.60	0.7257	1.01	0.8438	1.42	0.9222
0.20	0.5793	0.61	0.7291	1.02	0.8461	1.43	0.9236
0.21	0.5832	0.62	0.7324	1.03	0.8485	1.44	0.9251
0.22	0.5871	0.63	0.7357	1.04	0.8508	1.45	0.9265
0.23	0.5910	0.64	0.7389	1.05	0.8531	1.46	0.9279
0.24	0.5948	0.65	0.7422	1.06	0.8554	1.47	0.9292
0.25	0.5987	0.66	0.7454	1.07	0.8577	1.48	0.9306
0.26	0.6026	0.67	0.7486	1.08	0.8599	1.49	0.9319
0.27	0.6064	0.68	0.7517	1.09	0.8621	1.50	0.9332
0.28	0.6103	0.69	0.7549	1.10	0.8643	1.51	0.9345
0.29	0.6141	0.70	0.7580	1.11	0.8665	1.52	0.9357
0.30	0.6179	0.71	0.7611	1.12	0.8686	1.53	0.9370
0.31	0.6217	0.72	0.7642	1.13	0.8708	1.54	0.9382
0.32	0.6255	0.73	0.7673	1.14	0.8729	1.55	0.9394
0.33	0.6293	0.74	0.7704	1.15	0.8749	1.56	0.9406
0.34	0.6331	0.75	0.7734	1.16	0.8770	1.57	0.9418
0.35	0.6368	0.76	0.7764	1.17	0.8790	1.58	0.9429
0.36	0.6406	0.77	0.7794	1.18	0.8810	1.59	0.9441
0.37	0.6443	0.78	0.7823	1.19	0.8830	1.60	0.9452
0.38	0.6480	0.79	0.7852	1.20	0.8849	1.61	0.9463
0.39	0.6517	0.80	0.7881	1.21	0.8869	1.62	0.9474
0.40	0.6554	0.81	0.7910	1.22	0.8888	1.63	0.9484

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TABLE B.10. P IS THE VALUE OF THE STANDARD NORMAL CUMULATIVE DISTRIBUTION AT Z (CONTINUED)

z	p	z	p	z	p	z	p
1.64	0.9495	2.05	0.9798	2.46	0.9931	2.87	0.9979
1.65	0.9505	2.06	0.9803	2.47	0.9932	2.88	0.9980
1.66	0.9515	2.07	0.9808	2.48	0.9934	2.89	0.9981
1.67	0.9525	2.08	0.9812	2.49	0.9936	2.90	0.9981
1.68	0.9535	2.09	0.9817	2.50	0.9938	2.91	0.9982
1.69	0.9545	2.10	0.9821	2.51	0.9940	2.92	0.9982
1.70	0.9554	2.11	0.9826	2.52	0.9941	2.93	0.9983
1.71	0.9564	2.12	0.9830	2.53	0.9943	2.94	0.9984
1.72	0.9573	2.13	0.9834	2.54	0.9945	2.95	0.9984
1.73	0.9582	2.14	0.9838	2.55	0.9946	2.96	0.9985
1.74	0.9591	2.15	0.9842	2.56	0.9948	2.97	0.9985
1.75	0.9599	2.16	0.9846	2.57	0.9949	2.98	0.9986
1.76	0.9608	2.17	0.9850	2.58	0.9951	2.99	0.9986
1.77	0.9616	2.18	0.9854	2.59	0.9952	3.00	0.9987
1.78	0.9625	2.19	0.9857	2.60	0.9953	3.01	0.9987
1.79	0.9633	2.20	0.9861	2.61	0.9955	3.02	0.9987
1.80	0.9641	2.21	0.9864	2.62	0.9956	3.03	0.9988
1.81	0.9649	2.22	0.9868	2.63	0.9957	3.04	0.9988
1.82	0.9656	2.23	0.9871	2.64	0.9959	3.05	0.9989
1.83	0.9664	2.24	0.9875	2.65	0.9960	3.06	0.9989
1.84	0.9671	2.25	0.9878	2.66	0.9961	3.07	0.9989
1.85	0.9678	2.26	0.9881	2.67	0.9962	3.08	0.9990
1.86	0.9686	2.27	0.9884	2.68	0.9963	3.09	0.9990
1.87	0.9693	2.28	0.9887	2.69	0.9964	3.10	0.9990
1.88	0.9699	2.29	0.9890	2.70	0.9965	3.11	0.9991
1.89	0.9706	2.30	0.9893	2.71	0.9966	3.12	0.9991
1.90	0.9713	2.31	0.9896	2.72	0.9967	3.13	0.9991
1.91	0.9719	2.32	0.9898	2.73	0.9968	3.14	0.9992
1.92	0.9726	2.33	0.9901	2.74	0.9969	3.15	0.9992
1.93	0.9732	2.34	0.9904	2.75	0.9970	3.16	0.9992
1.94	0.9738	2.35	0.9906	2.76	0.9971	3.17	0.9992
1.95	0.9744	2.36	0.9909	2.77	0.9972	3.18	0.9993
1.96	0.9750	2.37	0.9911	2.78	0.9973	3.19	0.9993
1.97	0.9756	2.38	0.9913	2.79	0.9974	3.20	0.9993
1.98	0.9761	2.39	0.9916	2.80	0.9974	3.21	0.9993
1.99	0.9767	2.40	0.9918	2.81	0.9975	3.22	0.9994
2.00	0.9772	2.41	0.9920	2.82	0.9976	3.23	0.9994
2.01	0.9778	2.42	0.9922	2.83	0.9977	3.24	0.9994
2.02	0.9783	2.43	0.9925	2.84	0.9977	3.25	0.9994
2.03	0.9788	2.44	0.9927	2.85	0.9978	3.26	0.9994
2.04	0.9793	2.45	0.9929	2.86	0.9979	3.27	0.9995

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TABLE B.10. P IS THE VALUE OF THE STANDARD NORMAL CUMULATIVE DISTRIBUTION AT Z (CONTINUED)

z	p	z	p	z	p	z	p
3.28	0.9995	3.46	0.9997	3.64	0.9999	3.82	0.9999
3.29	0.9995	3.47	0.9997	3.65	0.9999	3.83	0.9999
3.30	0.9995	3.48	0.0997	3.66	0.9999	3.84	0.9999
3.31	0.9995	3.49	0.9998	3.67	0.9999	3.85	0.9999
3.32	0.9995	3.50	0.9998	3.68	0.9999	3.86	0.9999
3.33	0.9996	3.51	0.9998	3.69	0.9999	3.87	0.9999
3.34	0.9996	3.52	0.9998	3.70	0.9999	3.88	0.9999
3.35	0.9996	3.53	0.9998	3.71	0.9999	3.89	0.9999
3.36	0.9996	3.54	0.9998	3.72	0.9999	3.90	1.0000
3.37	0.9996	3.55	0.9998	3.73	0.9999	3.91	1.0000
3.38	0.9996	3.56	0.9998	3.74	0.9999	3.92	1.0000
3.39	0.9997	3.57	0.9998	3.75	0.9999	3.93	1.0000
3.40	0.9997	3.58	0.9998	3.76	0.9999	3.94	1.0000
3.41	0.9997	3.59	0.9998	3.77	0.9999	3.95	1.0000
3.42	0.9997	3.60	0.9998	3.78	0.9999	3.96	1.0000
3.43	0.9997	3.61	0.9998	3.79	0.9999	3.97	1.0000
3.44	0.9997	3.62	0.9999	3.80	0.9999	3.98	1.0000
3.45	0.9997	3.63	0.9999	3.81	0.9999	3.99	1.0000

2.10.7 Next, calculate the following differences for each ordered observation:

$$D_i+ = (i/n) - p_i$$

$$D_i- = p_i - [(i-1)/n]$$

The differences for the example are listed in Table B.9.

2.10.8 Obtain the maximum of the D_i+, and denote it as D+. Obtain the maximum of the D_i-, and denote it as D-. For the example, D+ = 0.0525, and D- = 0.0597.

2.10.9 Next, obtain the maximum of D+ and D-, and denote it as D. For the example, D = 0.0597.

2.10.10 The test statistic, D*, is calculated as follows:

$$D^* = D(\sqrt{n} - 0.01 + \frac{0.85}{\sqrt{n}})$$

For the example, D* = 0.4684.

2.10.11 The decision rule for the two tailed test is to compare the critical value from Table B.11 to the computed D*. If the computed value is greater than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 is 1.035. The calculated value, 0.4684, is not greater than the critical value. Thus, the conclusion of the test is that the data are normally distributed.

2.10.12 In general, if the data fail the test for normality, a transformation such as the log transformation may normalize the data. After transforming the data, repeat the Kolmogorov "D" test for normality.

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TABLE B.11. CRITICAL VALUES FOR THE KOLMOGOROV "D" TEST

Alpha Level	Critical Value
0.010	1.035
0.025	0.955
0.050	0.895
0.100	0.819
0.150	0.775

3. TEST FOR HOMOGENEITY OF VARIANCE

3.1 For Dunnett's Procedure and the t test with Bonferroni's adjustment, the variances of the data obtained from each toxicant concentration and the control are assumed to be equal. Bartlett's Test is a formal test of this assumption. In using this test, it is assumed that the data are normally distributed.

3.2 The data used in this example are growth data from a Fathead Minnow Larval Survival and Growth Test, and are the same data used in Appendices C and D. These data are listed in Table B.12, together with the calculated variance for the control and each toxicant concentration.

TABLE B.12. FATHEAD LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR BARTLETT'S TEST FOR HOMOGENEITY OF VARIANCE

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.711	0.646	0.669	0.629	0.650
B	0.662	0.626	0.669	0.680	0.558
C	0.718	0.723	0.694	0.513	0.606
D	0.767	0.700	0.676	0.672	0.508
Mean($\bar{Y}_i$)	0.714	0.674	0.677	0.624	0.580
S_i^2	0.0018	0.0020	0.0001	0.0059	0.0037
I	1	2	3	4	5

3.3 The test statistic for Bartlett's Test (Snedecor and Cochran, 1980) is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i)\ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each toxicant concentration and control

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p = number of levels of toxicant concentration including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations

n_i = the number of replicates for concentration i.

$$\bar{S}^2 = \frac{\left(\sum_{i=1}^{p} V_i S_i^2\right)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3(p-1)]^{-1}\left[\sum_{i=1}^{p} \frac{1}{V_i} - \left(\sum_{i=1}^{p} V_i\right)^{-1}\right]$$

3.4 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value is obtained from a table of the chi-square distribution for p - 1 degrees of freedom and a significance level of 0.01. If B is less than the critical value then the variances are assumed to be equal.

3.5 For the data in this example, $V_i = 3$, p = 5, $\bar{S}^2 = 0.0027$, and C = 1.133. The calculated B value is:

$$B = \frac{(15)[\ln(0.0027)] - 3\sum_{i=1}^{p} \ln(S_i^2)}{1.133}$$

$$= \frac{15(-5.9145) - 3(-32.4771)}{1.133}$$

$$= 7.691$$

3.6 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value for the test is 13.277 for a significance level of 0.01. Since B = 7.691 is less than the critical value of 13.277, conclude that the variances are not different.

4. TRANSFORMATIONS OF THE DATA

4.1 When the assumptions of normality and/or homogeneity of variance are not met, transformations of the data may remedy the problem, so that the data can be analyzed by parametric procedures, rather than by nonparametric technique such as Steel's Many-one Rank Test or Wilcoxon's Rank Sum Test. Examples of transformations include log, square root, arc sine square root, and reciprocals. After the data have been transformed, Shapiro-Wilk's and Bartlett's tests should be performed on the transformed observations to determine whether the assumptions of normality and/or homogeneity of variance are met.

4.2 ARC SINE SQUARE ROOT TRANSFORMATION (USEPA, 1993)

4.2.1 For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the ith treatment is proportional to P_i (1 - P_i), where P_i is the expected proportion for the treatment. This clearly violates the homogeneity of variance assumption required by parametric procedures such as Dunnett's Procedure or the t test with Bonferroni's adjustment, since the existence of a treatment effect implies different values of P_i for different treatments, i. Also, when the observed proportions are based on small samples, or when P_i is

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close to zero or one, the normality assumption may be invalid. The arc sine square root (arc sine$\sqrt{P}$) transformation is commonly used for such data to stabilize the variance and satisfy the normality requirement.

4.2.2 Arc sine transformation consists of determining the angle (in radians) represented by a sine value. In the case of arc sine square root transformation of mortality data, the proportion of dead (or affected) organisms is taken as the sine value, the square root of the sine value is calculated, and the angle (in radians) for the square root of the sine value is determined. Whenever the proportion dead is 0 or 1, a special modification of the arc sine square root transformation must be used (Bartlett, 1937). An explanation of the arc sine square root transformation and the modification is provided below.

4.2.3 Calculate the response proportion (RP) at each effluent concentration, where:

RP = (number of surviving or "unaffected" organisms)/(number exposed)

<u>Example:</u> If 12 of 20 animals in a given treatment replicate survive:

RP = 12/20

= 0.60

4.2.4 Transform each RP to its arc sine square root, as follows:

4.2.4.1 For RPs greater than zero or less than one:

Angle (radians) = arc sine$\sqrt{RP}$

<u>Example:</u> If RP = 0.60:

Angle = arc sine$\sqrt{0.60}$

= arc sine 0.7746

= 0.8861 radians

4.2.4.2 Modification of the arc sine square root when RP = 0:

Angle (in radians) = arc sine $\sqrt{1/4N}$

Where: N = Number of animals/treatment replicate

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Example: If 20 animals are used:

Angle = arc sine $\sqrt{1/80}$

= arc sine 0.1118

= 0.1120 radians

4.2.4.3 Modification of the arc sine square root when RP = 1.0:

Angle = 1.5708 radians - (radians for RP = 0)

Example: Using above value:

Angle = 1.5708 - 0.1120

= 1.4588 radians

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APPENDIX C

DUNNETT'S PROCEDURE

1. MANUAL CALCULATIONS

1.1 Dunnett's Procedure (Dunnett, 1955; Dunnett, 1964) is used to compare each concentration mean with the control mean to decide if any of the concentrations differ from the control. This test has an overall error rate of alpha, which accounts for the multiple comparisons with the control. It is based on the assumptions that the observations are independent and normally distributed and that the variance of the observations is homogeneous across all concentrations and control (see Appendix B for a discussion on validating the assumptions). Dunnett's Procedure uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance. Dunnett's Procedure can only be used when the same number of replicate test vessels have been used at each concentration and the control. When this condition is not met, a t test with Bonferroni's adjustment is used (see Appendix D).

1.2 The data used in this example are growth data from a Fathead Minnow Larval Survival and Growth Test, and are the same data used in Appendices B and D. These data are listed in Table C.1.

TABLE C.1. FATHEAD MINNOW, *PIMEPHALES PROMELAS,* LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR DUNNETT'S PROCEDURE

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.711	0.517	0.602	0.566	0.455
B	0.662	0.501	0.669	0.612	0.502
C	0.646	0.723	0.694	0.410	0.606
D	0.690	0.560	0.676	0.672	0.254
Mean($\bar{Y}_i$)	0.677	0.575	0.660	0.565	0.454
Total(T_i)	2.709	2.301	2.641	2.260	1.817

1.3 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, using the following formulas:

Where: p = number of effluent concentrations including:

$$SST = \sum_{ij} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSB = \sum_{i} T_i^2/n_i - G^2/N$$ Between Sum of Squares

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$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations; $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

Y_{ij} = the jth observation for concentration i

1.4 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 4$

$N = 20$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 2.709$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 2.301$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.641$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.260$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 1.817$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 11.728$

$$SST = \sum_{ij} Y_{ij}^2 - G^2/N$$

$$= 7.146 - (11.728)^2/20$$

$$= 0.2687$$

$$SSB = \sum_i T_i^2/n_i - G^2/N$$

$$= \tfrac{1}{4}(28.017 - 11.728)^2/20$$

$$= 0.1270$$

$$SSW = SST - SSB$$

$$= 0.2687 - 0.1270$$

$$= 0.1417$$

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1.5 Summarize these data in the ANOVA table (Table C.2).

TABLE C.2. ANOVA TABLE FOR DUNNETT'S PROCEDURE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

1.6 Summarize data for ANOVA (Table C.3).

TABLE C.3. COMPLETED ANOVA TABLE FOR DUNNETT'S PROCEDURE

Source	df	SS	Mean Square
Between	5 - 1 = 4	0.1270	0.0318
Within	20 - 5 = 15	0.1417	0.0094
Total	19	0.2687	

1.7 To perform the individual comparisons, calculate the t statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1)+(1/n_i)}}$$

Where: $\bar{Y}_i$ = mean for concentration i

$\bar{Y}_1$ = mean for the control

S_w = square root of the within mean square

n_1 = number of replicates in the control

n_i = number of replicates for concentration i.

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1.8 Table C.4 includes the calculated t values for each concentration and control combination.

TABLE C.4. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.487
64	3	0.248
128	4	1.633
256	5	3.251

1.9 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison (2.36), with an overall alpha level of 0.05, 15 degrees of freedom and four concentrations excluding the control is read from the table of Dunnett's "T" values (Table C.5; this table assumes an equal number of replicates in all treatment concentrations and the control). The mean weight for concentration i is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since T_5 is greater than 2.36, the 256 µg/L concentration has significantly lower growth than the control. Hence the NOEC and LOEC for growth are 128 µg/L and 256 µg/L, respectively.

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TABLE C.5. DUNNETT'S "T" VALUES (Miller, 1981)

	(One-tailed)d'_k																	
k / v	α = .05									α = 0.1								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.58	2.85	2.98	3.08	3.16	3.24	3.30	3.37	3.90	4.21	4.43	4.50	4.73	4.85	4.94	5.03
6	1.94	2.34	2.56	2.71	2.83	2.92	3.00	3.07	3.12	3.14	3.61	4.88	4.07	4.21	4.33	4.43	4.51	4.39
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01	3.00	3.42	3.56	3.83	3.96	4.07	4.15	4.23	4.30
8	1.86	2.22	2.42	2.55	2.56	2.74	2.81	2.87	2.92	2.90	3.20	3.51	3.67	3.79	3.18	3.96	4.03	4.09
9	1.83	2.18	2.37	2.50	2.60	2.68	2.75	2.81	2.86	2.82	3.19	3.40	3.55	3.86	3.75	3.82	3.89	3.94
10	1.81	2.15	2.34	2.47	2.56	2.64	2.70	2.76	2.81	2.76	3.11	3.31	3.45	3.56	3.64	3.71	3.78	3.83
11	1.80	2.13	2.31	2.44	2.53	2.60	2.67	2.72	2.77	2.72	3.06	3.25	3.38	3.46	3.56	3.63	3.69	3.74
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.59	2.74	2.68	3.01	3.19	3.32	3.42	3.50	3.56	3.62	3.67
13	1.77	2.09	2.27	2.39	2.48	2.55	2.61	2.68	2.71	2.65	2.97	3.15	3.27	3.37	3.44	3.91	3.56	3.61
14	1.76	2.08	2.25	2.37	2.46	2.53	2.59	2.64	2.69	2.62	2.94	3.11	3.23	3.32	3.40	3.46	3.51	3.56
15	1.75	2.07	2.24	2.36	2.44	2.51	2.57	2.62	2.67	2.60	2.91	3.08	3.20	3.29	3.36	3.42	3.47	3.52
16	1.75	2.06	2.23	2.34	2.43	2.50	2.56	2.61	2.65	2.58	2.38	3.05	3.17	3.28	3.33	3.39	3.44	3.48
17	1.74	2.05	2.22	2.33	2.42	2.49	2.54	2.59	2.64	2.57	2.86	3.03	3.14	3.23	3.30	3.36	3.41	3.45
18	1.73	2.04	2.21	2.32	2.41	2.48	2.53	2.58	2.62	2.55	2.84	3.01	3.12	3.21	3.27	3.33	3.38	3.40
19	1.73	2.03	2.20	2.31	2.40	2.47	2.52	2.57	2.61	2.54	2.83	2.99	3.10	3.18	3.25	3.31	3.36	3.40
20	1.72	2.03	2.19	2.30	2.30	2.46	2.51	2.56	2.60	2.53	2.81	2.97	3.08	3.17	3.23	3.29	3.34	3.40
24	1.71	2.01	3.17	2.28	2.36	2.43	2.48	2.53	2.57	2.40	2.77	2.92	3.03	3.11	3.17	3.22	3.27	3.31
30	1.70	1.99	2.15	2.25	2.33	2.40	2.45	2.50	2.54	2.46	2.72	2.87	2.97	3.05	3.11	3.16	3.21	3.24
40	1.68	1.97	2.13	2.23	2.31	2.37	2.42	2.47	2.51	2.42	2.68	2.32	2.92	2.99	3.06	3.10	3.14	3.18
60	1.67	1.95	2.10	2.21	2.28	2.35	2.39	2.44	2.48	2.39	2.64	2.78	2.87	2.94	3.08	3.04	3.06	3.12
120	1.86	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45	2.36	2.60	2.73	2.82	2.90	2.94	2.90	3.03	3.06
α	1.64	1.92	2.06	2.16	2.23	2.29	2.34	2.33	2.42	2.33	2.56	2.68	2.77	2.34	2.90	2.93	2.97	3.00

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1.10 To quantify the sensitivity of the test, the minimum significant difference (MSD) may be calculated. The formula is as follows:

$$MSD = d\,S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = critical value for the Dunnett's Procedure

S_w = the square root of the within mean square

n = the number of replicates at each concentration, assuming an equal number of replicates at all treatment concentrations

n_1 = number of replicates in the control

For example:

$$MSD = 2.36(0.097)[3\sqrt{(1/4)+(1/4)}] = 2.36(0.097)(\sqrt{2/4})$$

$$= 2.36\ (0.097)(0.707)$$

$$= 0.162$$

1.11 For this set of data, the minimum difference between the control mean and a concentration mean that can be detected as statistically significant is 0.087 mg. This represents a decrease in growth of 24% from the control.

1.11.1 If the data have not been transformed, the MSD (and the percent decrease from the control mean that it represents) can be reported as is.

1.11.2 In the case where the data have been transformed, the MSD would be in transformed units. In this case carry out the following conversion to determine the MSD in untransformed units.

1.11.2.1 Subtract the MSD from the transformed control mean. Call this difference D. Next, obtain untransformed values for the control mean and the difference, D.

$$MSD_u = control_u - D_u$$

Where: MSD_u = the minimum significant difference for untransformed data

$Control_u$ = the untransformed control mean

D_u = the untransformed difference

1.11.2.2 Calculate the percent reduction from the control that MSD_u represents as:

$$\text{Percent Reduction} = \frac{MSD_u}{Control_u} \times 100$$

1.11.3 An example of a conversion of the MSD to untransformed units, when the arc sine square root transformation was used on the data, follows:

Step 1. Subtract the MSD from the transformed control mean. As an example, assume the data in Table C.1 were transformed by the arc sine square root transformation. Thus:

$0.677 - 0.162 = 0.515$

Step 2. Obtain untransformed values for the control mean (0.677) and the difference (0.515) obtained in Step 1 above.

$[\text{Sine } (0.677)]^2 = 0.392$
$[\text{Sine } (0.515)]^2 = 0.243$

Step 3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values obtained in Step 2.

$MSD_u = 0.392 - 0.243 = 0.149$

In this case, the MSD would represent a 38.0% decrease in survival from the control [(0.149/0.392)(100)].

2. COMPUTER CALCULATIONS

2.1 This computer program incorporates two analyses: an analysis of variance (ANOVA), and a multiple comparison of treatment means with the control mean (Dunnett's Procedure). The ANOVA is used to obtain the error value. Dunnett's Procedure indicates which toxicant concentration means (if any) are statistically different from the control mean at the 5% level of significance. The program also provides the minimum difference between the control and treatment means that could be detected as statistically significant, and tests the validity of the homogeneity of variance assumption by Bartlett's Test. The multiple comparison is performed based on procedures described by Dunnett (1955).

2.2 The source code for the Dunnett's program is structured into a series of subroutines, controlled by a driver routine. Each subroutine has a specific function in the Dunnett's Procedure, such as data input, transforming the data, testing for equality of variances, computing p values, and calculating the one-way analysis of variance.

2.3 The program compares up to seven toxicant concentrations against the control, and can accommodate up to 50 replicates per concentration.

2.4 If the number of replicates at each toxicant concentration and control are not equal, a t test with Bonferroni's adjustment is performed instead of Dunnett's Procedure (see Appendix D).

2.5 The program was written in IBM-PC FORTRAN by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled executable version of the program can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

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2.6 DATA INPUT AND OUTPUT

2.6.1 Reproduction data from a daphnid, *Ceriodaphnia dubia,* survival and reproduction test (Table C.6) are used to illustrate the data input and output for this program.

TABLE C.6. SAMPLE DATA FOR DUNNETT'S PROGRAM *CERIODAPHNIA DUBIA* REPRODUCTION DATA

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	27	32	39	27	10
2	30	35	30	34	13
3	29	32	33	36	7
4	31	26	33	34	7
5	16	18	36	31	7
6	15	29	33	27	10
7	18	27	33	33	10
8	17	16	27	31	16
9	14	35	38	33	12
10	27	13	44	31	2

2.6.2 Data Input

2.6.2.1 When the program is entered, the user is asked to select the type of data to be entered:

1. Response proportions, like survival or fertilization proportions.
2. Counts and measurements, like offspring counts, cystocarp counts or weights.

2.6.2.2 After the type of data is chosen, the user has the following options:

1. Create a data file
2. Edit a data file
3. Perform analysis on existing data set
4. Stop

2.6.2.3 When Option 1 (Create a data file) is selected for counts and measurements, the program prompts the user for the following information:

1. Number of concentrations, including control
2. For each concentration:
   - number of observations
   - data for each observation

2.6.2.4 After the data have been entered, the user may save the file on a disk, and the program returns to the menu (see below).

2.6.2.5 Sample data input is shown in Figure C.1.

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```
                    EMSL Cincinnati Dunnett Software
                              Version 1.5

      1)    Create a data file
      2)    Edit a data file
      3)    Perform ANOVA on existing data
      4)    Stop

Your choice ? 1

Number of groups, including control ? 5

Number of observations for group  1 ? 10

Enter the data for group  1 one observation at a time.

NO.  1?  27

NO.  2?  30

NO.  3?  29

NO.  4?  31

NO.  5?  16

NO.  6?  15

NO.  7?  18

NO.  8?  17

NO.  9?  14

NO. 10?  27

Number of observations for group  2  ?  10

Do you wish to save the data on disk  ?y

Disk file for output  ?  cerio
```

Figure C.1. Sample Data Input for Dunnett's Program for Reproduction Data from Table C.6.

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2.6.3 Program Output

2.6.3.1 When Option 3 (Perform analysis on existing data set) is selected from the menu, the user is asked to select the transformation desired, and indicate whether they expect the means of the test groups to be less or greater than the mean for the control group (see Figure C.2).

2.6.3.2 Summary statistics (Figure C.3) for the raw and transformed data, if applicable, the ANOVA table, results of Bartlett's Test, the results of the multiple comparison procedure and the minimum detectable difference are included in the program output.

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Version 1.5

1) Create a data file
2) Edit a data file
3) Perform analysis on existing data set
4) Stop

Your choice ? 3

File name ? cerio

Available Transformations

1) no transform
2) square root
3) log10

Your choice ? 1

Dunnett's test as implemented in this program is
a one-sided test. You must specify the direction
the test is to be run; that is, do you expect the
means for the test groups to be less than or
greater than the mean for the control group mean.

Direction for Dunnett's test : L=less than, G=greater than ? L

Figure C.2. Example of Choosing Option 3 from the Menu of the Dunnett Program.

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Ceriodaphnia Reproduction Data from Table C.6

Summary Statistics and ANOVA

Transformation = None

Group	n	Mean	s.d.	CV%
1 = control	10	22.4000	6.9314	30.9
2	10	26.3000	8.0007	30.4
3	10	34.6000	4.8351	14.0
4	10	31.7000	2.9458	9.3
5*	10	9.4000	3.8930	41.4

*) the mean for this group is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -5.628560
This difference corresponds to -25.13 percent of control

Between concentrations
Sum of squares = 3887.880000 with 4 degrees of freedom.

Error mean square = 31.853333 with 45 degrees of freedom.

Bartlett's test p-value for equality of variances = .029

Do you wish to restart the program ?

Figure C.3. Example of Program Output for the Dunnett's Program Using the Reproduction Data from Table C.6.

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APPENDIX D

T TEST WITH BONFERRONI'S ADJUSTMENT

1. The t test with Bonferroni's adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus, Dunnett's Procedure is a more powerful test.

2. The t test with Bonferroni's adjustment is based on the same assumptions of normality and homogeneity of variance as Dunnett's Procedure (see Appendix B for testing these assumptions), and, like Dunnett's Procedure, uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance.

3. An example of the use of the t test with Bonferroni's adjustment is provided below. The data used in the example are the same as in Appendix C, except that the third replicate from the 256 µg/L concentration is presumed to have been lost. Thus, Dunnett's Procedure cannot be used. The weight data are presented in Table D.1.

TABLE D.1. FATHEAD MINNOW, *PIMEPHALES PROMELAS,* LARVAL GROWTH DATA (WEIGHT IN MG) USED FOR THE T-TEST WITH BONFERRONI'S ADJUSTMENT

		NaPCP Concentration (µg/L)			
Replicate	Control	32	64	128	256
A	0.711	0.517	0.602	0.566	0.455
B	0.662	0.501	0.669	0.612	0.502
C	0.646	0.723	0.694	0.410	(LOST)
D	0.690	0.560	0.676	0.672	0.254
Mean($\bar{Y}$)	0.677	0.575	0.660	0.565	0.404
Total(T_i)	2.709	2.301	2.641	2.260	1.211

3.1 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, using the following formulas:

Where: p = number of effluent concentrations including the control

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

$SST = \sum_{ij} Y_{ij}^2 - G^2/N$ Total Sum of Squares

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$$SSB = \sum_i T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

Where: G = The grand total of all sample observations; $G = \sum_{i=1}^{P} T_i$

T_i = The total of the replicate measurements for concentration i

Y_{ij} = The jth observation for concentration i

3.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 4$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 2.709$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 2.301$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 2.641$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 2.260$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 1.211$
$G = T_1 + T_2 + T_3 + T_4 + T_5 = 11.122$

$$SSB = \sum_i T_i^2/n_i - G^2/N$$

$$= 6.668 - (11.122)^2/19$$

$$= 0.158$$

$$SST = \sum_{ij} Y_{ij}^2 - G^2/N$$

$$= 6.779 - (11.122)^2/19$$

$$= 0.269$$

$$SSW = SST - SSB$$

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= 0.269 - 0.158

= 0.111

3.3 Summarize these data in the ANOVA table (Table D.2):

TABLE D.2. ANOVA TABLE FOR BONFERRONI'S ADJUSTMENT

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

3.4 Summarize these data in the ANOVA table (Table D.3):

TABLE D.3. COMPLETED ANOVA TABLE FOR THE T-TEST WITH BONFERRONI'S ADJUSTMENT

Source	df	SS	Mean Square
Between	5 - 1 = 4	0.158	0.0395
Within	19 - 5 = 14	0.111	0.0029
Total	18	0.269	

3.5 To perform the individual comparisons, calculate the t statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1)+(1/n_i)}}$$

Where: $\bar{Y}_i$ = mean for each concentration

$\bar{Y}_1$ = mean for the control

S_w = square root of the within mean square

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n_1 = number of replicates in the control.

n_i = number of replicates for concentration i.

3.6 Table D.4 includes the calculated t values for each concentration and control combination.

TABLE D.4. CALCULATED T VALUES

NaPCP Concentration (µg/L)	i	t_i
32	2	1.623
64	3	0.220
128	4	1.782
256	5	4.022

3.7 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison (2.510), with an overall alpha level of 0.05, fourteen degrees of freedom and four concentrations excluding the control, was obtained from Table D.5. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Since t_5 is greater than 2.510, the 256 µg/L concentration has significantly lower growth than the control. Hence the NOEC and LOEC for growth are 128 µg/L and 256 µg/L, respectively.

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TABLE D.5. CRITICAL VALUES FOR "T" FOR THE T TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
1	6.314	12.707	19.002	25.452	31.821	38.189	44.556	50.924	57.290	63.657
2	2.920	4.303	5.340	6.206	6.965	7.649	8.277	8.861	9.408	9.925
3	2.354	3.183	3.741	4.177	4.541	4.857	5.138	5.392	5.626	5.841
4	2.132	2.777	3.187	3.496	3.747	3.961	4.148	4.315	4.466	4.605
5	2.016	2.571	2.912	3.164	3.365	3.535	3.681	3.811	3.927	4.033
6	1.944	2.447	2.750	2.969	3.143	3.288	3.412	3.522	3.619	3.708
7	1.895	2.365	2.642	2.842	2.998	3.128	3.239	3.336	3.422	3.500
8	1.860	2.307	2.567	2.752	2.897	3.016	3.118	3.206	3.285	3.356
9	1.834	2.263	2.510	2.686	2.822	2.934	3.029	3.111	3.185	3.250
10	1.813	2.229	2.406	2.634	2.764	2.871	2.961	3.039	3.108	3.170
11	1.796	2.301	2.432	2.594	2.719	2.821	2.907	2.981	3.047	3.106
12	1.783	2.179	2.404	2.561	2.681	2.730	2.863	2.935	2.998	3.055
13	1.771	2.161	2.380	2.533	2.651	2.746	2.827	2.897	2.950	3.013
14	1.762	2.145	2.360	2.510	2.625	2.718	2.797	2.864	2.924	2.977
15	1.754	2.132	2.343	2.490	2.603	2.694	2.771	2.837	2.895	2.947
16	1.746	2.120	2.329	2.473	2.584	2.674	2.749	2.814	2.871	2.921
17	1.740	2.110	2.316	2.459	2.567	2.655	2.729	2.793	2.849	2.899
18	1.735	2.101	2.305	2.446	2.553	2.640	2.712	2.775	2.830	2.879
19	1.730	2.094	2.295	2.434	2.540	2.626	2.697	2.759	2.813	2.861
20	1.725	2.086	2.206	2.424	2.528	2.613	2.684	2.745	2.798	2.846
21	1.721	2.080	2.278	2.414	2.518	2.602	2.672	2.732	2.785	2.832
22	1.718	2.074	2.271	2.406	2.509	2.592	2.661	2.721	2.773	2.819
23	1.714	2.069	2.264	2.398	2.500	2.583	2.651	2.710	2.762	2.808
24	1.711	2.064	2.258	2.391	2.493	2.574	2.642	2.701	2.752	2.797
25	1.709	2.060	2.253	2.385	2.486	2.566	2.634	2.692	2.743	2.788
26	1.706	2.056	2.248	2.379	2.479	2.559	2.627	2.684	2.734	2.779
27	1.704	2.052	2.243	2.374	2.473	2.553	2.620	2.677	2.727	2.771
28	1.702	2.049	2.239	2.369	2.468	2.547	2.613	2.670	2.720	2.764

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TABLE D.5. CRITICAL VALUES FOR "T" FOR THE T TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED (CONTINUED)

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
29	1.700	2.046	2.235	2.364	2.463	2.541	2.607	2.664	2.713	2.757
30	1.698	2.043	2.231	2.360	2.458	2.536	2.602	2.658	2.707	2.750
31	1.696	2.040	2.228	2.356	2.453	2.531	2.597	2.652	2.701	2.745
32	1.694	2.037	2.224	2.352	2.449	2.527	2.592	2.647	2.696	2.739
33	1.693	2.035	2.221	2.349	2.445	2.523	2.587	2.643	2.691	2.734
34	1.691	2.033	2.219	2.346	2.442	2.519	2.583	2.638	2.686	2.729
35	1.690	2.031	2.216	2.342	2.438	2.515	2.579	2.634	2.682	2.724
36	1.689	2.029	2.213	2.340	2.435	2.512	2.575	2.630	2.678	2.720
37	1.688	2.027	2.211	2.337	2.432	2.508	2.572	2.626	2.674	2.716
38	1.686	2.025	2.209	2.334	2.429	2.505	2.568	2.623	2.670	2.712
39	1.685	2.023	2.207	2.332	2.426	2.502	2.565	2.619	2.667	2.708
40	1.684	2.022	2.205	2.329	2.424	2.499	2.562	2.616	2.663	2.705
50	1.676	2.009	2.189	2.311	2.404	2.478	2.539	2.592	2.638	2.678
60	1.671	2.001	2.179	2.300	2.391	2.463	2.324	2.576	2.621	2.661
70	1.667	1.995	2.171	2.291	2.381	2.453	2.513	2.564	2.609	2.648
80	1.665	1.991	2.166	2.285	2.374	2.446	2.505	2.556	2.600	2.639
90	1.662	1.987	2.162	2.280	2.369	2.440	2.499	2.549	2.593	2.632
100	1.661	1.984	2.158	2.276	2.365	2.435	2.494	2.544	2.588	2.626
110	1.659	1.982	2.156	2.273	2.361	2.432	2.490	2.540	2.583	2.622
120	1.658	1.980	2.153	2.270	2.358	2.429	2.487	2.536	2.580	2.618
Infinite	1.645	1.960	2.129	2.242	2.327	2.394	2.450	2.498	2.540	2.576

d.f. = Degrees of freedom for MSE (Mean Square Error) from ANOVA.
K = Number of concentrations to be compared to the control.

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APPENDIX E

STEEL'S MANY-ONE RANK TEST

1. Steel's Many-one Rank Test is a nonparametric test for comparing treatments with a control. This test is an alternative to Dunnett's Procedure, and may be applied to data when the normality assumption has not been met. Steel's Test requires equal variances across the treatments and the control, but it is thought to be fairly insensitive to deviations from this condition (Steel, 1959). The tables for Steel's Test require an equal number of replicates at each concentration. If this is not the case, use Wilcoxon's Rank Sum Test, with Bonferroni's adjustment (see Appendix F).

2. For an analysis using Steel's Test, for each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to the observation. (Extensive ties would invalidate this procedure). The sum of the ranks within each concentration and within the control is then calculated. To determine if the response in a concentration is significantly different from the response in the control, the minimum rank sum for each concentration and control combination is compared to the significant values of rank sums given later in this section. In this table, k equals the number of treatments excluding the control and n equals the number of replicates for each concentration and the control.

3. An example of the use of this test is provided below. The test employs reproduction data from a *Ceriodaphnia dubia* 7-day, chronic test. The data are listed in Table E.1. Significant mortality was detected via Fisher's Exact Test in the 50% effluent concentration. The data for this concentration is not included in the reproduction analysis.

TABLE E.1. EXAMPLE OF STEEL'S MANY-ONE RANK TEST: DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, 7-DAY CHRONIC TEST

Effluent Concentration	Replicate 1	2	3	4	5	6	7	8	9	10	No. Live Adults
Control	20	26	26	23	24	27	26	23	27	24	10
3%	13	15	14	13	23	26	0	25	26	27	9
6%	18	22	13	13	23	22	20	22	23	22	10
12%	14	22	20	23	20	23	25	24	25	21	10
25%	9	0	9	7	6	10	12	14	9	13	8
50%	0	0	0	0	0	0	0	0	0	0	0

4. For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign ranks (1, 2, 3,..., 16) to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to each tied observation.

5. An example of assigning ranks to the combined data for the control and 3% effluent concentration is given in Table E.2. This ranking procedure is repeated for each control and concentration combination. The complete set of rankings is listed in Table E.3. The ranks are then summed for each effluent concentration, as shown in Table E.4.

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TABLE E.2. EXAMPLE OF STEEL'S MANY-ONE RANK TEST: ASSIGNING RANKS TO THE CONTROL AND 3% EFFLUENT CONCENTRATION

Rank	Number of Young Produced	Control or % Effluent
1	0	3
2.5	13	3
2.5	13	3
4	14	3
5	15	3
6	20	Control
8	23	Control
8	23	Control
8	23	3
10.5	24	Control
10.5	24	Control
12	25	3
15	26	Control
15	26	Control
15	26	Control
15	26	3
15	26	3
19	27	Control
19	27	Control
19	27	3

TABLE E.3. TABLE OF RANKS

Replicate (Organism)	Control[1]		Effluent Concentration (%) 3		6		12		25	
1	20	(6,4.5,3,11)	13	(2.5)	18	(3)	14	(1)	9	(5)
2	26	(15,17,17,17)	15	(5)	22	(7.5)	22	(6)	0	(1)
3	26	(15,17,17,17)	14	(4)	13	(1.5)	20	(3)	9	(5)
4	23	(8,11.5,8.5,12.5)	13	(2.5)	13	(1.5)	23	(8.5)	7	(3)
5	24	(10.5,14.5,12,14.5)	23	(8)	23	(11.5)	20	(3)	6	(2)
6	27	(19,19.5,19.5,19.5)	26	(15)	22	(7.5)	23	(8.5)	10	(7)
7	26	(15,17,17,17)	0	(1)	20	(4.5)	25	(14.5)	12	(8)
8	23	(8,11.5,8.5,12.5)	25	(12)	22	(7.5)	24	(12)	14	(10)
9	27	(19,19.5,19.5,19.5)	26	(15)	23	(11.5)	25	(14.5)	9	(5)
10	24	(10.5,14.5,12,14.5)	27	(19)	22	(7.5)	21	(5)	13	(9)

[1] Control ranks are given in the order of the concentration with which they were ranked.

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TABLE E.4. RANK SUMS

Effluent Concentration (%)	Rank Sum
3	84
6	64
12	76
25	55

6. For this set of data, determine if the reproduction in any of the effluent concentrations is significantly lower than the reproduction by the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the reproduction of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the reproduction would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank in a test with four concentrations and ten replicates is 76 (see Table E.5 , for R=4).

7. Comparing the rank sums in Table E.4 to the appropriate critical rank, the 6%, 12% and 25% effluent concentrations are found to be significantly different from the control. Thus the NOEC and LOEC for reproduction are 3% and 6%, respectively.

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TABLE E.5. SIGNIFICANT VALUES OF RANK SUMS: JOINT CONFIDENCE COEFFICIENTS OF 0.95 (UPPER) and 0.99 (LOWER) FOR ONE-SIDED ALTERNATIVES (Steel, 1959)

	k = number of treatments (excluding control)							
n	2	3	4	5	6	7	8	9
4	11	10	10	10	10	--	--	--
	--	--	--	--	--	--	--	--
5	18	17	17	16	16	16	16	15
	15	--	--	--	--	--	--	--
6	27	26	25	25	24	24	24	23
	23	22	21	21	--	--	--	--
7	37	36	35	35	34	34	33	33
	32	31	30	30	29	29	29	29
8	49	48	47	46	46	45	45	44
	43	42	41	40	40	40	39	39
9	63	62	61	60	59	59	58	58
	56	55	54	53	52	52	51	51
10	79	77	76	75	74	74	73	72
	71	69	68	67	66	66	65	65
11	97	95	93	92	91	90	90	89
	87	85	84	83	82	81	81	80
12	116	114	112	111	110	109	108	108
	105	103	102	100	99	99	98	98
13	138	135	133	132	130	129	129	128
	125	123	121	120	119	118	117	117
14	161	158	155	154	153	152	151	150
	147	144	142	141	140	139	138	137
15	186	182	180	178	177	176	175	174
	170	167	165	164	162	161	160	160
16	213	209	206	204	203	201	200	199
	196	192	190	188	187	186	185	184
17	241	237	234	232	231	229	228	227
	223	219	217	215	213	212	211	210
18	272	267	264	262	260	259	257	256
	252	248	245	243	241	240	239	238
19	304	299	296	294	292	290	288	287
	282	278	275	273	272	270	268	267
20	339	333	330	327	325	323	322	320
	315	310	307	305	303	301	300	299

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APPENDIX F

WILCOXON RANK SUM TEST

1. Wilcoxon's Rank Sum Test is a nonparametric test, to be used as an alternative to Steel's Many-one Rank Test when the number of replicates are not the same at each concentration. A Bonferroni's adjustment of the pairwise error rate for comparison of each concentration versus the control is used to set an upper bound of alpha on the overall error rate, in contrast to Steel's Many-one Rank Test, for which the overall error rate is fixed at alpha. Thus, Steel's Test is a more powerful test.

2. An example of the use of the Wilcoxon Rank Sum Test is provided in Table F.1. The data used in the example are the same as in Appendix E, except that two males are presumed to have occurred, one in the control and one in the 12% effluent concentration. Thus, there is unequal replication for the reproduction analysis.

3. For each concentration and control combination, combine the data and arrange the values in order of size, from smallest to largest. Assign ranks to the ordered observations (a rank of 1 to the smallest, 2 to the next smallest, etc.). If ties in rank occur, assign the average rank to each tied observation.

TABLE F.1. EXAMPLE OF WILCOXON'S RANK SUM TEST: DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, 7-DAY CHRONIC TEST

Effluent Concentration	Replicate 1	2	3	4	5	6	7	8	9	10	No. Live Adults
Cont	M	26	26	23	24	27	26	23	27	24	10
3%	13	15	14	13	23	26	0	25	26	27	9
6%	18	22	13	13	23	22	20	22	23	22	10
12%	14	22	20	23	M	23	25	24	25	21	10
25%	9	0	9	7	6	10	12	14	9	13	8
50%	0	0	0	0	0	0	0	0	0	0	0

4. An example of assigning ranks to the combined data for the control and 3% effluent concentration is given in Table F.2. This ranking procedure is repeated for each of the three remaining control versus test concentration combinations. The complete set of ranks is listed in Table F.3. The ranks are then summed for each effluent concentration, as shown in Table F.4.

5. For this set of data, determine if the reproduction in any of the effluent concentrations is significantly lower than the reproduction by the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum for the control. Thus, compare the rank sums for the reproduction of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the reproduction would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank in a test with four concentrations and nine replicates in the control is 72 for those concentrations with ten replicates, and 60 for those concentrations with nine replicates (see Table F.5, for K = 4).

6. Comparing the rank sums in Table F.4 to the appropriate critical rank, the 6%, 12% and 25% effluent concentrations are found to be significantly different from the control. Thus, the NOEC and LOEC for reproduction are 3% and 6%, respectively.

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TABLE F.2. EXAMPLE OF WILCOXON'S RANK SUM TEST: ASSIGNING RANKS TO THE CONTROL AND EFFLUENT CONCENTRATIONS

Rank	Number of Young Produced	Control or % Effluent
1	0	3
2.5	13	3
2.5	13	3
4	14	3
5	15	3
7	23	Control
7	23	Control
7	23	3
9.5	24	Control
9.5	24	Control
11	25	3
14	26	Control
14	26	Control
14	26	Control
14	26	3
14	26	3
18	27	Control
18	27	Control
18	27	3

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TABLE F.3. TABLE OF RANKS

Replicate (Organism)	Control[1]		Effluent Concentration (%) 3	6	12	25
1	M		13 (2.5)	18 (3)	14 (1)	9 (5)
2	26	(14,16,15,16)	15 (5)	22 (6.5)	22 (4)	0 (1)
3	26	(14,16,15,16)	14 (4)	13 (1.5)	20 (2)	9 (5)
4	23	(7,10.5,6.5,11.5)	13 (2.5)	13 (1.5)	23 (6.5)	7 (3)
5	24	(9.5,13.5,10,13.5)	23 (7)	23 (10.5)	M	6 (2)
6	27	(18,18.5,17.5,18.5)	26 (14)	22 (6.5)	23 (6.5)	10 (7)
7	26	(14,16,15,16)	0 (1)	20 (4)	25 (12.5)	12 (8)
8	23	(7,10.5,6.5,11.5)	25 (11)	22 (6.5)	24 (10)	14 (10)
9	27	(18,18.5,17.5,18.5)	26 (14)	23 (10.5)	25 (12.5)	9 (5)
10	24	(9.5,13.5,10,13.5)	27 (18)	22 (6.5)	21 (3)	13 (9)

[1] Control ranks are given in the order of the concentration with which they were ranked.

TABLE F.4. RANK SUMS

Effluent Concentration	Rank Sum	No. of Replicates	Critical Rank Sum
3	79	10	72
6	57	10	72
12	58	9	60
25	55	10	72

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TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
1	3	6	10	16	23	30	39	49	59
	4	6	11	17	24	32	41	51	62
	5	7	12	19	26	34	44	54	66
	6	8	13	20	28	36	46	57	69
	7	8	14	21	29	39	49	60	72
	8	9	15	23	31	41	51	63	72
	9	10	16	24	33	43	54	66	79
	10	10	17	26	35	45	56	69	82
2	3	--	--	15	22	29	38	47	58
	4	--	10	16	23	31	40	49	60
	5	6	11	17	24	33	42	52	63
	6	7	12	18	26	34	44	55	66
	7	7	13	20	27	36	46	57	69
	8	8	14	21	29	38	49	60	72
	9	8	14	22	31	40	51	62	75
	10	9	15	23	32	42	53	65	78
3	3	--	--	--	21	29	37	46	57
	4	--	10	16	22	30	39	48	59
	5	--	11	17	24	32	41	51	62
	6	6	11	18	25	33	43	53	65
	7	7	12	19	26	35	45	56	68
	8	7	13	20	28	37	47	58	70
	9	7	13	21	29	39	49	61	73
	10	8	14	22	31	41	51	63	76

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TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
4	3	--	--	--	21	28	37	46	56
	4	--	--	15	22	30	38	48	59
	5	--	10	16	23	31	40	50	61
	6	6	11	17	24	33	42	52	64
	7	6	12	18	26	34	44	55	67
	8	7	12	19	27	36	46	57	69
	9	7	13	20	28	38	48	60	72
	10	7	14	21	30	40	50	62	75
5	3	--	--	--	--	28	36	46	56
	4	--	--	15	22	29	38	48	58
	5	--	10	16	23	31	40	50	61
	6	--	11	17	24	32	42	52	63
	7	6	11	18	25	34	43	54	66
	8	6	12	19	27	35	45	56	68
	9	7	13	20	28	37	47	59	71
	10	7	13	21	29	39	49	61	74
6	3	--	--	--	--	28	36	45	56
	4	--	--	15	21	29	38	47	58
	5	--	10	16	22	30	39	49	60
	6	--	11	16	24	32	41	51	63
	7	6	11	17	25	33	43	54	65
	8	6	12	18	26	35	45	56	68
	9	6	12	19	27	37	47	58	70
	10	7	13	20	29	38	49	60	73
7	3	--	--	--	--	--	36	45	56
	4	--	--	--	21	29	37	47	58
	5	--	--	15	22	30	39	49	60
	6	--	10	16	23	32	41	51	62
	7	--	11	17	25	33	43	53	65
	8	6	11	18	26	35	44	55	67
	9	6	12	19	27	36	46	58	70
	10	7	13	20	28	38	48	60	72

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TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicate Per Effluent Concentration							
		3	4	5	6	7	8	9	10
8	3	--	--	--	--	--	36	45	55
	4	--	--	--	21	29	37	47	57
	5	--	--	15	22	30	39	49	59
	6	--	10	16	23	31	40	51	62
	7	--	11	17	24	33	42	53	64
	8	6	11	18	25	34	44	55	67
	9	6	12	19	27	36	46	57	69
	10	6	12	19	28	37	48	59	72
9	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	30	39	48	59
	6	--	10	16	23	31	40	50	62
	7	--	10	17	24	33	42	52	64
	8	--	11	18	25	34	44	55	66
	9	6	11	18	26	35	46	57	69
	10	6	12	19	28	37	47	59	71
10	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	29	38	48	59
	6	--	10	16	23	31	40	50	61
	7	--	10	16	24	32	42	52	64
	8	--	11	17	25	34	43	54	66
	9	6	11	18	26	35	45	56	68
	10	6	12	19	27	37	47	58	71

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APPENDIX G

FISHER'S EXACT TEST

1. Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) is a statistical method based on the hypergeometric probability distribution that can be used to test if the proportion of successes is the same in two Bernoulli (binomial) populations. When used with the *Ceriodaphnia dubia* data, it provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a Bernoulli population. Additionally, since it is a conservative test, a pair-wise comparison error rate of 0.05 is suggested rather that an experiment-wise error rate.

2. The basis for Fisher's Exact Test is a 2×2 contingency table. However, in order to use this table the contingency table must be arranged in the format shown in Table G.1. From the 2×2 table, set up for the control and the concentration you wish to compare, you can determine statistical significance by looking up a value in the table provided later in this section.

TABLE G.1. FORMAT FOR CONTINGENCY TABLE

	Number of		
	Successes	Failures	Number of Observations
Row 1	a	A - a	A
Row 2	b	B - b	B
Total	a + b	[(A + B) - a - b]	A + B

3. Arrange the table so that the total number of observations for row one is greater than or equal to the total for row two (A $ B). Categorize a success such that the proportion of successes for row one is greater than or equal to the proportion of successes for row two (a/A $ b/B). For the *Ceriodaphnia dubia* survival data, a success may be 'alive' or 'dead', whichever causes a/A $ b/B. The test is then conducted by looking up a value in the table of significance levels of b and comparing it to the b value given in the contingency table. The table of significance levels of b is Table G.5. Enter Table G.5 in the section for A, subsection for B, and the line for a. If the b value of the contingency table is equal to or less than the integer in the column headed 0.05 in Table G.5, then the survival proportion for the effluent concentration is significantly different from the survival proportion for the control. A dash or absence of entry in Table G.5 indicates that no contingency table in that class is significant.

4. To illustrate Fisher's Exact Test, a set of survival data (Table G.2) from the daphnid, *Ceriodaphnia dubia*, survival and reproduction test will be used.

5. For each control and effluent concentration construct a 2x2 contingency table.

6. For the control and effluent concentration of 1% the appropriate contingency table for the test is given in Table G.3.

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TABLE G.2. EXAMPLE OF FISHER'S EXACT TEST: *CERIODAPHNIA DUBIA* MORTALITY DATA

Effluent Concentration (%)	No. Dead	Total[1]
Control	1	9
1	0	10
3	0	10
6	0	10
12	0	10
25	10	10

[1] Total number of live adults at the beginning of the test.

7. Since 10/10 > 8/9, the category 'alive' is regarded as a success. For A = 10, B = 9 and, a = 10, under the column headed 0.05, the value from Table G.5 is b = 5. Since the value of b (b = 8) from the contingency table (Table G.3), is greater than the value of b (b = 5) from Table G.5, the test concludes that the proportion of survival is not significantly different for the control and 1% effluent.

8. The contingency tables for the combinations of control and effluent concentrations of 3%, 6%, 12% are identical to Table G.3. The conclusion of no significant difference in the proportion of survival for the control and the level of effluent would also remain the same.

9. For the combination of control and 25% effluent, the contingency table would be constructed as Table G.4. The category 'dead' is regarded as a success, since 10/10 > 1/9. The b value (b = 1) from the contingency table (Table G.4) is less than the b value (b = 5) from the table of significance levels of b (Table G.5). Thus, the percent mortality for 25% effluent is significantly greater than the percent mortality for the control. Thus, the NOEC and LOEC for survival are 12% and 25%, respectively.

TABLE G.3. 2×2 CONTINGENCY TABLE FOR CONTROL AND 1% EFFLUENT

	Number of		Number of Observations
	Alive	Dead	
1% Effluent	10	0	10
Control	8	1	9
Total	18	1	19

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Table G.4. 2x2 CONTINGENCY TABLE FOR CONTROL AND 25% EFFLUENT

	Number of		Number of Observations
	Dead	Alive	
25% Effluent	10	0	10
Control	1	8	9
Total	11	8	19

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TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1]

	α	0-05	0-025	0-01	0-005
A=3 B=3	3	0 $_{-050}$			——
A=4 B=4	4	1 $_{-014}$	1 $_{-014}$	——	——
3	4	0 $_{-029}$	——	——	——
A=5 B=5	5	1 $_{-024}$	1 $_{-024}$	0 $_{-004}$	0 $_{-004}$
	4	0 $_{-024}$	1 $_{-024}$	——	——
4	5	1 $_{-048}$	0 $_{-008}$	0 $_{-008}$	——
	4	0 $_{-040}$	——	——	——
3	5	0 $_{-018}$	0 $_{-018}$	——	——
2	5	0 $_{-048}$	——	——	——
A=6 B=6	6	2 $_{-030}$	1 $_{-008}$	1 $_{-008}$	0 $_{-001}$
	5	1 $_{-040}$	0 $_{-008}$	0 $_{-008}$	——
	4	0 $_{-030}$	——	——	——
5	6	1 $_{-015}^{+}$	0 $_{-015}^{+}$	0 $_{-002}$	0 $_{-002}$
	5	0 $_{-013}$	0 $_{-013}$	——	——
	4	0 $_{-045}^{+}$	——	——	——
4	6	1 $_{-033}$	0 $_{-005}^{-}$	0 $_{-005}^{-}$	0 $_{-005}^{-}$
	5	0 $_{-024}$	0 $_{-024}$	——	——
3	6	0 $_{-012}$	0 $_{-012}$	——	——
	5	0 $_{-048}$	——	——	——
2	6	0 $_{-036}$	——	——	——
A=7 B=7	7	3 $_{-035}^{-}$	2 $_{-010}^{+}$	1 $_{-002}$	1 $_{-002}$
	6	1 $_{-015}^{-}$	1 $_{-015}^{-}$	0 $_{-002}$	0 $_{-002}$
	5	1 $_{-010}^{+}$	0 $_{-010}^{+}$	——	——
	4	0 $_{-035}$-	——	——	——
6	7	2 $_{-021}$	2 $_{-021}$	1 $_{-005}^{-}$	1 $_{-005}^{-}$
	6	1 $_{-025}^{+}$	0 $_{-004}$	0 $_{-004}$	0 $_{-004}$
	5	0 $_{-016}$	0 $_{-016}$	——	——
	4	0 $_{-049}$	——	——	——
5	7	2 $_{-045}^{+}$	1 $_{-010}^{+}$	0 $_{-001}$	0 $_{-001}$
	6	1 $_{-045}^{+}$	0 $_{-008}$	0 $_{-008}$	——
	5	0 $_{-027}$	——	——	——
	7	1 $_{-024}$	1 $_{-024}$	——	0 $_{-003}$
	6	0 $_{-015}^{+}$	0 $_{-015}^{+}$	0 $_{-003}$	——
	5	0 $_{-045}^{+}$	——	——	——
	7	0 $_{-008}$	0 $_{-008}$	0 $_{-008}$	——
	6	0 $_{-033}$	——	——	——
	7	0 $_{-028}$	——	——	——

	α	0-05	0-025	0-01	0-005
A=8	8	4 $_{-038}$	3 $_{-013}$	2 $_{-003}$	2 $_{-003}$
	7	2 $_{-020}$	2 $_{-020}$	1 $_{-005}^{+}$	0 $_{-001}$
	6	1 $_{-020}$	1 $_{-020}$	0 $_{-003}$	0 $_{-003}$
	5	0 $_{-013}$	0 $_{-013}$		
	4	0 $_{-038}$			
7	8	3 $_{-026}$	2 $_{-007}$	2 $_{-007}$	1 $_{-001}$
	7	2 $_{-035}^{-}$	1 $_{-009}$	1 $_{-009}$	0 $_{-001}$
	6	1 $_{-032}$	0 $_{-006}$	0 $_{-006}$	
	5	0 $_{-019}$	0 $_{-019}$		
6	8	2 $_{-015}^{-}$	2 $_{-015}^{-}$	1 $_{-003}$	1 $_{-003}$
	7	1 $_{-016}$	1 $_{-016}$	0 $_{-002}$	0 $_{-002}$
	6	0 $_{-009}$	0 $_{-009}$	0 $_{-009}$	
	5	0 $_{-028}$	——	——	
5	8	2 $_{-035}^{-}$	1 $_{-007}$	1 $_{-007}$	0 $_{-001}$
	7	1 $_{-032}$	0 $_{-005}^{-}$	0 $_{-005}^{-}$	0 $_{-005}^{-}$
	6	0 $_{-016}$	0 $_{-016}$	——	
	5	0 $_{-044}$	——	——	
4	8	1 $_{-018}$	1 $_{-018}$	0 $_{-002}$	0 $_{-002}$
	7	0 $_{-010}^{+}$	0 $_{-010}^{+}$	——	
	6	0 $_{-030}$	——	——	
3	8	0 $_{-006}$	0 $_{-006}$	0 $_{-006}$	
	7	0 $_{-024}$	0 $_{-024}$	——	
2	8	0 $_{-022}$	0 $_{-022}$	——	
A=9	9	5 $_{-041}$	4 $_{-015}^{-}$	3 $_{-005}^{-}$	3 $_{-005}^{-}$
	8	3 $_{-025}^{-}$	3 $_{-025}^{-}$	2 $_{-008}$	1 $_{-002}$
	7	2 $_{-028}$	1 $_{-008}$	1 $_{-008}$	0 $_{-001}$
	6	1 $_{-025}^{-}$	1 $_{-025}^{-}$	0 $_{-005}^{-}$	0 $_{-005}^{-}$
	5	0 $_{-015}^{-}$	0 $_{-015}^{-}$	——	
	4	0 $_{-041}$	——	——	
8	9	4 $_{-029}$	3 $_{-009}$	3 $_{-009}$	2 $_{-002}$
	8	3 $_{-043}$	2 $_{-013}$	1 $_{-003}$	1 $_{-003}$
	7	2 $_{-044}$	1 $_{-012}$	0 $_{-002}$	0 $_{-002}$
	6	1 $_{-036}$	0 $_{-007}$	0 $_{-007}$	
	5	0 $_{-020}$	0 $_{-020}$	——	
7	9	3 $_{-019}$	3 $_{-019}$	2 $_{-005}$	2 $_{-005}^{-}$
	8	2 $_{-024}$	2 $_{-024}$	1 $_{-006}$	0 $_{-001}$
	7	1 $_{-020}$	1 $_{-020}$	0 $_{-003}$	0 $_{-003}$
	6	0 $_{-010}^{+}$	0 $_{-010}^{+}$	——	
	5	0 $_{-029}$	——	——	
6	9	3 $_{-044}$	2 $_{-011}$	1 $_{-002}$	1 $_{-002}$
	8	2 $_{-047}$	1 $_{-011}$	0 $_{-001}$	0 $_{-001}$
	7	1 $_{-035}^{-}$	0 $_{-006}$	0 $_{-006}$	
	6	0 $_{-017}$	0 $_{-017}$	——	
	5	0 $_{-042}$	——	——	

[1] The table shows:(1) In bold type, for given a, A and B, the value of b ([a) which is just significant at the probability level quoted (one-tailed test); and (2) In small type, for given A, B and r = a + b, the exact probability (if there is independence) that b is equal to or less than the integer shown in bold type. From Pearson and Hartley (1962).

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TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability			
		0-05	0-025	0-01	0-005
A=9 B=5	9	2 -027	1 -005⁻	1 -005⁻	1 -005⁻
	8	1 -023	1 -023	0 -003	0 -003
	7	0 -	0 -010⁺	——	——
	6	0 -028	——	——	——
4	9	1 -014	1 -014	0 -001	0 -001
	8	0 -007	0 -007	0 -007	——
	7	0 -021	0 -021	——	——
	6	0 -049	——	0 -005	——
3	9	1 -	0 -005⁻	0 -005⁻	0 -005⁻
	8	0 -018	0 -018	——	——
	7	0 -	——	——	——
2	9	0 -018	0 -018	——	——
A=10 B=10	10	6 -043	5 -016	4 -005⁺	3 -002
	9	4 -029	3 -010⁻	3 -010	2 -003
	8	3 -	2 -012	1 -003	1 -003
	7	2 -	1 -010⁻	1 -010⁻	0 -002
	6	1 -029	0 -005⁺	0 -005⁺	——
	5	0 -016	0 -016	——	——
	4	0 -043	——	——	——
9	10	5 -033	4 -011	3 -003	3 -003
	9	4 -	3 -017	2 -005⁻	2 -005⁻
	8	2 -019	2 -019	1 -004	1 -004
	7	1 -	1 -015⁻	0 -002	0 -002
	6	1 -040	0 -008	0 -008	——
	5	0 -022	0 -022	——	——
8	10	4 -023	4 -023	3 -007	2 -002
	9	3 -032	2 -009	2 -009	1 -002
	8	2 -031	1 -008	1 -008	0 -001
	7	1 -023	1 -023	0 -004	0 -004
	6	0 -011	0 -011	——	——
	5	0 -029	——	——	——
7	10	3 -	3 -015⁻	2 -003	2 -003
	9	2 -018	2 -018	1 -004	1 -004
	8	1 -013	1 -013	0 -002	0 -002
	7	1 -036	0 -006	0 -006	——
	6	0 -017	0 -017	——	——
	5	0 -041	——	——	——
6	10	3 -036	2 -008	2 -008	1 -001
	9	2 -036	1 -008	1 -008	0 -001
	8	1 -024	1 -024	0 -003	0 -003
	7	0 -	0 -010⁺	——	——
	6	0 -026	——	——	——
5	10	2 -022	2 -022	1 -004	1 -004
	9	1 -017	1 -017	0 -002	0 -002
	8	1 -	0 -007	0 -007	——
	7	0 -	0 -019	——	——
	6	0 -	——	——	——

	α	Probability			
		0-05	0-025	0-01	0-005
A=10 B=4	10	1 -011	1 -011	0 -001	0 -001
	9	1 -041	0 -005⁻	0 -005⁻	0 -005⁻
	8	0 -	0 -015⁻	——	——
	7	0 -	——	——	——
3	10	1 -038	0 -003	0 -003	0 -003
	9	0 -014	0 -014	——	——
	8	0 -	——	——	——
2	10	0 -	0 -015⁺	——	——
	9	0 -	——	——	——
A=11 B=11	11	7 -	6 -018	5 -006	4 -002
	10	5 -032	4 -012	3 -004	3 -004
	9	4 -040	3 -015⁻	2 -004	2 -004
	8	3 -043	2 -015⁻	1 -004	1 -004
	7	2 -040	1 -012	0 -002	0 -002
	6	1 -032	0 -006	0 -006	——
	5	0 -018	0 -018	——	——
	4	0 -	——	——	——
10	11	6 -	5 -012	4 -004	4 -004
	10	4 -021	4 -021	3 -007	2 -002
	9	3 -024	3 -024	2 -007	1 -002
	8	2 -023	2 -023	1 -006	0 -001
	7	1 -017	1 -017	0 -003	0 -003
	6	1 -043	0 -009	0 -009	——
	5	0 -023	0 -023	——	——
9	11	5 -026	4 -008	4 -008	3 -002
	10	4 -038	3 -012	2 -003	2 -003
	9	3 -040	2 -012	1 -003	1 -003
	8	2 -	1 -009	1 -009	0 -001
	7	1 -	1 -025⁻	0 -004	0 -004
	6	0 -012	0 -012	——	——
	5	0 -030	——	——	——
8	11	4 -018	4 -018	3 -005⁻	3 -005⁻
	10	3 -024	3 -024	2 -006	1 -001
	9	2 -022	2 -022	1 -005⁻	1 -005⁻
	8	1 -	1 -015⁻	0 -002	0 -002
	7	1 -037	0 -007	0 -007	——
	6	0 -017	0 -017	——	——
	5	0 -040	——	——	——
7	11	4 -043	3 -011	2 -002	2 -002
	10	3 -047	2 -013	1 002	1 -002
	9	2 -039	1 -009	1 -009	0 -001
	8	1 -	1 -025⁻	0 -004	0 -004
	7	0 -	0 -010⁺	——	——
	6	0 -	0 -025⁻	——	——
6	11	3 -	2 006	2 -006	1 -001
	10	2 -	1 -005⁺	1 -005⁺	0 -001
	9	1 -	1 -018	0 -002	0 -002

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TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability			
		0-05	0-025	0-01	0-005
A=11 B=6	8	1 -043	0 -007	0 -007	——
	7	0 -017	0 -017	——	——
	6	0 -037	——	——	——
5	11	2 -018	2 -018	1 -003	1 -003
	10	1 -013	1 -013	0 -001	0 -001
	9	1 -036	0 -005⁻	0 -005⁻	0 -005⁻
	8	0 -013	0 -013	——	——
	7	0 -029	——	——	——
4	11	1 -009	1 -009	1 -009	0 -001
	10	1 -033	0 -004	0 -004	0 -004
	9	0 -011	0 -011	——	——
	8	0 -026	——	——	——
3	11	1 -033	0 -003	0 -003	0 -003
	10	0 -011	0 -011	——	——
	9	0 -027	——	——	——
2	11	0 -013	0 -013	——	——
	10	0 -038	——	——	——
A=12 B=12	12	8 -047	7 -019	6 -007	5 -002
	11	6 -034	5 -014	4 -005⁻	4 -005⁻
	10	5 -045⁻	4 -018	3 -006	2 -002
	9	4 -050⁻	3 -020	2 -006	1 -001
	8	3 -050⁻	2 -018	1 -005⁻	1 -005⁻
	7	2 -045⁻	1 -014	0 -002	0 -002
	6	1 -034	0 -007	0 -007	——
	5	0 -019	0 -019	——	——
	4	0 -047	——	——	——
11	12	7 -037	6 -014	5 -005⁻	5 -005⁻
	11	5 -024	5 -024	4 -008	3 -002
	10	4 -029	3 -010⁺	2 -003	2 -003
	9	3 -030	2 -009	2 -009	1 -002
	8	2 -026	1 -007	1 -007	0 -001
	7	1 -019	1 -019	0 -003	0 -003
	6	1 -045⁻	0 -009	0 -009	——
	5	0 -024	0 -024	——	——
10	12	6 -029	5 -010	5 -010⁻	4 -003
	11	5 -043	4 -015⁺	3 -005⁻	3 -005⁻
	10	4 -048	3 -017	2 -005⁻	2 -005⁻
	9	3 -046	2 -015⁻	1 -004	1 -004
	8	2 -038	1 -010⁺	0 -002	0 -002
	7	1 -026	0 -005⁻	0 -005⁻	0 -005-
	6	0 -012	0 -012	——	——
	5	0 -030	——	——	——
9	12	5 -021	5 -021	4 -006	3 -002
	11	4 -029	3 -009	3 -009	2 -002
	10	3 -029	2 -008	2 -008	1 -002
	9	2 -024	2 -024	1 -006	0 -001
	8	1 -016	1 -016	0 -002	0 -002

	α	Probability			
		0-05	0-025	0-01	0-005
A=12 B=9	7	1 -037	0 -007	0 -007	——
	6	0 -017	0 -017	——	——
	5	0 -039	——	——	——
8	12	5 -049	4 -014	3 -004	3 -004
	11	3 -018	3 -018	2 -004	2 -004
	10	2 -015⁺	2 -015⁺	1 -003	1 -003
	9	2 -040	1 -010⁻	1 -010⁻	0 -001
	8	1 -025⁻	1 -025⁻	0 -004	0 -004
	7	0 -010⁺	0 -010⁺	——	——
	6	0 -024	0 -024	——	——
7	12	4 -036	3 -009	3 -009	2 -002
	11	3 -038	2 -010⁻	2 -010⁻	1 -002
	10	2 -029	1 -006	1 -006	0 -001
	9	1 -017	1 -017	0 -002	0 -002
	8	1 -040	0 -007	0 -007	——
	7	0 -016	0 -016	——	——
	6	0 -034	——	——	——
6	12	3 -025⁻	3 -025⁻	2 -005⁻	2 -005⁻
	11	2 -022	2 -022	1 -004	1 -004
	10	1 -013	1 -013	0 -002	0 -002
	9	1 -032	0 -005⁻	0 -005⁻	0 -005⁻
	8	0 -011	0 -011	——	——
	7	0 -025⁻	0 -025⁻	——	——
	6	0 -050⁻	——	——	——
5	12	2 -015⁻	2 -015	1 -002	1 -002
	11	1 -010⁻	1 -010⁻	1 -010⁻	0 -001
	10	1 -028	0 -003	0 -003	0 -003
	9	0 -009	0 -009	0 -009	——
	8	0 -020	0 -020	——	——
	7	0 -041	——	——	——
4	12	2 -050	1 -007	1 -007	0 -001
	11	1 -027	0 -003	0 -003	0 -003
	10	0 -008	0 -008	0 -008	——
	9	0 -019	0 -019	——	——
	8	0 -038	——	——	——
3	12	1 -029	0 -002	0 -002	0 -002
	11	0 -009	0 -009	0 -009	——
	10	0 -022	0 -022	——	——
	9	0 -044	——	——	——
2	12	0 -011	0 -011	——	——
	11	0 -033	——	——	——
A=13 B=13	13	9 -048	8 -020	7 -007	6 -003
	12	7 -037	6 -015⁺	5 -006	4 -002
	11	6 -048	5 -021	4 -008	3 -002
	10	4 -024	4 -024	3 -008	2 -002
	9	3 -024	3 -024	2 -008	1 -002
	8	2 -021	2 -021	1 -006	0 -001

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TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability			
		0-05	0-025	0-01	0-005
A=13 B=13	7	2 -048	1 -015+	0 -003	0 -003
	6	1 -037	0 -007	0 -007	——
	5	0 -020	0 -020	——	——
	4	0 -048		——	——
12	1	8 -039	7 -015-	6 -005+	5 -002
	1	6 -027	5 -010-	5 -010-	4 -003
	1	5 -033	4 -013	3 -004	3 -004
	1	4 -036	3 -013	2 -004	2 -004
	9	3 -034	2 -011	1 -003	1 -003
	8	2 -029	1 -008	1 -008	0 -001
	7	1 -020	1 -020	0 -004	0 -004
	6	1 -046	0 -010-	0 -010-	——
	5	0 -024	0 -024	——	——
11	1	7 -031	6 -011	5 -003	5 -003
	1	6 -048	5 -018	4 -006	3 -002
	1	4 -021	4 -021	3 -007	2 -002
	1	3 -021	3 -021	2 -006	1 -001
	9	3 -050-	2 -017	1 -004	1 -004
	8	2 -040	1 -011	0 -002	0 -002
	7	1 -027	0 -005-	0 -005-	0 -005-
	6	0 -013	0 -013	——	——
		0 -030		——	——
10	1	6 -024	6 -024	5 -007	4 -002
	1	5 -035-	4 -012	3 -003	3 -003
	1	4 -037	3 -012	2 -003	2 -003
	1	3 -033	2 -010+	1 -002	1 -002
	9	2 -026	1 -006	1 -006	0 -001
	8	1 -017	1 -017	0 -003	0 -003
	7	1 -038	0 -007	0 -007	——
	6	0 -017	0 -017	——	——
	5	0 -038		——	——
9	1	5 -017	5 -017	4 -005-	4 -005-
	1	4 -023	4 -023	3 -007	2 -001
	1	3 -022	3 -022	2 -006	1 -001
	1	2 -017	2 -017	1 -004	1 -004
	9	2 -040	1 -010+	0 -001	0 -001
	8	1 -025-	1 -025-	0 -004	0 -004
	7	0' -010+	0 -010+	——	——
	6	0 -023	0 -023	——	——
	5	0 -049		——	——
8	1	5 -042	4 -012	3 -003	3 -003
	1	4 -047	3 -014	2 -003	2 -003
	1	3 -041	2 -011	1 -002	1 -002
	1	2 -029	1 -007	1 -007	0 -001
	9	1 -017	1 -017	0 -002	0 -002
	8	1 -037	0 -006	0 -006	——
	7	0 -015-	0 -015-	——	——
	6	0 -032		——	——
7	1	4 -031	3 -007	3 -007	2 -001
	1	3 -031	2 -007	2 -007	1 -001

	α	Probability			
		0-05	0-025	0-01	0-005
A=13 B=7	11	2 -022	2 -022	1 -004	1 -004
	10	1 -012	1 -012	0 -002	0 -002
	9	1 -029	0 -004	0 -004	0 -004
	8	0 -010+	0 -010+	——	——
	7	0 -022	0 -022	——	——
	6	0 -044	——	——	——
6	13	3 -021	3 -021	2 -004	2 -004
	12	2 -017	2 -017	1 -003	1 -003
	11	2 -046	1 -010-	1 -010-	0 -001
	10	1 -024	1 -024	0 -003	0 -003
	9	1 -050-	0 -008	0 -008	——
	8	0 -017	0 -017	——	——
	7	0 -034	——	——	——
5	13	2 -012	2 -012	1 -002	1 -002
	12	2 -044	1 -008	1 -008	0 -001
	11	1 -022	1 -022	0 -002	0 -002
	10	1 -047	0 -007	0 -007	——
	9	0 -015-	0 -015-	——	——
	8	0 -029	——	——	——
4	13	2 -044	1 -006	1 -006	0 -000
	12	1 -022	1 -022	0 -002	0 -002
	11	0 -006	0 -006	0 -006	——
	10	0 -015-	0 -015-	——	——
	9	0 -029	——	——	——
3	13	1 -025	1 -025	0 -002	0 -002
	12	0 -007	0 -007	0 -007	——
	11	0 -018	0 -018	——	——
	10	0 -036	——	——	——
2	13	0 -010-	0 -010-	0 -010-	——
	12	0 -029	——	——	——
A=14	14	10 -049	9 -020	8 -008	7 -003
	13	8 -038	7 -016	6 -006	5 -002
	12	6 -023	6 -023	5 -009	4 -003
	11	5 -027	4 -011	3 -004	3 -004
	10	4 -028	3 -011	2 -003	2 -003
	9	3 -027	2 -009	2 -009	1 -002
	8	2 -023	2 -023	1 -006	0 -001
	7	1 -016	1 -016	0 -003	0 -003
	6	1 -038	0 -008	0 -008	——
	5	0 -020	0 -020	——	——
	4	0 -049	——	——	——
13	14	9 -041	8 -016	7 -006	6 -002
	13	7 -029	6 -011	5 -004	5 -004
	12	6 -037	5 -015+	4 -005+	3 -002
	11	5 -041	4 -017	3 -006	2 -001
	10	4 -041	3 -016	2 -005-	2 -005-
	9	3 -038	2 -013	1 -003	1 -003
	8	2 -031	1 -009	1 -009	0 -001

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TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=14	7	1 -021	1 -021	0 -004	0 -004	A=14 B=7	14	4 -026	3 -006	3 -006	2 -001
	6	1 -048	0 -010+	——	——		13	3 -025	2 -006	2 -006	1 -001
	5	0 -025-	0 -025-	——	——		12	2 -017	2 -017	1 -003	1 -003
12	1	8 -033	7 -012	6 -004	6 -004		11	2 -041	1 -009	1 -009	0 -001
	1	6 -021	6 -021	5 -007	4 -002		10	1 -021	1 -021	0 -003	0 -003
	1	5 -025+	4 -009	4 -009	3 -003		9	1 -043	0 -007	0 -007	——
	1	4 -026	3 -009	3 -009	2 -002		8	0 -015-	0 -015-	——	——
	1	3 -024	3 -024	2 -007	1 -002		7	0 -030	——	——	——
	9	2 -019	2 -019	1 -005-	1 -005-	6	14	3 -018	3 -018	2 -003	2 -003
	8	2 -042	1 -012	0 -002	0 -002		13	2 -014	2 -014	1 -002	1 -002
	7	1 -028	0 -005+	0 -005+	——		12	2 -037	1 -007	1 -007	0 -001
	6	0 -013	0 -013	——	——		11	1 -018	1 -018	0 -002	0 -002
	5	0 -030	——	——	——		10	1 -038	0 -005+	0 -005+	——
11	1	7 -026	6 -009	6 -009	5 -003		9	0 -012	0 -012	——	——
	1	6 -039	5 -014	4 -004	4 -004		8	0 -024	0 -024	——	——
	1	5 -043	4 -016	3 -005-	3 -005-		7	0 -044	——	——	——
	1	4 -042	3 -015-	2 -004	2 -004	5	14	2 -010+	2 -010+	1 -001	1 -001
	1	3 -036	2 -011	1 -003	1 -003		13	2 -037	1 -006	1 -006	0 -001
	9	2 -027	1 -007	1 -007	0 -001		12	1 -017	1 -017	0 -002	0 -002
	8	1 -017	1 -017	0 -003	0 -003		11	1 -038	0 -005-	0 -005-	0 -005-
	7	1 -038	0 -007	0 -007	——		10	0 -011	0 -011	——	——
	6	0 -017	0 -017	——	——		9	0 -022	0 -022	——	——
	5	0 -038	——	——	——		8	0 -040	——	——	——
10	1	6 -020	6 -020	5 -006	4 -002	4	14	2 -039	1 -005-	1 -005-	1 -005-
	1	5 -028	4 -009	4 -009	3 -002		13	1 -019	1 -019	0 -002	0 -002
	1	4 -028	3 -009	3 -009	2 -002		12	1 -044	0 -005-	0 -005-	0 -005-
	1	3 -024	3 -024	2 -007	1 -001		11	0 -011	0 -011	——	——
	1	2 -018	2 -018	1 -004	1 -004		10	0 -023	0 -023	——	——
	9	2 -040	1 -011	0 -002	0 -002		9	0 -041	——	——	——
	8	1 -024	1 -024	0 -004	0 -004	3	14	1 -022	1 -022	0 -001	0 -001
	7	0 -010-	0 -010-	0 -010-	——		13	0 -006	0 -006	0 -006	——
	6	0 -022	0 -022	——	——		12	0 -015-	0 -015-	——	——
	5	0 -047	——	——	——		11	0 -029	——	——	——
9	1	6 -047	5 -014	4 -004	4 -004	2	14	0 -008	0 -008	0 -008	——
	1	4 -018	4 -018	3 -005-	3 -005-		13	0 -025	0 -025	——	——
	1	3 -017	3 -017	2 -004	2 -004		12	0 -050	——	——	——
	1	3 -042	2 -012	1 -002	1 -002						
	1	2 -029	1 -007	1 -007	0 -001						
	9	1 -017	1 -017	0 -002	0 -002						
	8	1 -036	0 -006	0 -006	——	A=15 B=15	15	11 -050-	10 -021	9 -008	8 -003
	7	0 -014	0 -014	——	——		14	9 -040	8 -018	7 -007	6 -003
	6	0 -030	——	——	——		13	7 -025+	6 -010+	5 -004	5 -004
8	1	5 -036	4 -010-	4 -010-	3 -002		12	6 -030	5 -013	4 -005-	4 -005-
	1	4 -039	3 -011	2 -002	2 -002		11	5 -033	4 -013	3 -005-	3 -005-
	1	3 -032	2 -008	2 -008	1 -001		10	4 -033	3 -013	2 -004	2 -004
	1	2 -022	2 -022	1 -005-	1 -005-		9	3 -030	2 -010+	1 -003	1 -003
	1	2 -048	1 -012	0 -002	0 -002		8	2 -025+	1 -007	1 -007	0 -001
	9	1 -026	0 -004	0 -004	0 -004		7	1 -018	1 -018	0 -003	0 -003
	8	0 -009	0 -009	0 -009	——		6	1 -040	0 -008	0 -008	——
	7	0 -020	0 -020	——	——		5	0 -021	0 -012	——	——
	6	0 -040	——	——	——		4	0 -050-	——	——	——

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TABLE G.5. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE) AND CORRESPONDING PROBABILITIES (SMALL TYPE)[1] (CONTINUED)

	α	Probability					α	Probability			
		0-05	0-025	0-01	0-005			0-05	0-025	0-01	0-005
A=15 B=14	15	10 -042	9 -017	8 -006	7 -002	A=15 B=9	13	4 -042	3 -013	2 -003	2 -003
	14	8 -031	7 -013	6 -005-	6 -005-		12	3 -032	2 -009	2 -009	1 -002
	13	7 -041	6 -017	5 -007	4 -002		11	2 -021	2 -021	1 -005-	1 -005-
	12	6 -046	5 -020	4 -007	3 -002		10	2 -045-	1 -011	0 -002	0 -002
	11	5 -048	4 -020	3 -007	2 -002		9	1 -024	1 -024	0 -004	0 -004
	10	4 -046	3 -018	2 -006	1 -001		8	1 -048	0 -009	0 -009	—
	9	3 -041	2 -014	1 -004	1 -004		7	0 -019	0 -019	—	—
	8	2 -033	1 -009	1 -009	0 -001		6	0 -037	—	—	—
	7	1 -022	1 -022	0 -004	0 -004	8	15	5 -032	4 -008	4 -008	3 -002
	6	1 -049	0 -011	—	—		14	4 -033	3 -009	3 -009	2 -002
	5	0 -025+	—	—	—		13	3 -026	2 -006	2 -006	1 -001
13	15	9 -035-	8 -013	7 -005-	7 -005-		12	2 -017	2 -017	1 -003	1 -003
	14	7 -023	7 -023	6 -009	5 -003		11	2 -037	1 -008	1 -008	0 -001
	13	6 -029	5 -011	4 -004	4 -004		10	1 -019	1 -019	0 -003	0 -003
	12	5 -031	4 -012	3 -004	3 -004		9	1 -038	0 -006	0 -006	—
	11	4 -030	3 -011	2 -003	2 -003		8	0 -013	0 -013	—	—
	10	3 -026	2 -008	2 -008	1 -002		7	0 -026	—	—	—
	9	2 -020	2 -020	1 -005+	0 -001		6	0 -050-	—	—	—
	8	2 -043	1 -013	0 -002	0 -002	7	15	4 -023	4 -023	3 -005-	3 -005-
	7	1 -029	0 -005+	0 -005+	—		14	3 -021	3 -021	2 -004	2 -004
	6	0 -013	0 -013	—	—		13	2 -014	2 -014	1 -002	1 -002
	5	0 -031	—	—	—		12	2 -032	1 -007	1 -007	0 -001
12	15	8 -028	7 -010-	7 -010-	6 -003		11	1 -015+	1 -015+	0 -002	0 -002
	14	7 -043	6 -016	5 -006	4 -002		10	1 -032	0 -005-	0 -005-	0 -005-
	13	6 -049	5 -019	4 -007	3 -002		9	0 -010+	0 -010+	—	—
	12	5 -049	4 -019	3 -006	2 -002		8	0 -020	0 -020	—	—
	11	4 -045+	3 -017	2 -005-	2 -005-		7	0 -038	—	—	—
	10	3 -038	2 -012	1 -003	1 -003	6	15	3 -015+	3 -015+	2 -003	2 -003
	9	2 -028	1 -007	1 -007	0 -001		14	2 -011	2 -011	1 -002	1 -002
	8	1 -018	1 -018	0 -003	0 -003		13	2 -031	1 -006	1 -006	0 -001
	7	1 -038	0 -007	0 -007	—		12	1 -014	1 -014	0 -002	0 -002
	6	0 -017	0 -017	—	—		11	1 -029	0 -004	0 -004	0 -004
	5	0 -037	—	—	—		10	0 -009	0 -009	0 -009	—
11	15	7 -022	7 -022	6 -007	5 -002		9	0 -017	0 -017	—	—
	14	6 -032	5 -011	4 -003	4 -003		8	0 -032	—	—	—
	13	5 -034	4 -012	3 -003	3 -003	5	15	2 -009	2 -009	2 -009	1 -001
	12	4 -032	3 -010+	2 -003	2 -003		14	2 -032	1 -005-	1 -005-	1 -005-
	11	3 -026	2 -008	2 -008	1 002		13	1 -014	1 -014	0 -001	0 -001
	10	2 -019	2 -019	1 -004	1 -004		12	1 -031	0 -004	0 -004	0 -004
	9	2 -040	1 -011	0 -002	0 -002		11	0 -008	0 -008	0 -008	—
	8	1 -024	1 -024	0 -004	0 -004		10	0 -016	0 -016	—	—
	7	1 -049	0 -010-	0 -010-	—		9	0 -030	—	—	—
	6	0 -022	0 -022	—	—	4	15	2 -035+	1 -004	1 -004	1 -004
	5	0 -046	—	—	—		14	1 -016	1 -016	0 -001	0 -001
10	15	6 -017	6 -017	5 -005-	5 -005-		13	1 -037	0 -004	0 -004	0 -004
	14	5 -023	5 -023	4 -007	3 -002		12	0 -009	0 -009	0 -009	—
	13	4 -022	4 -022	3 -007	2 -001		11	0 -018	0 -018	—	—
	12	3 -018	3 -018	2 -005-	2 -005-		10	0 -033	—	—	—
	11	3 -042	2 -013	1 -003	1 -003	3	15	1 -020	1 -020	0 -001	0 -001
	10	2 -029	1 -007	1 -007	0 -001		14	0 -005-	0 -005-	0 -005-	0 -005-
	9	1 -016	0 -016	0 -002	0 -002		13	0 -012	0 -012	—	—
	8	1 -034	0 -006	0 -006	—		12	0 -025-	0 -025-	—	—
	7	0 -013	1 -013	—	—		11	0 -043	—	—	—
	6	0 -028	—	—	—	2	15	0 -007	0 -007	0 -007	—
9	15	6 -042	5 -012	4 -003	4 -003		14	0 -022	0 -022	—	—
	14	5 -047	4 -015-	3 -004	3 -004		13	0 -044	—	—	—

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APPENDIX H

SINGLE CONCENTRATION TOXICITY TEST - COMPARISON OF CONTROL WITH 100% EFFLUENT OR RECEIVING WATER

1. To statistically compare a control with one concentration, such as 100% effluent or the instream waste concentration, a t-test is the recommended analysis. The t-test is based on the assumptions that the observations are independent and normally distributed and that the variances of the observations are equal between the two groups.

2. Shapiro Wilk's test may be used to test the normality assumption (see Appendix B for details). If the data do not meet the normality assumption, the nonparametric test, Wilcoxon's Rank Sum Test, may be used to analyze the data. An example of this test is given in Appendix F. Since a control and one concentration are being compared, the K = 1 section of Table F.5 contains the needed critical values.

3. The F test for equality of variances is used to test the homogeneity of variance assumption. When conducting the F test, the alternative hypothesis of interest is that the variances are not equal.

4. To make the two-tailed F test at the 0.01 level of significance, put the larger of the two variances in the numerator of F.

$$F = \frac{S_1^2}{S_2^2} \text{ where } S_1^2 > S_2^2$$

5. Compare F with the 0.005 level of a tabled F value with $n_1 - 1$ and $n_2 - 1$ degrees of freedom, where n_1 and n_2 are the number of replicates for each of the two groups.

6. A set of *Ceriodaphnia dubia* reproduction data from an effluent screening test will be used to illustrate the F test. The raw data, mean and variance for the control and 100% effluent are given in Table H.1.

TABLE H.1. *CERIODAPHNIA DUBIA* REPRODUCTION DATA FROM AN EFFLUENT SCREENING TEST

	Replicate											
	1	2	3	4	5	6	7	8	9	10	$\bar{X}$	S^2
Control	36	38	35	35	28	41	37	33	.	.	35.4	14.5
100% Effluent	23	14	21	7	12	17	23	8	18	.	15.9	36.6

7. Since the variability of the 100% effluent is greater than the variability of the control, S^2 for the 100% effluent concentration is placed in the numerator of the F statistic and S^2 for the control is placed in the denominator.

$$F = \frac{36.61}{14.55}$$

8. There are 9 replicates for the effluent concentration and 8 replicates for the control. Thus, the numerator degrees of freedom is 8 and the denominator degrees of freedom is 7. For a two-tailed test at the 0.01 level of

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significance, the critical F value is obtained from a table of the F distribution (Snedecor and Cochran, 1980). The critical F value for this test is 8.68. Since 2.52 is not greater than 8.68, the conclusion is that the variances of the control and 100% effluent are homogeneous.

9. EQUAL VARIANCE T-TEST

9.1 To perform the t-test, calculate the following test statistic:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where: $\bar{Y}_1$ = Mean for the control

$\bar{Y}_2$ = Mean for the effluent concentration

$$S_p = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}$$

S_1^2 = Estimate of the variance for the control

S_2^2 = Estimate of the variance for the effluent concentration

n_1 = Number of replicates for the control

n_2 = Number of replicates for the effluent concentration

9.2 Since we are usually concerned with a decreased response from the control, such as a decrease in survival or a decrease in reproduction, a one-tailed test is appropriate. Thus, compare the calculated t with a critical t, where the critical t is at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the calculated t exceeds the critical t, the mean responses are declared different.

9.3 Using the data from Table H.1 to illustrate the t-test, the calculation of t is as follows:

$$t = \frac{35.4 - 15.9}{5.13\sqrt{\frac{1}{8} + \frac{1}{9}}} = 7.82$$

Where:

$$S_p = \sqrt{\frac{(8 - 1)14.5 + (9 - 1)36.6}{(8 + 9 - 2)}} = 5.13$$

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9.4 For an 0.05 level of significance test with 15 degrees of freedom the critical t is 1.754 (Note: Table D.5 for K = 1 includes the critical t values for comparing two groups). Since 7.82 is greater than 1.754, the conclusion is that the reproduction in the 100% effluent concentration is significantly lower than the control reproduction.

10. UNEQUAL VARIANCE T-TEST

10.1 If the F test for equality of variance fails, the t-test is still a valid test. However, the denominator of the t statistic is adjusted as follows:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where: $\bar{Y}_1$ = Mean for the control

$\bar{Y}_2$ = Mean for the effluent concentration

S_1^2 = Estimate of the variance for the control

S_2^2 = Estimate of the variance for the effluent concentration

n_1 = Number of replicates for the control

n_2 = Number of replicates for the effluent concentration

10.2 Additionally, the degrees of freedom for the test are adjusted using the following formula:

$$df' = \frac{(n_1 - 1)(n_2 - 1)}{(n_2 - 1)C^2 + (1 - C)^2(n_1 - 1)}$$

Where:

$$C = \frac{\frac{S_1^2}{n_1}}{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

10.3 The modified degrees of freedom is usually not an integer. Common practice is to round down to the nearest integer.

10.4 The t-test is then conducted as the equal variance t-test. The calculated t is compared to the critical t at the 0.05 significance level with the modified degrees of freedom. If the calculated t exceeds the critical t, the mean responses are found to be statistically different.

APPENDIX I

PROBIT ANALYSIS

1. This program calculates the EC1 and EC50 (or LC1 and LC50), and the associated 95% confidence intervals.

2. The program is written in IBM PC Basic for the IBM compatible PC by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled, executable version of the program can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

2.1 Data input is illustrated by a set of total mortality data (Figure I.1) from a fathead minnow embryo-larval survival and teratogenicity test. The program requests the following input:

1. Desired output of abbreviated (A) or full (F) output? (Note: only abbreviated output is shown below.)
2. Output designation (P = printer, D = disk file).
3. Title for the output.
4. The number of exposure concentrations.
5. Toxicant concentration data.

2.2 The program output for the abbreviated output includes the following:

1. A table of the observed proportion responding and the proportion responding adjusted for the controls (see Figure I.2).
2. The calculated chi-square statistic for heterogeneity and the tabular value. This test is one indicator of how well the data fit the model. The program will issue a warning when the test indicates that the data do not fit the model.
3. Estimated LC1 and LC50 values and associated 95% confidence intervals (see Figure I.2).

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USEPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Do you wish abbreviated (A) or full (F) input/output? A
Output to printer (P) or disk file (D)? P
Title ? Example of Probit Analysis

Number responding in the control group = ? 2
Number of animals exposed in the concurrent control group = ? 20
Number of exposure concentrations, exclusive of controls ? 5

Input data starting with the lowest exposure concentration

Concentration = ? 0.5
Number responding = ? 2
Number exposed = ? 20

Concentration = ? 1.0
Number responding = ? 1
Number exposed = ? 20

Concentration = ? 2.0
Number responding = ? 4
Number exposed = ? 20

Concentration = ? 4.0
Number responding = ? 16
Number exposed = ? 20

Concentration = ? 8.0
Number responding = ? 20
Number exposed = ? 20

Number	Number Conc.	Number Resp.	Exposed
1	0.5000	2	20
2	1.0000	1	20
3	2.0000	4	20
4	4.0000	16	20
5	8.0000	20	20

Do you wish to modify your data ? N

The number of control animals which responded = 2
The number of control animals exposed = 20
Do you wish to modify these values ? N

Figure I.1. Sample Data Input for USEPA Probit Analysis program, Version 1.5.

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Example of Probit Analysis

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	20	2	0.1000	0.0000
0.5000	20	2	0.1000	0.0174
1.0000	20	1	0.0500	-.0372
2.0000	20	4	0.2000	0.1265
4.0000	20	16	0.8000	0.7816
8.0000	20	20	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 0.441
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 7.815

Example of Probit Analysis

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	Lower 95% Confidence	Upper Limits
LC/EC 1.00	1.346	0.453	1.922
LC/EC 50.00	3.018	2.268	3.672

Figure I.2. USEPA Probit Analysis Program Used for Calculating LC/EC Values, Version 1.5.

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APPENDIX J

SPEARMAN-KARBER METHOD

1. The Spearman-Karber Method is a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978). The Spearman-Karber Method estimates the mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Spearman-Karber Method is recommended when partial mortalities occur in the test solutions, but the data do not fit the Probit model.

4. To calculate the LC50 using the Spearman-Karber Method, the following must be true: 1) the smoothed adjusted proportion mortality for the lowest effluent concentration (not including the control) must be zero, and 2) the smoothed adjusted proportion mortality for the highest effluent concentration must be one.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed adjusted proportion mortalities must be between zero and one.

6. The Spearman-Karber Method is illustrated below using a set of mortality data from a Fathead Minnow Larval Survival and Growth test. These data are listed in Table J.1.

TABLE J.1. EXAMPLE OF SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25%	2	0.05
12.5%	0	0.00
25.0%	0	0.00
50.0%	26	0.65
100.0%	40	1.00

7. Let $p_0, p_1, ..., p_k$ denote the observed response proportion mortalities for the control and k effluent concentrations. The first step is to smooth the p_i if they do not satisfy p_0 # p_1 # ... # p_k. The smoothing process replaces any adjacent p_i's that do not conform to p_0 # p_1 # ... # p_k with their average. For example, if p_i is less than p_{i-1} then:

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$$p_{i\&1}^{s} = p_i^{s} = \frac{(p_i \% p_{i\&1})}{2}$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

7.1 For the data in this example, because the observed mortality proportions for the control and the 6.25% effluent concentration are greater than the observed response proportions for the 12.5% and 25.0% effluent concentrations, the responses for these four groups must be averaged:

$$p_0^{s} = p_1^{s} = p_2^{s} = p_3^{s} = \frac{0.05\%0.05\%0.00\%0.00}{4} = \frac{0.10}{4} = 0.025$$

7.2 Since $p_4 = 0.65$ is larger than p_3^s, set $p_4^s = 0.65$. Similarly, $p_5 = 1.00$ is larger than p_4^s, so set $p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table J.2.

8. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: $p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$

p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

8.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_0^{a} = p_1^{a} = p_2^{a} = p_3^{a} = \frac{p_1^{s}\&p_0^{s}}{1\&p_0^{s}} = \frac{0.025\&0.025}{1\&0.025} = \frac{0.0}{0.975} = 0.0$$

$$p_4^{a} = \frac{p_4^{s}\&p_0^{s}}{1\&p_0^{s}} = \frac{0.650\&0.025}{1\&0.025} = \frac{0.0625}{0.975} = 0.641$$

$$p_5^{a} = \frac{p_5^{s}\&p_0^{s}}{1\&p_0^{s}} = \frac{1.000\&0.025}{1\&0.025} = \frac{0.975}{0.975} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table J.2. A plot of the smoothed, adjusted data is shown in Figure J.1.

9. Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k\&1} \frac{(p_{i\%1}^{a})(x_i \% x_{i\%1})}{2}$$

Where: p_i^a = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control.

9.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

$$
\begin{aligned}
m = \; & [(0.000 - 0.000)\,(0.7959 + 1.0969)]/2 + \\
& [(0.000 - 0.000)\,(1.0969 + 1.3979)]/2 + \\
& [(0.641 - 0.000)\,(1.3979 + 1.6990)]/2 + \\
& [(1.000 - 0.641)\,(1.6990 + 2.0000)]/2 \\
= \; & 1.656527
\end{aligned}
$$

TABLE J.2. EXAMPLE OF SPEARMAN-KARBER METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.05	0.025	0.000
6.25%	0.05	0.025	0.000
12.5%	0.00	0.025	0.000
25.0%	0.00	0.025	0.000
50.0%	0.65	0.650	0.641
100.0%	1.00	1.000	1.000

10. Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^a(1-p_i^a)(X_{i+1}-X_{i-1})^2}{4(n_i-1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control.

10.1 For this example, the estimated variance of m, V(m), is calculated as follows:

$$
\begin{aligned}
V(m) = \; & (0.000)(1.000)(1.3979 - 0.7959)^2/4(39) + \\
& (0.000)(1.000)(1.6990 - 1.0969)^2/4(39) + \\
& (0.641)(0.359)(2.0000 - 1.3979)^2/4(39) \\
= \; & 0.00053477
\end{aligned}
$$

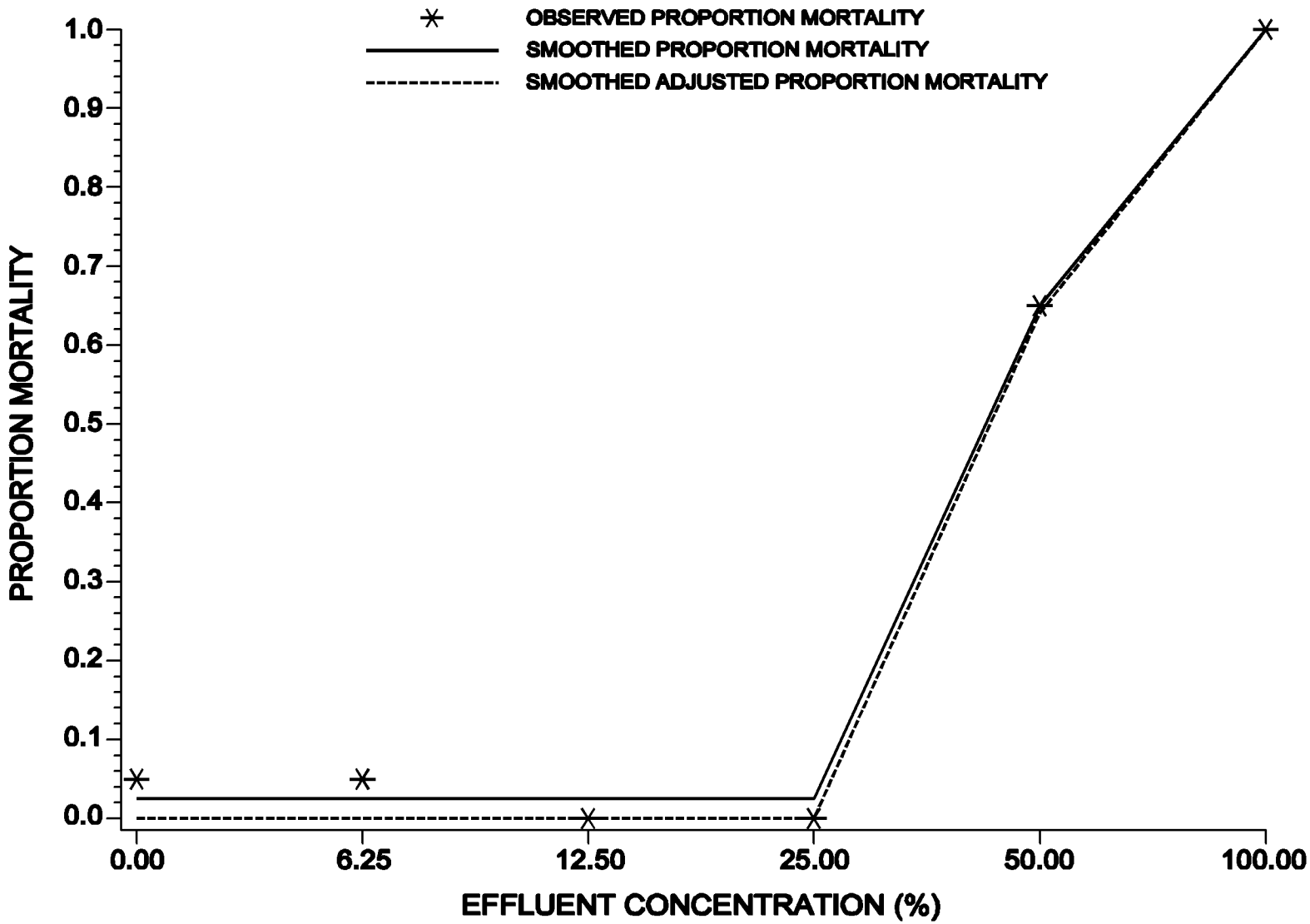

Figure J.1. Plot of the smoothed, adjusted data for the fathead minnow larval survival and growth test.

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11. Calculate the 95% confidence interval for m: $m \pm 2.0\sqrt{V(m)}$

11.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.656527 \pm 2\sqrt{0.00053477} \text{ ' } (1.610277,\ 1.702777)$$

12. The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking base_{10} antilogs of the above values.

12.1 For this example, the estimated LC50 is calculated as follows:

LC50 = antilog(m) = antilog(1.656527) = 45.3%.

12.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: antilog(1.610277) = 40.8%

upper limit: antilog(1.702777) = 50.4%

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APPENDIX K

TRIMMED SPEARMAN-KARBER METHOD

1. The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber Method, a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton et al, 1977). Appendix The Trimmed Spearman-Karber Method estimates the trimmed mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Trimmed Spearman-Karber Analysis is recommended only when the requirements for the Probit Method and the Spearman-Karber Method are not met.

4. To calculate the LC50 using the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed, adjusted, observed proportion mortalities must be between zero and one.

6. Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \# p_1 \# ... \# p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \# p_1 \# ... \# p_k$, with their average. For example, if p_i is less than p_{i-1} then:

Where: $p^s_{i-1} = p^s_i = (p_i + p_{i-1})/2$

p^s_i = the smoothed observed proportion mortality for effluent concentration i.

7. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

Where: $p^a_i = (p^s_i - p^s_0) / (1 - p^s_0)$

p^s_0 = the smoothed observed proportion mortality for the control

p^s_i = the smoothed observed proportion mortality for effluent concentration i.

8. Calculate the amount of trim to use in the estimation of the LC50 as follows:

Where: Trim = $\max(p^a_1, 1-p^a_k)$

p^a_1 = the smoothed, adjusted proportion mortality for the lowest effluent concentration, exclusive of the control

p^a_k = the smoothed, adjusted proportion mortality for the highest effluent concentration

k = the number of effluent concentrations, exclusive of the control.

The minimum trim should be calculated for each data set rather than using a fixed amount of trim for each data set.

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9. Due to the intensive nature of the calculation for the estimated LC50 and the calculation of the associated 95% confidence interval using the Trimmed Spearman-Karber Method, it is recommended that the data be analyzed by computer.

10. A computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed Spearman-Karber Method, can be obtained from EMSL-Cincinnati by sending a written request to EMSL, 3411 Church Street, Cincinnati, OH 45244.

11. The Trimmed Spearman-Karber program automatically performs the following functions:

a. Smoothing.
b. Adjustment for mortality in the control.
c. Calculation of the necessary trim.
d. Calculation of the LC50.
e. Calculation of the associated 95% confidence interval.

12. To illustrate the Trimmed Spearman-Karber method using the Trimmed Spearman-Karber computer program, a set of data from a Fathead Minnow Larval Survival and Growth test will be used. The data are listed in Table K.1.

TABLE K.1. EXAMPLE OF TRIMMED SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	0	0.00
12.5	2	0.05
25.0	0	0.00
50.0	0	0.00
100.0	32	0.80

12.1 The program requests the following input (Figure K.1):

a. Output destination (D = disk file, P = printer).
b. Control data.
c. Data for each toxicant concentration.

12.2 The program output includes the following (Figure K.2):

a. A table of the concentrations tested, number of organisms exposed, and mortalities.
b. The amount of trim used in the calculation.
c. The estimated LC50 and the associated 95% confidence interval.

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```
A:>spearman

 TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5

ENTER DATE OF TEST:
1
ENTER TEST NUMBER:
2
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
 L
ENTER TEST SPECIES NAME:
Fathead minnow
ENTER TOXICANT  NAME:
Effluent
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT:
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
 40
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
2
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL;  MAX = 10):
5
ENTER THE  5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
6.25  12.5  25  50  100
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:        40

ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
Days
ENTER DURATION OF TEST:
7
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:        0 2 0 0 32

WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
 y
```

Figure K.1. Example input for Trimmed Spearman-Karber Method.

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TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1 TEST NUMBER: 2 DURATION: 7 Days
TOXICANT: effluent
SPECIES: fathead minnow

RAW DATA: Concentration (%)	Number Exposed	Mortalities
.00	40	2
6.25	40	0
12.50	40	2
25.00	40	0
50.00	40	0
100.00	40	32

SPEARMAN-KARBER TRIM: 20.41%

SPEARMAN-KARBER ESTIMATES: LC50: 77.28
95% CONFIDENCE LIMITS
ARE NOT RELIABLE.

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure K.2. Example output for Trimmed Spearman-Karber Method.

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APPENDIX L

GRAPHICAL METHOD

1. The Graphical Method is used to calculate the LC50. It is a mathematical procedure which estimates the LC50 by linearly interpolating between points of a plot of observed percent mortality versus the base 10 logarithm ($\log_{10}$) of percent effluent concentration. This method does not provide a confidence interval for the LC50 estimate and its use is only recommended when there are no partial mortalities. The only requirement for the Graphical Method is that the observed percent mortalities bracket 50%.

2. For an analysis using the Graphical Method the data must first be smoothed and adjusted for mortality in the control replicates. The procedure for smoothing and adjusting the data is detailed in the following steps.

3. The Graphical Method is illustrated below using a set of mortality data from an Fathead Minnow Larval Survival and Growth test. These data are listed in Table L.1.

TABLE L.1. EXAMPLE OF GRAPHICAL METHOD: MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST (40 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.05
6.25	0	0.00
12.5	0	0.00
25.0	0	0.00
50.0	40	1.00
100.0	40	1.00

4. Let $p_0, p_1, ..., p_k$ denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \# p_1 \# ... \# p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \# p_1 \# ... \# p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i\&1}^{s} \; ' \; p_i^{s} \; ' \; (p_i \% p_{i\&1})/2$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

4.1 For the data in this example, because the observed mortality proportions for the 6.25%, 12.5%, and 25.0% effluent concentrations are less than the observed response proportion for the control, the values for these four groups must be averaged:

$$p_0^{s} \; ' \; p_1^{s} \; ' \; p_2^{s} \; ' \; p_3^{s} \; ' \; \frac{0.05\%0.00\%0.00\%0.00}{4} \; ' \; \frac{0.05}{4} \; ' \; 0.0125$$

4.2 Since $p_4 = p_5 = 1.00$ are larger then 0.0125, set $p_4^s = p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table L.2.

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TABLE L.2. EXAMPLE OF GRAPHICAL METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration %	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.05	0.0125	0.00
6.25	0.00	0.0125	0.00
12.5	0.00	0.0125	0.00
25.0	0.00	0.0125	0.00
50.0	1.00	1.0000	1.00
100.0	1.00	1.0000	1.00

5. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

5.1 Because the smoothed observed proportion mortality for the control group is greater than zero, the responses must be adjusted using Abbott's formula, as follows:

$$p_0^a = p_1^a = p_2^a = p_3^a = \frac{p_1^s - p_0^s}{1 - p_0^s} = \frac{0.0125 - 0.0125}{1 - 0.0125} = \frac{0.0}{0.9875} = 0.0$$

$$p_4^a = p_5^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{1.00 - 0.0125}{1 - 0.0125} = \frac{0.9875}{0.9875} = 1.00$$

A table of the smoothed, adjusted response proportions for the effluent concentrations are shown in Table L.2.

5.2 Plot the smoothed, adjusted data on 2-cycle semi-log graph paper with the logarithmic axis (the y axis) used for percent effluent concentration and the linear axis (the x axis) used for observed percent mortality. A plot of the smoothed, adjusted data is shown in Figure L.1.

6. Locate the two points on the graph which bracket 50% mortality and connect them with a straight line.

7. On the scale for percent effluent concentration, read the value for the point where the plotted line and the 50% mortality line intersect. This value is the estimated LC50 expressed as a percent effluent concentration.

7.1 For this example, the two points on the graph which bracket the 50% mortality line (0% mortality at 25% effluent, and 100% mortality at 50% effluent) are connected with a straight line. The point at which the plotted line intersects the 50% mortality line is the estimated LC50. The estimated LC50 = 35% effluent.

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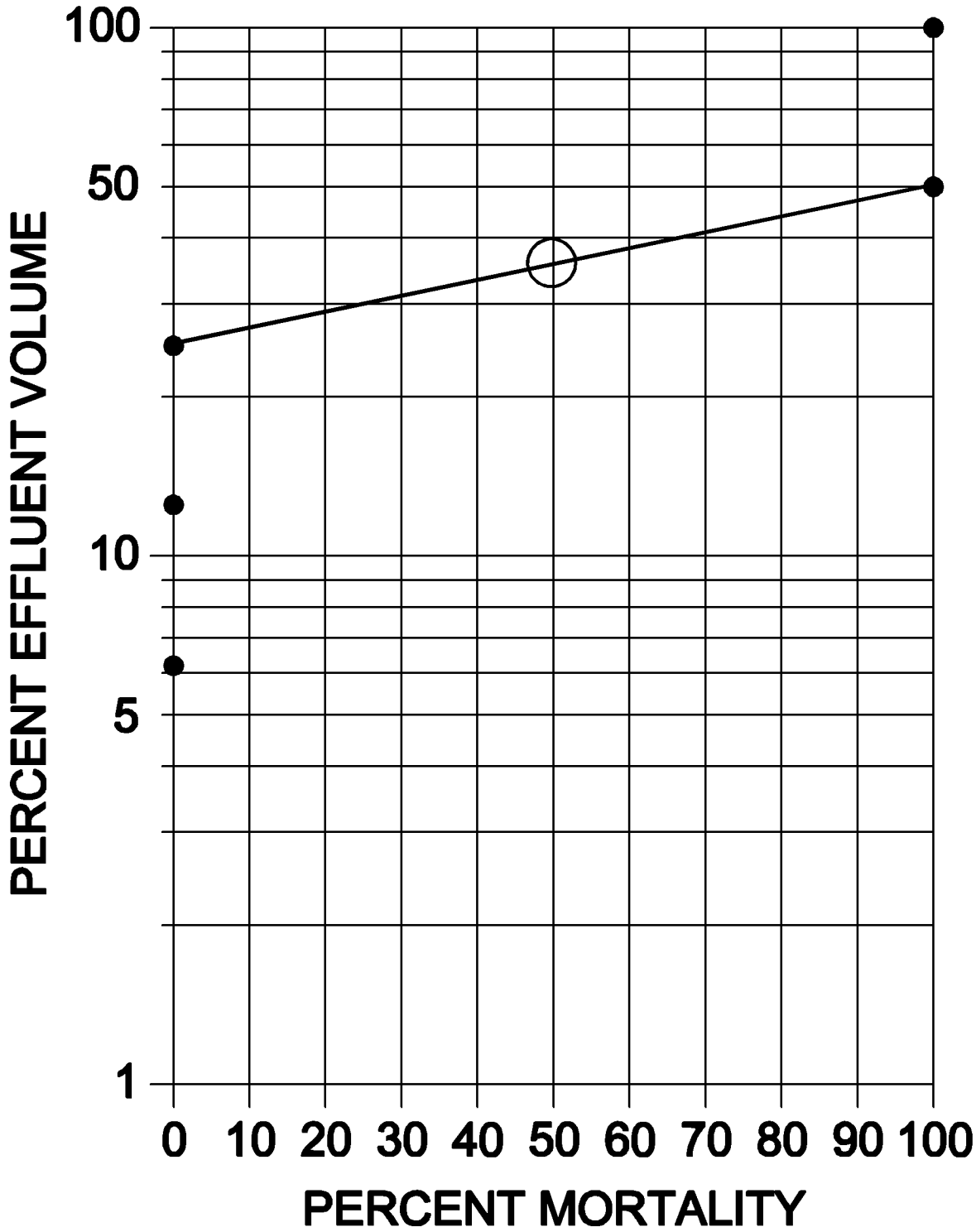

Figure L.1 Plot of the smoothed adjusted response proportions for fathead minnow, *Pimephales promelas*, survival data.

APPENDIX M

LINEAR INTERPOLATION METHOD

1. GENERAL PROCEDURE

1.1 The Linear Interpolation Method is used to calculate a point estimate of the effluent or other toxicant concentration that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms (Inhibition Concentration, or IC). The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests, and the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test, and a mean and coefficient of variation for the endpoints of multiple tests.

1.2 The Linear Interpolation Method assumes that the responses (1) are monotonically non-increasing, where the mean response for each higher concentration is less than or equal to the mean response for the previous concentration, (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. If the data are not monotonically nonincreasing, they are adjusted by smoothing (averaging). In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. Also, no assumption is made about the distribution of the data except that the data within a group being resampled are independent and identically distributed.

2. DATA SUMMARY AND PLOTS

2.1 Calculate the mean responses for the control and each toxicant concentration, construct a summary table, and plot the data.

3. MONOTONICITY

3.1 If the assumption of monotonicity of test results is met, the observed response means ($\bar{Y}_i$) should stay the same or decrease as the toxicant concentration increases. If the means do not decrease monotonically, the responses are "smoothed" by averaging (pooling) adjacent means.

3.2 Observed means at each concentration are considered in order of increasing concentration, starting with the control mean ($\bar{Y}_1$). If the mean observed response at the lowest toxicant concentration ($\bar{Y}_2$) is equal to or smaller than the control mean ($\bar{Y}_1$), it is used as the response. If it is larger than the control mean, it is averaged with the control, and this average is used for both the control response (M_1) and the lowest toxicant concentration response (M_2). This mean is then compared to the mean observed response for the next higher toxicant concentration ($\bar{Y}_3$). Again, if the mean observed response for the next higher toxicant concentration is smaller than the mean of the control and the lowest toxicant concentration, it is used as the response. If it is higher than the mean of the first two, it is averaged with the first two, and the mean is used as the response for the control and two lowest concentrations of toxicant. This process is continued for data from the remaining toxicant concentrations. A numerical example of smoothing the data is provided below. (Note: Unusual patterns in the deviations from monotonicity may require an additional step of smoothing). Where $\bar{Y}_i$ decrease monotonically, the $\bar{Y}_i$ become M_i without smoothing.

4. LINEAR INTERPOLATION METHOD

4.1 The method assumes a linear response from one concentration to the next. Thus, the ICp is estimated by linear interpolation between two concentrations whose responses bracket the response of interest, the (p) percent reduction from the control.

4.2 To obtain the estimate, determine the concentrations C_J and C_{J+1} which bracket the response M_1 (1 - p/100), where M_1 is the smoothed control mean response and p is the percent reduction in response relative to the control

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response. These calculations can easily be done by hand or with a computer program as described below. The linear interpolation estimate is calculated as follows:

$$ICp \;'\; C_J \,\%\, [\; M_1 \;(1 \;\&\; p/100) \;\&\; M_J \;] \;\; \frac{(C_{J\%1} \;\&\; C_J)}{(M_{J\%1} \;\&\; M_J)}$$

Where: C_J = tested concentration whose observed mean response is greater than $M_1(1 - p/100)$.

C_{J+1} = tested concentration whose observed mean response is less than $M_1(1 - p/100)$.

M_1 = smoothed mean response for the control.

M_J = smoothed mean response for concentration J.

M_{J+1} = smoothed mean response for concentration J + 1.

p = percent reduction in response relative to the control response.

ICp = estimated concentration at which there is a percent reduction from the smoothed mean control response. The ICp is reported for the test, together with the 95% confidence interval calculated by the ICPIN.EXE program described below.

4.3 If the C_J is the highest concentration tested, the ICp would be specified as *greater than* C_J. If the response at the lowest concentration tested is used to extrapolate the ICp value, the ICp should be expressed as a *less than the lowest test concentration.*

5. CONFIDENCE INTERVALS

5.1 Due to the use of a linear interpolation technique to calculate an estimate of the ICp, standard statistical methods for calculating confidence intervals are not applicable for the ICp. This limitation is avoided by use a technique known as the bootstrap method as proposed by Efron (1982) for deriving point estimates and confidence intervals.

5.2 In the Linear Interpolation Method, the smoothed response means are used to obtain the ICp estimate reported for the test. The bootstrap method is used to obtain the 95% confidence interval for the true mean. In the bootstrap method, the test data Y_{ji} is randomly resampled with replacement to produce a new set of data Y_{ji}*, that is statistically equivalent to the original data, but a new and slightly different estimate of the ICp (ICp*) is obtained. This process is repeated at least 80 times (Marcus and Holtzman, 1988) resulting in multiple "data" sets, each with an associate ICp* estimate. The distribution of the ICp* estimates derived from the sets of resampled data approximates the sampling distribution of the ICp estimate. The standard error of the ICp is estimated by the standard deviation of the individual ICp* estimates. Empirical confidence intervals are derived from the quantiles of the ICp* empirical distribution. For example, if the test data are resampled a minimum of 80 time, the empirical 2.5% and the 97.5% confidence limits are approximately the second smallest and second largest ICp* estimates (Marcus and Holtzman, 1988).

5.3 The width of the confidence intervals calculated by the bootstrap method is related to the variability of the data. When confidence intervals are wide, the reliability of the IC estimate is in question. However, narrow intervals do not necessarily indicate that the estimate is highly reliable, because of undetected violations of assumptions and the fact that the confidence limits based on the empirical quantiles of a bootstrap distribution of 80 samples may be unstable.

5.4 The bootstrapping method of calculating confidence intervals is computationally intensive. For this reason, all of the calculations associated with determining the confidence intervals for the ICp estimate have been incorporated into a computer program. Computations are most easily done with a computer program such as the revision of the

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BOOTSTRP program (USEPA, 1988; USEPA, 1989) which is now called "ICPIN" which is described below in subsection 7.

6. **MANUAL CALCULATIONS**

6.1 DATA SUMMARY AND PLOTS

6.1.1 The data used in this example are the *Ceriodaphnia dubia* reproduction data used in the example in Section 13. Table M.1 includes the raw data and the mean reproduction for each concentration. Data are included for all animals tested regardless of death of the organism. If an animal died during the test without producing young, a zero is entered. If death occurred after producing young, the number of young produced prior to death is entered. A plot of the data is provided in Figure M.1.

TABLE M.1. *CERIODAPHNIA DUBIA* REPRODUCTION DATA

		Effluent Concentration (%)				
Replicate	Control	1.56	3.12	6.25	12.5	25.0
1	27	32	39	27	10	0
2	30	35	30	34	13	0
3	29	32	33	36	7	0
4	31	26	33	34	7	0
5	16	18	36	31	7	0
6	15	29	33	27	10	0
7	18	27	33	33	10	0
8	17	16	27	31	16	0
9	14	35	38	33	12	0
10	27	13	44	31	2	0
Mean ($\bar{Y}_i$)	22.4	26.3	34.6	31.7	9.4	0
i	1	2	3	4	5	6

6.2 MONOTONICITY

6.2.1 As can be seen from the plot in Figure M.1, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

6.2.2 Starting with the control mean $\bar{Y}_1 = 22.4$ and $\bar{Y}_2 = 26.3$, we see that $\bar{Y}_1 < \bar{Y}_2$. Calculate the smoothed means:

$$M_1 = M_2 = (\bar{Y}_1 + \bar{Y}_2)/2 = 24.35$$

6.2.3 Since $\bar{Y}_3 = 34.6$ is larger than M_2, average $\bar{Y}_3$ with the previous concentrations:

6.2.4 Additionally, $\bar{Y}_4 = 31.7$ is larger than M_3, and is pooled with the first three means. Thus,

$$M_1 = M_2 = M_3 = M_4 = (M_1 + M_2 + M_3 + \bar{Y}_4)/4 = 28.7$$

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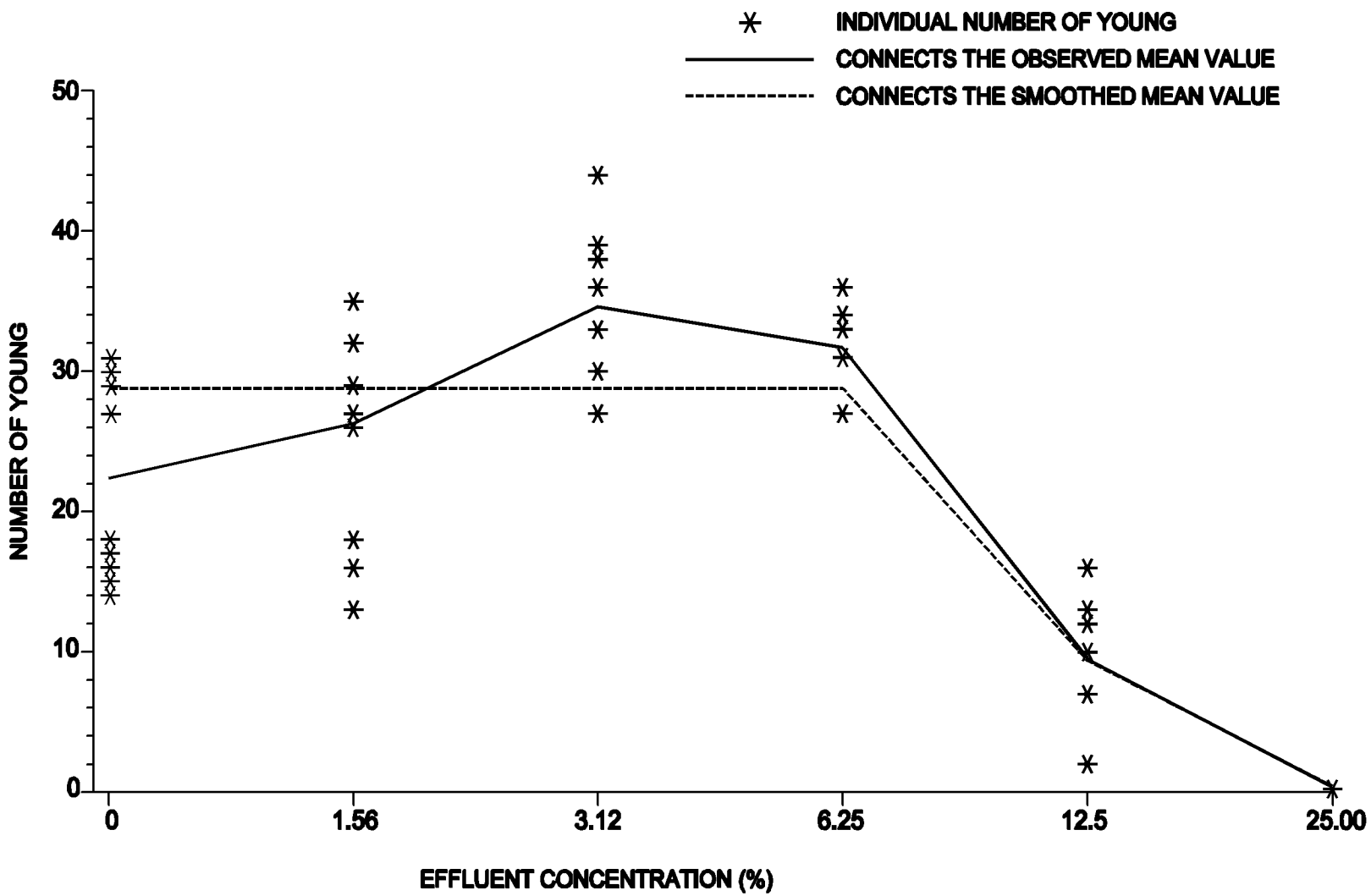

Figure M.1. Plot of raw data, observed means, and smoothed means for the daphnid, *Ceriodaphnia dubia*, reproductive data.

TABLE M.2. *CERIODAPHNIA DUBIA* REPRODUCTION MEAN RESPONSE AFTER SMOOTHING

Effluent Concentration %	i	Response Mean (Y_i) (Young/female)	Smoothed Mean (M_i) (Young/female)
Control	1	22.4	28.75
1.56	2	26.3	28.75
3.12	3	34.6	28.75
6.25	4	31.7	28.75
12.5	5	9.4	9.40
25.0	6	0.0	0.00

6.2.5 Since $M_4 > Y_5 = 9.4$, set $M_5 = 9.4$. Likewise, $M_5 > Y_6 = 0$ and M_6 becomes 0. Table M.2 contains the smoothed means and Figure M.1 gives a plot of the smoothed response curve.

6.3 LINEAR INTERPOLATION

6.3.1 Estimates of the IC25 and IC50 are calculated using the Linear Interpolation Method. A 25% reduction in reproduction, compared to the controls, would result in a mean reproduction of 21.56 young per adult, where $M_1(1-p/100) = 28.75(1-25/100)$. A 50% reduction in reproduction, compared to the controls, would result in a mean reproduction of 14.38 young per adult, where $M_1(1-p/100) = 28.75(1-50/100)$. Examining the smoothed means and their associated concentrations (Table M.2), the two effluent concentrations bracketing the reproduction of 21.56 young per adult are $C_4 = 6.25\%$ effluent and $C_5 = 12.5\%$ effluent. The two effluent concentrations bracketing a response of 14.38 young per adult are also $C_4 = 6.25\%$ effluent and $C_5 = 12.5\%$ effluent.

6.3.2 Using Equation 1 from 4.2, the estimate of the IC25 is calculated as follows:

$$ICp = C_J\% \left[M_1 (1 - p/100) - M_J \right] \frac{(C_{J\%1} - C_J)}{(M_{J\%1} - M_J)}$$

$$IC25 = 6.25\% \left[28.75 (1 - 25/100) - 28.75\right] \frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

$$= 8.57\% \text{ effluent}$$

6.3.3 Using the equation from section 4.2, the estimate of the IC50 is calculated as follows:

$$ICp = C_J\% \left[M_1 (1 - p/100) - M_J \right] \frac{(C_{J\%1} - C_J)}{(M_{J\%1} - M_J)}$$

$$IC50 = 6.25\% \left[28.75 (1 - 50/100) - 28.75\right] \frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

$$= 10.89\% \text{ effluent}$$

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6.4 CONFIDENCE INTERVALS

6.4.1 Confidence intervals for the ICp are derived using the bootstrap method. As described above, this method involves randomly resampling the individual observations and recalculating the ICp at least 80 times, and determining the mean ICp, standard deviation, and empirical 95% confidence intervals. For this reason, the confidence intervals are calculated using a computer program called ICPIN. This program is described below and is available to carry out all the calculations of both the interpolation estimate (ICp) and the confidence intervals.

7. COMPUTER CALCULATIONS

7.1 The computer program, ICPIN, prepared for the Linear Interpolation Method was written in TURBO PASCAL for IBM compatible PCs. The program (version 2.0) has been modified by Computer Science Corporation, Duluth, MN with funding provided by the Environmental Research Laboratory, Duluth, MN (Norberg-King, 1993). The program was originally developed by Battelle Laboratories, Columbus, OH through a government contract supported by the Environmental Research Laboratory, Duluth, MN (USEPA, 1988). To obtain the program and supporting documentation, send a written request to EMSL-Cincinnati at 3411 Church Street, Cincinnati, OH 45244.

7.2 The ICPIN.EXE program performs the following functions: 1) it calculates the observed response means (Y_i) (response means); 2) it calculates the standard deviations; 3) checks the responses for monotonicity; 4) calculates smoothed means (M_i) (pooled response means) if necessary; 5) uses the means, M_i, to calculate the initial ICp of choice by linear interpolation; 6) performs a user-specified number of bootstrap resamples between 80 and 1000 (as multiples of 40); 7) calculates the mean and standard deviation of the bootstrapped ICp estimates; and 8) provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven (Norberg-King, 1993).

7.3 For the IC*p* calculation, up to twelve treatments can be used (which includes the control). There can be up to 40 replicates per concentration, and the program does not require an equal number of replicates per concentration. The value of p can range from 1% to 99%.

7.4 DATA INPUT

7.4.1 Data is entered directly into the program onscreen. A sample data entry screen in shown in Figure M.2. The program documentation provides guidance on the entering and analysis of data for the Linear Interpolation Method (Norberg-King, 1993).

7.4.2 The user selects the ICp estimate desired (e.g., IC25 or IC50) and the number of resamples to be taken for the bootstrap method of calculating the confidence intervals. The program has the capability of performing any number of resamples from 80 to 1000 as multiples of 40. However, Marcus and Holtzman (1988) recommend a minimum of 80 resamples for the bootstrap method be used and at least 250 resamples are better (Norberg-King, 1993).

7.5 DATA OUTPUT.

7.5.1 The program output includes the following (Figures M.3 and M.4):

1. A table of the concentration identification, the concentration tested and raw data response for each replicate and concentration.
2. A table of test concentrations, number of replicates, concentration (units), response means ($\bar{Y}_i$), standard deviations for each response mean, and the pooled response means (smoothed means; M_i).
3. The linear interpolation estimate of the ICp using the means (M_i). *Use this value for the ICp estimate.*
4. The mean ICp and standard deviation from the bootstrap resampling.
5. The confidence intervals calculated by the bootstrap method for the ICp. Provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is

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over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven.

7.6 ICPIN program output for the analysis of the *Ceriodaphnia dubia* reproduction data in Table M.1 is provided in Figures M.3 and M.4.

7.6.1 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 8.57% effluent. The empirical 95% confidence intervals for the true mean were 8.30% to 8.85% effluent.

7.6.2 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 10.89% effluent. The empirical 95% confidence intervals for the true mean were 10.36% to 11.62% effluent.

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ICp Data Entry/Edit Screen Current File:

Conc. ID	1	2	3	4	5	6
Conc. Tested						
Conc. Tested						
Response 1						
Response 2						
Response 3						
Response 4						
Response 5						
Response 6						
Response 7						
Response 8						
Response 9						
Response 10						
Response 11						
Response 12						
Response 13						
Response 14						
Response 15						
Response 16						
Response 17						
Response 18						
Response 19						
Response 20						

F10 for Command Menu Use arrow Keys to Switch Fields

Figure M.2. ICp data entry/edit screen. Twelve concentrating identifications can be used. Data for concentrations are entered in columns 1 through 6. For concentrations 7 through 12 and responses 21-40 the data is entered in additional fields of the same screen.

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Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: app M Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration: 7-d
DATA FILE: cerioman.icp
OUTPUT FILE: cerioman.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 8.5715 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 8.6014 Standard Deviation: 0.1467
Original Confidence Limits: Lower: 8.3040 Upper: 8.8496
Resampling time in Seconds: 2.53 Random Seed: -1652543090

Figure M.3. Example of ICPIN program output for the IC25.

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Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent:
Test Start Date: app M Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration: 7-d
DATA FILE: cerioman.icp
OUTPUT FILE: cerioman.i50

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 10.8931 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 10.9108 Standard Deviation: 0.3267
Original Confidence Limits: Lower: 10.3618 Upper: 11.6201
Resampling time in Seconds: 2.58 Random Seed: 340510286

Figure M.4. Example of ICPIN program output for the IC50.

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Part III

Environmental Protection Agency

40 CFR Parts 110 and 300
National Oil and Hazardous Substances Pollution Contingency Plan; Proposed Rule

ENVIRONMENTAL PROTECTION AGENCY

40 CFR Parts 110 and 300

[EPA–HQ–OPA–2006–0090; FRL–9689–9–OSWER]

RIN 2050–AE87

National Oil and Hazardous Substances Pollution Contingency Plan

AGENCY: U.S. Environmental Protection Agency (EPA).

ACTION: Proposed rule.

SUMMARY: The Environmental Protection Agency (EPA or the Agency) proposes to amend the requirements in Subpart J of the National Oil and Hazardous Substances Pollution Contingency Plan (NCP) that govern the use of dispersants, other chemical and biological agents, and other spill mitigating substances when responding to oil discharges into waters of the United States (U.S.). The proposal addresses the efficacy, toxicity, environmental monitoring of dispersants, and other chemical and biological agents, as well as public, state, local, and federal officials' concerns regarding their use. Specifically, the proposal amends the Subpart J regulatory requirements for the NCP Product Schedule (Schedule) by adding new listing criteria, revising the efficacy and toxicity testing protocols, and clarifying the evaluation criteria for removing products from the Schedule. The Agency also proposes amended requirements for the authorities, notifications, monitoring, and data reporting when using chemical or biological agents in response to oil discharges to the navigable waters of the United States and adjoining shorelines, the waters of the contiguous zone, and the high seas beyond the contiguous zone in connection with activities under the Outer Continental Shelf Lands Act, activities under the Deepwater Port Act of 1974, or activities that may affect natural resources belonging to, appertaining to, or under the exclusive management authority of the United States, including resources under the Magnuson Fishery Conservation and Management Act of 1976. These requirements are anticipated to encourage the development of safer and more effective spill mitigating products, and would better target the use of these products to reduce the risks to human health and the environment. Further, the amendments are intended to ensure that On-Scene Coordinators (OSCs), Regional Response Teams (RRTs), and Area Committees have sufficient information to support agent preauthorization or authorization of use decisions.

DATES: Comments must be received on or before April 22, 2015.

ADDRESSES: Submit your comments, identified by Docket ID No. EPA–HQ–OPA–2006–0090, by one of the following methods:

- *Federal Rulemaking Portal: http://www.regulations.gov.* Follow the on-line instructions for submitting comments.
- *Mail:* The mailing address of the docket for this rulemaking is EPA Docket Center (EPA/DC), Docket ID No. EPA–HQ–OPA–2006–0090, 1200 Pennsylvania Avenue NW., Washington, DC 20460.
- *Hand Delivery:* Such deliveries are only accepted during the Docket's normal hours of operation, and special arrangements should be made for deliveries of boxed information.

Instructions: Direct your comments to Docket ID No. EPA–HQ–OPA–2006–0090. EPA's policy is that all comments received will be included in the public docket without change and may be made available online at *http://www.regulations.gov,* including any personal information provided, unless the comment includes information claimed to be Confidential Business Information (CBI) or other information whose disclosure is restricted by statute. Do not submit information that you consider to be CBI or otherwise protected through *http://www.regulations.gov.* The *http://www.regulations.gov* Web site is an ''anonymous access'' system, which means EPA will not know your identity or contact information unless you provide it in the body of your comment. If you submit an electronic comment, EPA recommends that you include your name and other contact information in the body of the comment and with any disk or CD–ROM you submit. If EPA cannot read your comment due to technical difficulties and cannot contact you for clarification, EPA may not be able to consider your comment. Electronic files should avoid the use of special characters, any form of encryption, and be free of any defects or viruses. Comments and suggestions regarding the scope of any future rulemaking should be clearly differentiated from comments specific to this proposal (*e.g.,* label Suggestions for Future Rulemaking and Comments on Current Proposal).

Docket: All documents in the docket are listed in the *http://www.regulations.gov* index. Although listed in the index, some information is not publicly available, *e.g.,* CBI or other information whose disclosure is restricted by statute. Certain other material, such as copyrighted material, will be publicly available only in hard copy. Publicly available docket materials are available either electronically in *http://www.regulations.gov* or in hard copy at the EPA Docket, EPA/DC, EPA West, Room 3334, 1301 Constitution Avenue NW., Washington, DC. The Public Reading Room is open from 8:30 a.m. to 4:30 p.m., Monday through Friday, excluding legal holidays. The telephone number for the Public Reading Room is 202–566–1744 to make an appointment to view the docket.

FOR FURTHER INFORMATION CONTACT: For general information, contact the Superfund, TRI, EPCRA, RMP, and Oil Information Center at 800–424–9346 or TDD at 800–553–7672 (hearing impaired). In the Washington, DC metropolitan area, contact the Superfund, TRI, EPCRA, RMP, and Oil Information Center at 703–412–9810 or TDD 703–412–3323. For more detailed information on this proposed rule contact Gregory Wilson at 202–564–7989 (*wilson.gregory@epa.gov*) or Vanessa Principe at 202–564–7913 (*principe.vanessa@epa.gov*). The contacts address is: U.S. Environmental Protection Agency, Office of Emergency Management, Regulations Implementation Division, 1200 Pennsylvania Avenue NW., Washington, DC 20460–0002, Mail Code 5104A, or visit the Office of Emergency Management Web site at *http://www.epa.gov/oem/.*

SUPPLEMENTARY INFORMATION: The contents of this preamble are:

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I. National Technology Transfer and Advancement Act
J. Executive Order 12898: Environmental Justice

I. General Information

In April 2010, the Deepwater Horizon underwater oil well blowout discharged significant quantities of oil into the Gulf of Mexico. The blowout discharged oil from one mile below the sea surface. Approximately one million gallons of dispersants over a three-month period were deployed on surface slicks over thousands of square miles of the Gulf, and approximately three quarters of a million gallons of dispersants were, for the first time, injected directly into the oil gushing from the well riser. This use of dispersants raised many questions about efficacy, toxicity, environmental trade-offs, and monitoring challenges that EPA seeks to address through the proposed revisions to Subpart J.

The proposed revisions to Subpart J address the use of dispersants and other chemical and biological agents to respond to oil discharges into waters of the U.S. Over the past several years, EPA's Office of Research and Development (ORD) has conducted research on improved laboratory protocols for dispersant and bioremediation efficacy, and revisions to Subpart J to address these new protocols were under consideration. As a result of this research and the Deepwater Horizon event, the new protocols in the proposed revisions, in addition to increasing the overall scientific soundness of the data, take into consideration not only the efficacy but also the toxicity, long-term environmental impacts, endangered species protection, and human health concerns raised during responses to oil discharges, including the Deepwater Horizon blowout. Additionally, area planning requirements for dispersant use authorization, toxicity thresholds and advanced monitoring techniques are also proposed. The proposed amendments are a major component of EPA's effort to inform the use of dispersants and other chemical or biological agents when responding to oil discharges. They incorporate lessons learned from the federal government's experiences in the Gulf, and address recommendations specific to agent testing and use in response to oil discharges from the National Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling Report to the President.

The proposed amendments would help to ensure that only products that perform effectively in laboratory testing would be listed on the NCP Product Schedule (Schedule) for use in mitigating the effects of oil discharges in the environment. Manufacturers would be required to provide more detailed product application materials, ecological toxicity data, and human health and safety information, including more detailed instructions for product application in the field. Prohibitions on using products in certain areas under certain conditions determined by On-Scene Coordinators (OSCs), Regional Response Teams (RRTs), and EPA would help ensure that first responders are better able to mitigate environmental effects when spills occur. The required submission of additional product toxicity information would aid OSCs and RRTs when evaluating specific product information and when deciding whether and which products to use to mitigate hazards to the environment and human health caused by discharges or threatened discharges of oil.

Specifically in this action, the Agency proposes, for the following areas:

- *Definitions.* Amend definitions for Bioremediation agents, Burning agents, Chemical agents, Dispersants, Sinking agents, Sorbents, and Surface washing agents; add new definitions for Bioaccumulation, Bioconcentration, Biodegradation, Biological agents, Bioremediation, Herding agents, Products, and Solidifiers; and remove definitions for Miscellaneous Oil Spill Control Agents (MOSCA) and Surface collecting agents.
- *General Requirements.* Revise to reflect new and amended regulatory definitions.
- *Authorization of Use.* Revise to clarify planning and preauthorization responsibilities, establish limitations and prohibitions on the use of certain agents, establish requirements for storage and use of agents, clarify authorities for requiring supplemental testing, monitoring and information on agents, establish requirements for agent recovery from the environment, and establish reporting requirements for agent use.
- *Monitoring the Use of Dispersant.* Establish monitoring requirements for dispersant use in response to major discharges and/or certain dispersant use situations.
- *General Product Information for Schedule Listing.* Revise and establish requirements, including designation of and testing for all product categories under which the listing is requested, Safety Data Sheets, sample product labels, shelf life, collection and recovery, persistence in the environment, storage and use conditions, physical and chemical properties, component identities, concentration limits on National Water Quality Criteria and Standards contaminants, laboratory accreditations, submission of all testing data and calculations, production capabilities, and any other data or certification informing the product's performance capabilities or environmental benefits.
- *Dispersant Testing and Listing Requirements.* Revise the efficacy testing methodology using a baffled flask test, establish new developmental and sub-chronic toxicity testing requirements, revise the acute toxicity testing methodologies, revise the listing criteria, and establish use limitations to saltwater environments.
- *Surface Washing Agent Testing and Listing Requirements.* Revise the acute toxicity testing methodology and listing requirements, establish efficacy testing requirements and listing criteria, and establish use limitations based on product testing for salt and/or freshwater environments.
- *Bioremediation Agent Testing and Listing Requirements.* Revise the efficacy and acute toxicity testing methodologies and listing criteria, establish exceptions for specified non-proprietary products, and establish use limitations based on product testing for salt and/or freshwater environments.
- *Solidifier and Herding Agent Testing and Listing Requirements.* Revise the acute toxicity testing methodology and listing criteria, and establish use limitations based on product testing for salt and/or freshwater environments.
- *Sorbent Requirements.* Establish a list of known, non-proprietary sorbents to be made publicly available in lieu of listing sorbents on the Schedule, and requirements for data and information for sorbent products with components other than those specifically identified in the rule.
- *Submissions of Confidential Business Information.* Revise the allowable confidential business information claims and reporting procedures.

• *Addition of a Product to the Schedule.* Revise the submission requirements including the package contents, EPA's review of submission package, request for review of decision, changes to a product listing, and transitioning of listed products from the current Schedule to the new Schedule.

• *Mandatory Product Disclaimer.* Revise the product disclaimer requirements.

• *Removal of a Product from the Schedule.* Revise the basis for removal and appeals process.

• *Appendix C to part 300.* Revise the requirements for product testing protocols and summary test data including new dispersant baffled flask efficacy and toxicity tests; new standard acute toxicity tests for bioremediation agents, surface washing agents, herding agents, and solidifiers; and revised bioremediation agent efficacy test.

• *Appendix E to Part 300—Oil Spill Response.* Remove this appendix from the NCP.

EPA estimates industry may incur total incremental costs of approximately $668,000 to $694,000 annually. The benefits of the Subpart J amendments are assessed qualitatively. Such benefits include, for example, greater clarity of regulatory requirements, as well as less toxic products. This action does not pose significant impacts on a substantial number of small entities. The Regulatory Impact analysis, which can be found in the docket, provides more detail on the cost methodology and benefits of this action.

COST OF THE PROPOSED RULE

	Annualized costs, 20 years	
	Annualized at 3%	Annualized at 7%
Costs	$667,610	$694,343

II. Entities Potentially Affected by This Proposed Rule

Industrial category	NAICS code
Chemical Manufacturing	325
Merchant Wholesalers, Nondurable Goods	424
Professional, Scientific, and Technical Services	541
Waste Management and Remediation Services	562
Oil and Gas Extraction	211

The list of potentially affected entities in the above table includes manufacturers and users of chemical and biological agents, and other oil spill mitigating devices and substances used as countermeasures against oil discharges. The Agency's goal is to provide a guide for readers to consider regarding entities that potentially could be affected by this action. However, this action may affect other entities not listed in this table. If you have questions regarding the applicability of this action to a particular entity, consult the person(s) listed in the preceding section entitled **FOR FURTHER INFORMATION CONTACT**.

III. Statutory Authority and Delegation of Authority

Under sections 311(d) and 311(j) of the Clean Water Act (CWA), as amended by section 4201 of the Oil Pollution Act of 1990 (OPA), Public Law 101–380, the President is directed to prepare and publish the National Oil and Hazardous Substances Pollution Contingency Plan (NCP) for removal of oil and hazardous substances. Specifically, section 311(d)(2)(G) requires the President to include a schedule identifying "dispersants, other chemicals, and other spill mitigating devices and substances, if any, that may be used in carrying out" the NCP. The authority of the President to implement this portion of the CWA is currently delegated to EPA in Executive Order 12777 (56 FR 54757, October 22, 1991). Subpart J of the NCP governs the use of dispersants, and any other chemical or biological agent to respond to oil discharges (40 CFR part 300 series 900).

IV. Background

A. Historical Background

The Council on Environmental Quality (CEQ) first published the National Oil and Hazardous Materials Pollution Contingency Plan in 1970 (35 FR 8508). Among its elements was Annex X—Schedule of Dispersants and other Chemicals to Treat Oil Spills. Annex X provided a basic regulatory framework that included authorization of use, restrictions, and information requirements to be submitted to the Federal Water Quality Administration (FWQA). This initial schedule advocated mechanical and other control methods, and the removal and proper disposal of oil from the environment. It also specified that dispersants might be used in accordance with the schedule if other control methods are judged to be inadequate or infeasible, and if certain information requirements and usage requirements were met. As a listing requirement, manufacturers had to provide the FWQA with methods for analyzing the chemical components in fresh and salt water, or reasons why such analytical methods could not be provided. Except to prevent or substantially reduce the hazard to human life or limb, or substantial hazard of fire to property, dispersants were restricted from use on or in any: Distillate fuel oil; spill of oil less than 200 barrels in quantity; shoreline; waters less than 100 feet deep; waters containing major populations or breeding or passage for species of fish or marine life which may be damaged or rendered commercially less marketable by exposure to the dispersant or dispersed oil; waters where the winds and/or currents are of such velocity and direction that the dispersed oil mixtures would likely, in the judgment of the FWQA, be carried to shore areas within 24 hours; and waters where such use may affect surface water supplies. The CEQ revised the National Oil and Hazardous Materials Pollution Contingency Plan, renaming it the National Oil and Hazardous Substances Pollution Contingency Plan (NCP) in 1971 (36 FR 16215). The NCP was amended again in 1971 (36 FR 18411) and 1972 (37 FR 28208), with no substantive changes to Annex X.

As a result of the Federal Water Pollution Control Act (Pub. L. 92–500) of 1972, CEQ again revised the NCP, including revisions to Annex X (38 FR 21887, August 13, 1973). The title of Annex X changed to the "*Schedule of Chemicals to Remove Oil & Hazardous Substances Discharges.*" While the Schedule still advocated mechanical and other control methods, and the removal and proper disposal of oil from the environment, it broadened its applicability to chemical agents, including dispersants. It also recognized separate authorizations of use for chemical agents on minor, medium and major discharges. In addition, the revised schedule required an official report from a recognized laboratory with a description of the analytical methods employed and results obtained in determining the chemical and biological characteristics of the chemical agent, but no longer required the submission of those analytical methods. Biological and burning agents were not part of the Schedule, which expressed caution on their use.

In 1975 CEQ again revised the NCP, including Annex X (40 FR 6282). Annex X was now the "*Schedule of Chemicals and Other Additives to Remove Oil & Hazardous Substances Discharges,*" and it was ". . . revised and expanded to provide more precise and definitive information, concerning substances which may be employed to remove discharges." Additionally, Executive Order 11735 (38 FR 21243, August 3, 1973) made EPA responsible for Annex X. Chemical agents or any other substance not specifically defined in the

Schedule were to be considered for use on a case-by-case basis. The Schedule advocated the development and utilization of mechanical control methods to remove or mitigate oil, and to remove, mitigate, or neutralize hazardous substances discharges from the environment, with subsequent proper disposal. The Schedule intended that no harmful quantities of any substance were to be applied to waters to remove or mitigate the effects of oil or hazardous substances discharges. The Schedule also provided procedures for authorization of use for different agent categories, and separate regulatory authorization of use for dispersants or other chemical agents was established for minor, medium, and major discharges. Product shelf life, toxicity and effectiveness, and analytical methods needed to obtain such data were among the technical data requirements. Similar provisions were tailored to surface collecting agents and biological additives.

In 1982, EPA amended the NCP; the revision included rewriting of Annex X as Subpart H of 40 CFR part 300 of the revised Plan (47 FR 31180). The Agency allowed OSCs to authorize the use of dispersants or other chemicals to treat discharges of oil, provided they were listed in the previous Annex X, with the following limitations:

- Authorization applies only to discharges of oil, not to releases of hazardous substances.
- OSCs may only authorize the use of dispersant or other chemicals on EPA's Acceptance list, which included the twenty-eight products tested and found acceptable for their intended purpose in the previous Annex X.
- State consultation provisions regarding the use of any dispersant or other chemicals in its waters required the OSC to obtain concurrence from the EPA representative to the RRT.

The new Subpart H of 40 CFR part 300 also provided for a case-by-case authorization by the EPA Administrator ("Administrator") or designee of the use of dispersants or other chemicals not on EPA's Acceptance list in treating oil discharges or hazardous substances releases. However, EPA did not include testing procedures or a process for designation of dispersants or other chemicals as acceptable for use.

In 1984, EPA published amendments to Subpart H, including adding Appendix C (49 FR 29192). The amendments specified testing and data requirements for listing of dispersants, surface collecting agents, or biological additives on the Schedule to ensure sufficient data was available for the OSC. Standardized testing procedures generated comparable data regarding the products' effectiveness and toxicity. Appendix C detailed the methods and types of apparatus to be used in carrying out the revised standard dispersant effectiveness and toxicity tests, as well as the format required for summary presentation of product test data. Listing of a product on the Schedule was neither a recommendation nor authorization for use on an oil discharge, but rather a confirmation that data submission requirements had been satisfied. EPA's listing on the Schedule did not confirm its safety or effectiveness or constitute an endorsement; in fact, a new requirement was established that either a written disclaimer to this effect or EPA's written disclaimer be included in all product technical literature or advertisements. Products previously listed under Annex X were included on the new Schedule as the previous data requirements were sufficient to permit OSCs to make informed decisions about product use.

The amendments also provided for OSC authorization for use of burning agents on a case-by-case basis with the concurrence of the EPA RRT representative and the States, and prohibited the use of sinking agents in waters of the United States. They encouraged advance planning by allowing the OSC to act without the concurrence of the RRT and affected States if these parties have approved a plan identifying the products that may be used in specific contexts. The amendments also allowed the OSC to authorize the use of any product (including products not on the Schedule) without obtaining the concurrence of the EPA representative to the RRT or the affected States if the use of a dispersant, surface collecting agent, or biological additive is necessary to prevent or substantially reduce hazard to human life, and there is not sufficient time to obtain concurrence. However, the OSC was to inform the EPA RRT representative and the affected States of the use of a product as soon as possible, and obtain their concurrences for the product's continued use in the situation once the threat to human life had subsided. These provisions were designed to eliminate delays in life threatening situations, such as spills of highly flammable petroleum products in harbors or near inhabited areas. Subpart H was re-designated as Subpart J with minor changes in 1990 (55 FR 8666), with those definitions present in Subpart H moved to Subpart A, and a new definition and data requirements for miscellaneous spill control agents added.

In 1994, EPA revised the NCP in response to the passage of the Oil Pollution Act in 1990 (59 FR 47384). The final rule significantly revised Subpart J to its current regulatory requirements with respect to authorization of use, data requirements, dispersant effectiveness and toxicity testing protocols, surface washing agent toxicity testing protocol, bioremediation agent effectiveness testing protocol, and requirements for adding products to the Schedule. As a result, several dispersants were re-classified as surface washing agents because they did not pass the dispersant efficacy test threshold, but were effective in removing oil from solid surfaces.

B. Current Statute and Regulation

Section 300.910 of Subpart J addresses the authorization of the use of products on the Schedule and specifies the conditions under which OSCs may authorize the use of dispersants, other chemicals, and other spill mitigating substances. Sections 300.915 and 300.920 describe the data requirements and the process for adding products to the Schedule. To list a product on the Schedule, Subpart J currently requires the submission of technical data on the product to EPA. Data on dispersants, surface collecting agents, surface washing agents, and miscellaneous oil spill control agents must include the results of the toxicity test set for these products in Appendix C of the NCP. Data on dispersants must also include the results of the dispersant effectiveness test, while bioremediation agents must include results of the bioremediation effectiveness test, also set forth in Appendix C. These tests are conducted at the expense of the product manufacturers and must be performed by laboratories experienced with Appendix C protocols.

The raw data and a summary of the results from these tests are then submitted to EPA, where they are reviewed to confirm that the data are complete and that the specified procedures were followed. The data requirements for placement of a product on the Schedule are designed to provide sufficient data for the OSCs to judge whether and in what quantities a product may be used to control a particular discharge. Inclusion of a product on the Schedule means only that the data submission requirements have been satisfied. The listing of a product on the Schedule does not mean that the product is recommended or authorized for use on any specific oil discharge nor does it imply that EPA has in any other way endorsed the product for the use listed or for other

uses. The standardized testing procedures set forth in Appendix C provide OSCs comparable data regarding the toxicity, effectiveness, and other characteristics of different products.

C. Advanced Response Planning

Under the current 300.910(a) regulation, RRTs and the Area Committees (ACs) are required to address, as part of their planning activities, the desirability of using appropriate dispersants, and other chemical or biological agents. The RRTs and ACs generally develop ''preauthorization plans'' which address the specific context in which products can be used under OSC direction. Preauthorization plans are approved with concurrences from EPA, Department of Interior (DOI) and Department of Commerce (DOC) natural resource trustees, and the state(s) with jurisdiction over the water to the area, which they apply. When a preauthorization plan approves in advance the use of certain products under specified circumstances, the OSC may authorize the use of the products without obtaining the specific concurrences described elsewhere in that section of the regulation. The use of chemical or biological agents is only one approach of many available, such as mechanical collection or *in-situ* burning, and decisions about their use should be weighed to achieve greater overall environmental protection.

To facilitate the best possible response, it is important that the regional-level and area-level contingency planning efforts of the RRTs and ACs, respectively, are coordinated closely with each other and are consistent. Section 300.910(a) authorizes the RRTs to review and either approve, disapprove, or approve with modification the preauthorization plans developed by Area Committees that addresses dispersants or other spill mitigating substances. This advanced planning has allowed the OSC to authorize the use of products without obtaining the specific concurrences, if the RRT representatives from EPA and the states with jurisdiction over waters to which a preauthorization plan applies, and DOC and DOI natural resource trustees approve their use in advance. The OSC primarily uses the Schedule to confirm if a product is listed, analyze toxicity and efficacy data, note worker health and safety precautions, understand proper product application, and compare one product to another in order to make the most informed decision on how to mitigate an oil discharge.

AC responsibilities include enhancing contingency planning; ensuring preplanning of joint federal, state, and local response efforts; and expediting decisions on the use of dispersants and other spill mitigating devices and substances. The Area Contingency Plan (ACP) must list the equipment, dispersants or other spill mitigating substances, and personnel available to ensure effective and immediate removal of a discharge. ACPs must also ensure the mitigation or prevention of a substantial threat of a discharge; provide a description of the procedures to be followed for obtaining an expedited decision regarding the use of dispersants (which may be addressed in applicable preauthorization plans); and identify the means to monitor use of chemical countermeasures. Many coastal ACPs include some type of preauthorization zones for dispersants, while most Regional Contingency Plans (RCPs) address other product categories, such as bioremediation and surface washing agents. RRTs, in cooperation with the states and federal agencies, have addressed the requirements for the conduct of in-situ burns (ISB) of oil discharges in their RCPs.

This planning has allowed consideration of chemical agent use on oil discharges as a viable response tool in combination with other mitigation measures. These agents have increasingly been considered and used in the field, as evidenced by research and case studies presented at national and international oil spill conferences, research and development funding from private and government stakeholders, RRT efforts to plan for their use, requests for EPA and other federal experts to advise field personnel on the use of such products, and by the response to the Deepwater Horizon oil spill. While many research efforts have helped to clarify issues and added information on the toxicity, efficacy, proper use, and human health impacts of these response technologies, uncertainties still arise. The Agency has an interest in resolving the issues that arise from the use of chemical and biological agents in spill responses, as its representatives are asked to concur with chemical and biological agent use for marine and freshwater spills.

The use of non-mechanical oil spill mitigating technologies, such as dispersants, surface washing agents, sorbents, solidifiers, bioremediation agents, and others are among the available oil response options that responders may consider in the United States and the world. The National Academies' National Research Council report titled ''Oil Spill Dispersants: Efficacy and Effects'' (2005), recommends that steps be taken to better support policymakers and spill responders faced with making choices regarding the use of dispersants as part of spill contingency planning efforts or during actual spills. The United States Coast Guard has promulgated new requirements for certain vessels to have only Schedule listed dispersant response capabilities while operating in designated dispersant preauthorization zones (74 FR 45004, August 31, 2009).

The proposed amendments are aimed at ensuring that chemical and biological agents have met efficacy and toxicity requirements, that product manufacturers provide important use and safety information, and that the planning and response community is equipped with the proper information to authorize and use the products in a judicious and effective manner.

V. This Action

This proposal addresses the efficacy, toxicity, environmental monitoring of dispersants, other chemical and biological agents, and other spill mitigating substances, as well as public, state, local, and federal officials concerns on their authorization and use. The sections below explain the proposed requirements and revisions, and EPA is requesting comments by section. Alternatives offered should include rationale and supporting information in order for the Agency to include the alternative in any final rule.

A. Discharge of Oil

The Agency is proposing revisions to harmonize 40 CFR part 110.4 with the definitions for chemical and biological agents proposed for Subpart J. The current language in §110.4 is specific to dispersants and emulsifiers. The proposal replaces the terms ''dispersants and emulsifiers'' with the broader terms of ''chemical agent'' and ''biological agent'' as proposed to be amended in §300.5. The proposed definition for chemical agents includes elements, compounds, or mixtures designed to facilitate the removal of oil from a contaminated environment and mitigate any deleterious effects. The proposed definition for biological agents includes microorganisms (typically bacteria, fungi, or algae) or biological catalysts, such as enzymes, able to enhance the biodegradation of a contaminated environment. Chemical and biological agents would include both the dispersants and emulsifiers cited in §110.4. By revising 40 CFR part 110, the Agency is clarifying that any chemical or biological agent added to a discharge of oil with the intent to circumvent any

provision of 40 CFR part 110 is prohibited. To further reflect the proposed revisions, the Agency is proposing to also amend the section title to ''Chemical or biological agents.'' EPA believes the proposed amendment is consistent with U.S. Coast Guard (USCG) regulations, which prohibit the discharge of chemicals or other substances into the sea that circumvent discharge conditions specified in their regulation (33 CFR 151.10(g)). EPA requests comment on these revisions.

B. Subpart A—Introduction

1. Definitions

The Agency is proposing revisions to §300.5 that amend the definitions for Bioremediation agents, Burning agents, Chemical agents, Dispersants, Sinking agents, and Sorbents. The Agency proposes to revise the term Surface washing agent and amend its definition. Additionally, the proposal includes new definitions for Bioaccumulation, Bioconcentration, Biodegradation, Biological agents, Bioremediation, Herding agents, Products, and Solidifiers. Finally, the Agency is removing the definitions for Miscellaneous Oil Spill Control Agent (MOSCA) and Surface collecting agents.

(a) Revised Definitions

Bioremediation agent—The Agency is proposing to revise the definition of bioremediation agents to identify as such biological agents and/or nutrient additives. These agents would be deliberately introduced into a contaminated environment to increase the rate of biodegradation, which in turn would assist in mitigating deleterious effects caused by contaminants. The proposed definition identifies as bioremediation agents microorganisms and enzymes. It also identifies nutrient additives such as fertilizers containing bio-available forms of nitrogen, phosphorus, and potassium. The proposed definition clarifies the current definition and adds specific examples of bioremediation agents. This clarification will help manufacturers of products in identifying the type of product, and hence, what testing requirements they will need to comply with to have a product listed on the Schedule.

Burning agents—The Agency proposes to revise the definition of burning agents to identify as such those additives that improve the combustibility of the materials to which they are applied. This could be achieved through either physical or chemical means. Burning agents include inorganic compounds in the form of gelling agents, such as aluminum soap. For example, the fuel used in helitorch systems is a mixture of powdered gelling agents with either gasoline, jet fuel, or a diesel/gas mixture, which are organic compounds.[1] The Agency believes both the inorganic gelling agent and the organic fuel (*e.g.,* gasoline) meet the burning agent definition by improving the combustibility of the materials to which they are applied through physical or chemical means. The Agency considered including ignition devices in the definition of burning agent because improving the combustibility of oil and igniting that oil could be considered one and the same. The intent would be to clarify the potential that not only substances, but also the devices holding those substances, be considered in the case-by-case authorization of use of such agents. The Agency rejected this approach since many devices either deliver the agent to the oil to be burned and do not enter the water, or are consumed in the burn along with the agent. The Agency requests comment on whether it should add ignition devices to the definition of a burning agent.

Chemical agents—The Agency proposes to revise the definition of chemical agents to identify as such those elements, compounds, or mixtures that are designed to facilitate the removal of oil. These agents may be used to mitigate deleterious effects of the oil on a contaminated environment. The proposed definition would include under the chemical agent category burning agents, dispersants, herding agents, sinking agents, solidifiers, surface washing agents, and those bioremediation agents that consist of nutrient additives. The proposed language reflects the distinction the Agency is now making between chemical and biological agents, allowing product manufacturers to better target the testing requirements and OSCs to better inform their authorization of agent use in specific situations. The proposal also removes from the definition agent categories that are either being eliminated, prohibited or amended to conform to the changes, as discussed below.

Dispersants—The Agency is proposing to revise the definition of dispersants to identify them as those agents that promote the formation of small droplets of oil in the water column by reducing the oil-water interfacial tension. Dispersants are proposed to be defined as typically mixtures of solvents, surfactants (including biosurfactants), and additives. The proposed definition specifically addresses the process through which these agents assist in mitigating the consequences of a discharge, clarifying for manufacturers which testing requirements they will be subject to when seeking to list a product on the Schedule.

Sinking agents—The Agency is proposing to revise the definition of sinking agents to identify them as those substances deliberately introduced into an oil discharge to submerge the oil to the bottom of a water body. The current definition is ambiguous in regards to how much sinking below the water surface would qualify as a sinking agent, as some submersion below the surface but generally not to the bottom of the water body can also be associated with other agents, such as dispersants. The proposed definition specifies these agents purposely sink the oil to the bottom of a water body. The Agency is prohibiting the use of sinking agents in the remediation of oil discharges in water because of their potential for causing adverse effects on benthic organisms vital to the food chain of the aquatic environment.

Sorbents—The Agency is proposing to revise the definition of sorbents to identify them as inert, insoluble substances that readily absorb and/or adsorb oil or hazardous substances. The proposed definition specifies that sorbents are not combined with or act as any other chemical or biological agent. The proposed definition also specifies that sorbents are generally collected and recovered from the environment and that they may be used in their natural bulk form, or as manufactured products in particulate form, sheets, rolls, pillows, or booms. The proposed definition identifies sorbents as substances consisting of: (1) Natural organic substances (*e.g.,* feathers, cork, peat moss, and cellulose fibers such as bagasse, corncobs, and straw); (2) inorganic/mineral compounds (*e.g.,* volcanic ash, perlite, vermiculite, zeolite, clay); and (3) synthetic compounds (*e.g.,* polypropylene, polyethylene, polyurethane, polyester). The proposed changes simplify the definition by removing the definitions of absorption and adsorption that are embedded in the current definition of sorbents. The Agency believes this is appropriate given these are generally recognized scientific terms and the proposal does not distinguish sorbents or in any way restrict their use based on whether they absorb or adsorb the oil. The definition also adds the ''natural'' qualifier to organic substances, indicating that organic substances that

[1] Fingas, Mervin, (Ed) (2011), Oil Spill Science and Technology, Gulf Professional Publishing, pp. 836, ISBN: 978–1–85617–943–0.

have been treated with other substances would not necessarily fall under this category of agents and should not immediately be considered a sorbent. It also expands on and simplifies the examples by removing the references to the type of birds that feathers could come from, by adding bagasse to the examples for natural organic substances, and by adding clay to the examples for inorganic/mineral compounds. The Agency requests comments on whether the definition is appropriate or if there are other materials that should also be included.

Additionally, the Agency requests comments on particulate materials (*e.g.,* clay) as sorbents. There is concern that particulate materials with densities greater than the seawater (or freshwater depending on where they may be used) have the potential to become sinking agents, settling to the seabed and posing potential risks to benthic organisms. This question is also relevant when considering emerging response technologies such as the use of particulate materials to form oil-mineral aggregates (OMAs) to promote dispersion. OMAs are stable microscopic entities formed when particulate materials interact with the oil droplets resulting in distinct oil and mineral phases. These fine mineral particles could be intentionally introduced by themselves to promote physical dispersion by preventing the oil to re-coalesce, or can also be used in conjunction with dispersants to enhance chemical dispersion. However, any particulate material that by itself, or when combined with oil (*e.g.,* OMA), results in overall densities less than the waters where it may be used, would not be prohibited as a sinking agent. The Agency requests comment on whether the use of sorbent materials in particulate form should be specified for use only in booms or other contained manufactured products, and whether there should be limitations on the authorization of use on water for sorbents in loose particulate form. Alternatively, the Agency also requests comment and supporting rationale on other approaches, including whether particulate materials in loose form, or specifically OMA technologies, should be excluded from the regulatory definition of sorbents.

Finally, EPA also requests comments on the qualifier phrase ''that are generally collected and recovered from the environment.'' For example, a natural organic and biodegradable sorbent (*e.g.,* bagasse) may not necessarily need to be removed after it has absorbed/adsorbed the oil when used in a wetland or salt marsh. Such removal may cause more harm than the oil itself due to trampling in the wetland or salt marsh. Once the oil is brought to the surface by the sorbent, biodegradation of both the oil and the sorbent can take place, especially if nutrients are added to enhance biodegradation. Another example would be the use of OMA technology to promote dispersion, which might not lend itself to collect or remove the aggregates from the environment. Thus, the Agency is requesting comment on whether testing and/or authorization of use requirements should be considered for particulate materials in loose form or OMA technologies that may be used in discharge situations where they would not be collected and recovered from the environment.

Surface washing agents—The Agency is proposing to revise the term surface washing agent and the definition for surface washing agents. The proposed definition would identify surface washing agents as those substances that separate oil from solid surfaces (*e.g.,* beaches, rocks, metals, or concrete) through a detergency mechanism. This detergency mechanism would lift and float the oil for collection and recovery from the environment. The use of these agents results in minimal dissolution, dispersion, or transfer of oil into the water column. The proposed changes revise the term from singular to plural to be consistent with the other agent definitions and clarifies that these agents are to be recovered from the environment along with the oil being treated.

(b) New Definitions

The Agency is proposing to add several new definitions that serve as the foundation for the new proposed biological agent classification: Bioaccumulation, Bioconcentration, Biodegradation, Biological agents, and Bioremediation. The proposed definitions include basic terminology and are consistent with definitions of these terms generally understood by the scientific community.

The Agency is also proposing new definitions for the terms herding agents and solidifiers. The proposed definitions address types of agents originally captured under the surface collecting agent or the miscellaneous oil spill control agent categories respectively, and are terms that are more commonly used in industry. The definitions more specifically describe the specific process through which the product affects the oil for the categories and are consistent with definitions of these terms generally understood by the scientific community.

Finally, the Agency is proposing a new definition for the term product to clarify the difference between a specific product and an agent type or category. All of the proposed new definitions can be found in the §300.5 of this action.

(c) Removed Definitions

The Agency is proposing to remove the definitions for surface collecting agent and for miscellaneous oil spill control agent (MOSCA). The surface collecting agent definition is being removed and replaced with a new herding agent definition to better reflect the common terminology used in industry for these agents. The MOSCA definition is being removed and replaced with a number of new and/or revised definitions for types of agents. The original MOSCA category was used as a catchall for all types of products that did not meet other agent definitions. As the Agency adds new, more stringent testing requirements for listing products on the Schedule, there is a need for more specific category definitions to assist manufacturers in determining which of those testing requirements apply to their products. The Agency believes it has identified categories that capture all products to be listed on the Schedule; we request comment on whether the MOSCA category should be retained, and whether the proposed categories are appropriate, including sufficient information and rationale to support the addition of any new categories.

C. Subpart J—Use of Dispersants, and Other Chemical and Biological Agents

1. General

EPA is proposing to amend §300.900 by revising paragraphs (a) and (c), and by adding paragraph (d) to reserve for ''Releases of Hazardous Substances''. The proposed revisions clarify that Subpart J addresses not only chemical agents, but also those agents that now fall under the newly proposed biological agent category. The revisions reaffirms the notion that Subpart J is not only comprised of a Schedule of chemical and biological agents, but also includes testing requirements and authorization of use procedures. Consistent with current Subpart J regulatory requirements the Agency is proposing to reserve a section for ''Releases of Hazardous Substances'' to take place of the current placeholder in §300.905, which is proposed to be removed. Based on all relevant circumstances, testing data and information, and in accordance with the authorization of use procedures (including the appropriate concurrences and consultations), the waters and

quantities in which a dispersant, or other chemical or biological agents may be safely used are to be determined in each case by the OSC. When taken together, these testing requirements, listing of agents and authorization of use procedures address the types of waters and the quantities of listed agents that may be used in response to discharges of oil or hazardous substances. The wide variability in waters, weather conditions, organisms living in the waters, and types of oil that might be discharged requires this approach.

2. Authorization of Use

Section 300.910 sets forth the provisions for the authorization of use of products on the Schedule in response to oil discharges. Subpart J does not state or imply that chemical or biological agents are preferred over other response options such as mechanical recovery devices. EPA believes that the circumstances surrounding oil discharges and the factors influencing the choice of response methods are many. During the DWH response, a priority countermeasures scheme was established to first use mechanical recovery via skimming/booming or in-situ burning followed by subsea dispersant and lastly surface dispersant use. Following DWH, EPA and the NRT issued Interim Actions regarding the use of dispersants on oil spills to NRT members and RRT co-chairs [2] and to EPA Regional Administrators [3] for consideration during response planning. While response actions are incident specific, the availability of response methods that address the specific discharge situation depends largely on contingency planning activities and on how these requirements are implemented through the RCPs, ACPs and vessel and facility response plans. In order for a response to be effective, the NCP requires coordination between the regulatory and planning entities responsible for all these response plans. The Agency believes that preauthorization or expedited decision making plans are critical elements of contingency planning activities. Regularly reviewing or revising preauthorization or expedited decision making plans provides those agencies in charge of preparedness planning the opportunity to identify and resolve concerns in advance, leading to quick and effective operations during removal actions. The Agency believes these proposed revisions to the authorization of use provisions will assist OSCs, RRTs, and ACs in their advanced planning activities as they consider response methods that result in the greatest environmental protection. The goal is to ensure that preauthorization or expedited decision making plans are developed and maintained to effectively support decisions by OSCs during removal actions. In carrying out advanced planning activities, the Agency believes the NRT can assist RRTs by providing guidance on national level issues that may arise during planning activities.

EPA is amending §300.910 by revising paragraphs (a) through (f); and by adding paragraphs (g), (h) and (i). EPA is not proposing major changes to the current authorities granted to OSCs, RRT representatives to the RRTs, States, DOC, DOI, or other National Response Team decision makers with regards to the authorization and application of chemical or biological agents. However, EPA recognizes that the planning for and prolonged use and monitoring of chemical agents, especially dispersants, may require additional planning activities and monitoring requirements. Thus, the Agency is proposing to reorganize this section; to add titles to the paragraphs for ease of use; to add several requirements addressing the storage and use of agents, notification of agent use and recovery from the environment; and to revise language to clarify established EPA policy. The proposed revisions and clarifications are highlighted for each paragraph under §300.910.

EPA is also confirming, consistent with the intent of the NCP, that use of chemical or biological agents in response to oil discharges to waters of the U.S. or its adjoining shorelines must be authorized by an OSC in accordance with Subpart J. The unauthorized use can result in violations of section 301 and 311 of the CWA. Section 301(a) makes unlawful "the discharge of any pollutant by any person," except in compliance with certain provisions of the CWA. In addition, section 311(b) establishes penalties for persons who fail or refuse to comply with any regulation issued under section 311(j) of the CWA.

(a) Use of Agents Identified on the Schedule on Oil Discharges Addressed by a Preauthorization Plan

The Agency is proposing revisions to §300.910(a) of Subpart J to address the preauthorized use of chemical and biological agents identified on the Schedule. The proposed revisions clarify the process for preauthorization, the responsibilities of all involved parties, and the factors to consider during the preauthorization process, including the authorization for the use of agents by the OSC at the time of a discharge. EPA is also proposing to reorganize paragraph (a) to provide greater clarity by making the regulatory text easier to read and follow. The Agency believes that the proposed revisions do not change its fundamental policies regarding roles of Federal, state and local representatives involved in planning for and responding to an oil discharge, but rather clarify the current requirements and further explain the responsibilities for each party. The Agency is also proposing added procedures and review requirements intended to ensure preauthorization plans are up-to-date so they are most effective when implemented in case of a discharge.

EPA believes RRTs and ACs must work together in order to effectively and successfully manage contingency planning. Thus, the proposed revisions continue to require that, as part of their planning activities, RRTs and ACs address in the preauthorization plan whether the use of chemical and biological agents listed on the Schedule on certain oil discharges is appropriate. The Agency believes that the proposed revision clarifies the meaning of the desirability of using appropriate chemical or biological agents. The Agency is removing ". . . the desirability of using appropriate burning agents." from paragraph (a) and addressing the use of burning agents under paragraph (c) to provide greater flexibility to OSCs for authorization of use.

Under the current paragraph (a), ACs are responsible for developing preauthorization plans. ACs are also responsible for developing ACPs, providing a forum to evaluate the environments within a jurisdiction and establishing protection priorities. The information gathered during the ACP development process should inform the development of preauthorization plans. Identified representatives from the RRTs are responsible for approving or requesting modifications of preauthorization plans developed by the ACs. ACs can advocate for local

[2] Tulis, Dana S., EPA Chair and Caplis, Captain John, USCG Vice-Chair, National Response Team, "Use of Dispersants on Oil Spills—Interim Actions;" Memorandum to NRT Members and RRT Co-Chairs; December 16, 2010.

[3] Stanislaus, Mathy; Assistant Administrator, OSWER EPA; "Revision of Area Contingency Plans/ Regional Contingency Plans Regarding Use of Dispersants on Oil Spills—Interim Actions;" Memorandum to EPA Regional Administrators, November 2, 2010.

concerns to be reflected in the preauthorization plans they develop, and the RRTs will decide if the plans are adequate and address region-wide and cross-regional issues. Since the RRTs should be in a position to provide guidance to ACs on common attributes within a region, EPA continues to believe RRTs and ACs should work together to develop preauthorization plans, particularly when identifying discharge situations where chemical or biological agents may be used. There may be circumstances where it is appropriate to allow either the RRTs and/or ACs to develop preauthorization plans. Preauthorization plans developed by an RRT would allow for these plans to better reflect overarching regional circumstances. Therefore, the Agency is proposing to include RRTs as another planning entity with responsibility for developing preauthorization plans, and is requesting comment on this change, and on the advantages or disadvantages of keeping the development of these plans at the AC level.

ACPs generally describe discharge situations for the geographical area for which they apply. Discharge situations developed as part of area contingency planning activities may consider likely scenarios from vessels, and onshore or offshore facilities. While RRTs and ACs should consider the discharge scenarios described in ACPs when identifying discharge situations in developing preauthorization plans, the Agency believes they should also have the flexibility to consider other discharge scenarios. The proposed language states preauthorization plans must specify limits for the quantities and duration of use, and use parameters for water depth, distance to shoreline, and proximity to populated areas for discharge situations identified in which agents may be used. The Agency believes that clearly stating the use parameters in a preauthorization plan will make it easier for planners to address concerns of preauthorizing agent use and in turn for responders to authorize their use.

In meeting these provisions, the preauthorization plans should document how both regional and logistical factors were addressed when establishing dispersant use limits and parameters. Regional factors include the likely sources and types of oil that might be discharged, various discharge scenarios, and the existence and location of environmentally sensitive resources or restricted areas that might be impacted by discharged oil. Logistical factors include inventory, storage locations and manufacturing capability of available agents, availability of equipment needed for agent use, availability of adequately trained operators, and the availability of appropriate means to monitor agent use. While the proposed revisions simplify the language and clarify the requirements, the Agency believes it is necessary to keep in place the fundamental elements that should be considered. The proposed factors generally parallel those under the current 300.910(a) regulation. Several revisions include identifying some factors as "regional" and others "logistical." The Agency is identifying "logistical" factors to ensure the availability of chemical and biological agents to address discharge situations identified in the preauthorization plan. The Agency added "various discharge scenarios" as a regional factor to be considered because preauthorization plans may cover more than one ACP with multiple discharge scenarios that RRTs and/or ACs may want to consider. The Agency also added the existence and location of "restricted areas" along with "environmentally sensitive resources" as a factor to consider. Environmentally sensitive resources would include fish, wildlife and their habitats, and other special areas of ecological sensitivity that may be adversely affected by a discharge. While "restricted areas" may include "environmentally sensitive resources" some areas may be restricted from certain activities because of biologically sensitive topographic features or critical habitats, such as submerged rock formations colonized by species (*e.g.,* coral) and the organisms they interact with and support. NOAA's Environmental Sensitivity Index maps, the Fish and Wildlife and Sensitive (FWS) Environments Plan in ACPs, or environmental impact statements, may contain relevant information on "environmentally sensitive resources" for the RRTs and ACs to consider.

The proposal revises the phrase "potential sources and types of oil that might be spilled" to "likely sources and types of oil that might be discharged." EPA believes the phrase "likely sources and types of oil" better focuses on the sources and types of oil specific to the preauthorization plan for which agents may be used. In addition, the proposal revises "spill" to "discharge" to be consistent with terminology in the NCP. The proposal also revises the phrase "the available means to monitor product application and effectiveness" to "means to monitor agent use in the environment" as the Agency believes it provides for additional flexibility for the RRTs and/or the ACs to consider the scope of the monitoring, and to include other endpoints beyond product application and effectiveness. The Agency is proposing to eliminate the "available" qualifier, as it believes it is unnecessary. Likewise, the Agency proposes to eliminate the "available" qualifier before "product and storage locations" and revise the phrase "product and storage location" to the broader "inventory, storage locations and manufacturing capability of available agents" to address lessons learned from the Deepwater Horizon incident, including the challenges posed by the potential sustained use of dispersants.

When developing preauthorization plans, RRTs and ACs should use the best available scientific information to assess environmental trade-offs, including those identified by conducting an ecological risk assessment. Environmental trade-offs should be considered in determining response options that provide the greatest environmental protection. The RRTs and ACs should identify the affected biological resources and their habitats likely to be negatively impacted, as well as those that are expected to benefit. The natural resource trustees are critical partners that can assist in conducting these analyses.

As previously stated, all members of the RRT are afforded an opportunity to review and provide input on a draft preauthorization plan. However, only the RRT representatives from EPA and the state(s) with jurisdiction over the waters and adjoining shorelines within the preauthorization plan area and the DOC and DOI natural resource trustees may approve, disapprove, or approve with modification the draft preauthorization plan. The Agency believes this remains the correct approach. Given preauthorization plans are developed during the contingency planning phase, the Agency believes that DOC and DOI natural resource trustee concurrence is preferred over just consultation because it provides for sufficient time to identify and resolve natural resource concerns. As noted in the 1994 NCP final rule, the requirement for concurrence during the advanced planning phase ensures trustee involvement in decision-making (59 FR 47398). Addressing in advance concerns that might otherwise slow the action ensures that operations during a removal action can be carried out quickly and effectively. EPA believes natural resource trustee concurrence with preauthorization plans satisfies the consultation obligation since the preauthorization plans specify the use parameters for chemical or biological

agents. Thus, the Agency is retaining this concurrence requirement for preauthorization plans.

The Agency is proposing to revise the "Approved preauthorization plans shall be included in the appropriate RCPs and ACPs" in the current §300.910(a) regulation to "RRTs and ACs shall, as appropriate, include applicable approved preauthorization plans in RCPs and ACPs." The Agency is proposing to modify the qualifier "approved" with "applicable" to clarify that RRTs and ACs need to include the preauthorization plans only in those RCPs and ACPs to which they apply, and remains consistent with current requirements.

The Agency considered adding specifically the EPA Administrator and the senior EPA representative to the NRT (*e.g.* NRT Chair) to §300.910(a) and other paragraphs to make clear the EPA Administrator's and senior EPA NRT representative's existing authority under section 311(d)(2)(G) of FWPCA and Executive Order 12777, along with the OSC to authorize any chemical or biological agent use. The Agency is clarifying that if the preauthorization plan is approved in advance for chemical or biological agent use under specified discharge circumstances, then the OSC may authorize the use of the agents on the Schedule for their intended purpose without the incident specific concurrences and consultations described in paragraphs (b) of this section unless otherwise directed by the Administrator in accordance with current concurrence authority. The Agency believes this clarification would not impede rapid decision-making on the part of the OSC, and that for the majority of discharge situations, the OSC will remain as the sole authorizing entity for discharge situations covered by preauthorization plans. Note that in situations like a spill of national significance (SONS) or an event of extended duration, the Administrator already has the authority for, and is likely to have a more direct role in chemical or biological agent use decisions. The authority, jurisdiction, and implementation provisions in the NCP flow from section 311 of the Clean Water Act and are reflected in Executive Order 12777. All authorities under CWA 311 are delegated either directly to the Administrator by Congress, or by Executive Order 12777 from the President to the Administrator. While the Administrator's authority may be further delegated through senior management on down to the RRT representative, the Administrator (and other delegatees) retain the authority to act. The mere delegation of authority does not prohibit the delegator from exercising said authority. However, given these situations are rare and that this is an existing authority, the Agency is not proposing a regulatory amendment to clarify the Administrator's authority at this time. We request comment on this issue.

The Agency is clarifying that chemical or biological agents may only be used for their intended use, given the different listing requirements proposed for the various categories of chemical or biological agents. For example, a chemical agent that is listed on the Schedule solely as a surface washing agent cannot be authorized for use as a dispersant, nor can a chemical agent that is listed on the Schedule solely as a dispersant for use under saltwater conditions be used in freshwater.

The Agency is proposing specific procedures for concurrence withdrawals, allowing agencies to do so if they believe the preauthorization plan no longer addresses or reflects existing situations if it were to be implemented. While an agency with concurrence authority may now decide to withdraw concurrence from an approved preauthorization plan, there are currently no set procedures to promptly address those situations. The proposal would require the RRT and the ACs to address the withdrawal of approval of the preauthorization plan within 30 days of the withdrawal, allowing an opportunity to address the concerns. The proposal also calls for the RRT to notify the NRT of the final status of the preauthorization plan within 30 days from the withdrawal. The Agency requests comments on whether this 30 day notification requirement should also include notification to the public. In the event of an Agency withdrawing its concurrence from an approved preauthorization plan, EPA believes the advanced planning process should continue with consideration for all the elements specified in paragraph (a) of this section. While the absence of a preauthorization plan requires that authorizations for agent use be conducted according to paragraph (b) of this section, the Agency continues to believe that preauthorization plans serve as a valuable advanced planning tool that provides a strong foundation to support decision-making and strongly encourages the resolution of any withdrawal.

Finally, EPA proposes a new requirement for RRTs and/or ACs to review, and revise as needed, preauthorization plans: (a) At least every 5 years to address revisions of the Schedule; (b) after a major discharge or a spill of national significance (SONS); (c) to reflect new listings of threatened and/or endangered species or; (d) after any other change such as a new or revised worst case discharge estimate that may impact the conditions under which the use of chemical and biological agents is preauthorized. A 5-year review cycle is consistent with facility response planning requirements; as those plans are revised and updated, it seems reasonable that preauthorization plans should be reviewed and revised accordingly. The Agency recognizes that development of preauthorization plans can be resource intensive; however, once developed, a periodic review and revision as needed should require much less effort. EPA welcomes comment on this timeframe and suggestions with supporting information for alternatives.

This review requirement is intended to ensure that preauthorization plans are actively maintained and updated to reflect revisions to the Schedule. Preauthorization plans, as well as the facility and vessel response plans reflected in them, may include information on products listed on the Schedule. A review at least every 5 years is expected to provide greater consistency not only between any Schedule revisions, but also between any ACPs, facility, and vessel response plans. For example, an ACP revision that results in a change in the worst-case discharge scenario could trigger a preauthorization plan review. Additionally, the requirement specifically includes plan review and revision requirements as appropriate to reflect new listings of threatened and/or endangered species that may occur. The EPA RRT representative, the DOC and DOI natural resource trustees, and, as appropriate, the RRT representative from the state(s) with jurisdiction over the waters of the area to which a preauthorization plan applies must review and either approve, approve with modification, or disapprove any revisions to the preauthorization plans. This review and approval is intended to focus on any revisions, and is not intended as a requirement for review and approval for those portions that do not require modifications.

(b) Use of Agents Identified on the Schedule on Oil Discharges Not Addressed by a Preauthorization Plan

The Agency is proposing revisions to §300.910(b) of Subpart J to address use of chemical or biological agents identified on the Schedule for discharge situations that have not been addressed in preauthorization plans. The proposed revisions clarify the authorities and responsibilities of all involved parties,

and the factors to consider when authorizing the use of listed chemical or biological agents in these situations. The Agency believes the proposed revisions do not change its fundamental policies regarding the roles of Federal, state and local representatives involved in an oil discharge response.

The proposed revisions maintain, with the appropriate concurrences and consultations, the OSC's authority to authorize the use of chemical or biological agents on the oil discharge, provided that the agents are listed on the NCP Product Schedule. The concurrence of the EPA representative to the RRT and, as appropriate, the concurrence of the RRT representatives from the states with jurisdiction over the waters and adjoining shorelines threatened by the release or discharge is maintained. The requirement for consultation with the DOC and DOI natural resource trustees is also maintained. However, the language is amended by removing "when practicable" with respect to consultation with the DOC and DOI natural resource trustees. The Agency believes that the case-by-case decision making should include consultations with natural resource trustees since these discharge situations may present unique challenges when selecting a response option that involves chemical or biological agents. While the Agency recognizes the time-critical nature of decision making during a response, advances in communication technology (*e.g.,* smart phones, email) provide OSCs with increased capabilities to communicate quickly. Therefore, the Agency believes it is reasonable to expect an OSC to be able to notify and explain the circumstances requiring use of the certain agents to natural resource trustees in a timely manner. Of note, while consultation with the trustees on removal actions as required by OPA does not equate to a concurrence requirement, the Agency believes that such concurrence is highly desirable. The Agency is also proposing to revise the term "navigable waters threatened" to "waters and adjoining shorelines threatened" to be consistent with the provisions in paragraph (a) of this section.

The preauthorization plan requirements in paragraph (a) proposes to remove the term "specific context" currently used and instead clearly establish what the term refers to. The proposal specifically identifies the parameters that must be considered by the OSC for authorizing agent use. Similar requirements are proposed under paragraph (b). Thus, in meeting the provisions of §300.910(b), the OSC must consider and document the parameters for the use of agents including, but not limited to, quantities to be used, the duration of use, the depth of water, the distance to shoreline and proximity to populated areas, and should address factors such as environmentally sensitive resources or restricted areas that might be impacted, agent inventory and storage locations, agent manufacturing capability, availability of equipment needed for agent use, availability of adequately trained operators and appropriate means to monitor agent use in the environment. These considerations are parallel to those proposed under paragraph (a) for preauthorization and planning purposes. While the Agency is not including other factors that may inform preauthorization planning development, such as various discharge scenarios, this does not mean these factors cannot or should not be considered if RRTs and/or ACs choose to develop expedited decision making plans. The Agency believes these are fundamental elements that would inform an assessment of the overall ecological risks for the OSC to consider when authorizing the use of chemical or biological agents.

Finally, while not required, EPA strongly recommends advanced planning for expedited decision making for cases where the discharge situation is not addressed in the preauthorization plans. Some RRTs have developed expedited approval guidelines that are not part of the preauthorization plans, but that offer an opportunity for advanced contingency planning by gathering information on the key parameters discussed above. Because discharge situations not covered by preauthorization plans need incident specific (*i.e.,* case-by-case) authorization concurrence,[4] expedited approval guidelines can be used to support expedited incident specific authorizations. For chemical or biological agents listed on the Schedule that are not authorized for use under a preauthorization plan, the ACs and RRTs should work together to outline the process for expedited authorization decisions regarding their use. It is important to note that while the NCP requires that the ACPs include procedures for expedited decisions, these procedures can include disapproving the use of agents, or approving the use of agents with certain operational conditions. For example, areas may be designated in which the use of certain agents or other discharge mitigating devices is prohibited, situations where limits are placed on the quantities of agents used, or situations that require certain monitoring requirements be in place.

(c) Burning Agents

The Agency is proposing to replace the current authorization of use for burning agents in §300.910(c) with a provision that provides greater flexibility to OSCs for authorizing the use of burning agents. Specifically, the Agency proposes that OSCs may authorize the use of burning agents for authorized in-situ burns. The proposed amendments recognize that relatively small quantities of burning agents are ignited prior to or immediately after they are introduced to an oil discharge. Furthermore, they are composed of substances that are expected to rapidly burn off during use, which serves to remove them from the water. The Agency also recognizes that ISB has become an important response option that is used more frequently and the proposed revisions would allow OSCs to authorize the use of burning agents for authorized burns. For example, a significant number of ISBs were conducted during the Deepwater Horizon oil spill,[5] and ISBs appear to be gaining a more prominent role as a response option in federal waters in remote locations, such as the Arctic. Therefore the Agency believes the proposed revisions better address OSC authorities for these situations, without compromising environmental concerns. Further, because of the nature of burning agents and the proposed revisions to the authorization of use for these products, the Agency continues to believe it is not necessary to require product submissions for burning agents. Thus, the proposal removes the provisions for burning agents under the current data requirements. The Agency requests comments on this approach.

(d) Exceptions

The proposed rule maintains the provision allowing OSCs to authorize the use of any agent, including products not on the Schedule, when it is determined that the use of the agent is necessary to prevent or substantially reduce a threat to human life. The proposed revisions do not change previous policy, but rather clarify the intent of the exception. The Agency believes that the protection of human life is the primary consideration in responding to an oil discharge. Life-

[4] *http://www.epaosc.org/sites/5083/files/rrt6_nearshore_dispersant_eap_031605.pdf.*

[5] *http://www.restorethegulf.gov/release/2011/08/19/operations-and-ongoing-response-august-17-2011.*

threatening oil discharges (*e.g.,* spills of highly flammable petroleum products in harbors or near inhabited areas) may occur at locations where chemical agents on the Schedule are not immediately available. The Agency believes that in such cases, an OSC must have the ability to use agents that, in his professional judgment, would effectively and expeditiously mitigate the threat to human life. Allowing this authorization to occur without the required concurrences for preauthorization or authorization of use for products on the Schedule under paragraphs (a) and (b) respectively, eliminates delays in responding to potentially life-threatening situations. The Agency is proposing to include "without obtaining the immediate concurrence" to clarify the scope of the exception. The proposed revisions are consistent with the intent of the current regulation which recognizes that once the threat to human life has subsided, the continued use of a product shall be in accordance with authorization of use paragraphs (a) and (b) of the section. In addition, this exception is intended for those extraordinary situations in which time is of the essence to mitigate the threat to human life; revising the language to replace "hazard" with "threat" clearly establishes this. The Agency emphasizes this authority is not intended to circumvent the authorization of use provisions in paragraphs (a) and (b) of this section, which serve to address all other situations.

The proposed revisions also specify that the OSC immediately notify, and document the reasons for the use of an agent to the EPA RRT representative and the affected states as soon as possible, and must obtain their concurrences where continued use of chemical or biological agents extends beyond 48 hours. The Agency believes that advances in communication technologies (*e.g.,* smart phones, email) provide OSCs the increased capabilities to communicate quickly. Therefore, it is reasonable to expect that the OSC notify and explain the circumstances requiring use of the agent to the designated EPA RRT representative and, as appropriate, the RRT representatives from the affected states and the DOC/DOI natural resources trustees within 48 hours. The Agency is requesting comments on these revisions, and specifically on the 48 hour timeframe within which the OSC shall be operating in accordance with authorization of use paragraphs (a) and (b) of the section.

(e) Prohibited Agents

The Agency is maintaining the current prohibition for the authorization of use of sinking agents, and is clarifying that this prohibition also applies to any other chemical agent, biological agent, or any substance that acts as a sinking agent when mixed with oil. While certain chemical and biological agents may submerge oil below the water surface (*e.g.* dispersants are designed to break up oil into small, near neutrally buoyant particles that are entrained in the water column between the surface and the bottom), they would not be considered "sinking agents" for purposes of the proposed definition and this prohibition, given that they do not completely submerge oil to the bottom of the water body when applied to an oil discharge. Sinking agents, when applied to oil discharges, function by sinking floating oil to the bottom of any body of water where used, potentially causing adverse effects on benthic organisms vital to the food chain of the aquatic environment. Additionally, the oil and these agents are very difficult to remove. The Agency has similar concerns regarding substances that could directly cause the oil to submerge to the bottom of the water body when used in an oil spill response, and thus it is specifically proposing to exclude their use.

The Agency is also proposing to add a prohibition from listing on the Schedule and from authorizing use of any chemical or biological agents that contain nonylphenol (NP) or nonylphenol ethoxylates (NPEs) as components. This prohibition reflects the Agency's concern for these substances, as presented in the *EPA Nonylphenol and Nonylphenol Ethoxylates Action Plan,* released August 18, 2010. The Action Plan specifically addresses nonylphenol (NP) and nonylphenol ethoxylates (NPEs). NP and NPEs are produced in large volumes, with uses that currently lead to widespread release to the aquatic environment. NP is persistent in the aquatic environment, moderately bioaccumulative, and extremely toxic to aquatic organisms. NP's main use is in the manufacture of NPEs. NPEs are nonionic surfactants that are used in a wide variety of industrial applications and consumer products. Many of these, such as laundry detergents, are "down-the-drain" applications. Some others, such as dust-control agents and deicers, lead to direct release to the environment. NPEs, though less toxic and persistent than NP, are also highly toxic to aquatic organisms, and, in the environment, degrade into NP. Both NP and NPEs have been found in environmental samples taken from freshwater, saltwater, groundwater, sediment, soil and aquatic biota. NP has also been detected in human breast milk, blood, and urine and is associated with reproductive and developmental effects in rodents. EPA has encouraged the ongoing voluntary phase-out of NPEs in industrial laundry detergents, and intends to evaluate how releases and exposures are mitigated through the phase-out action prior to taking any final regulatory action under the Toxic Substances Control Act.[6] The Agency believes this prohibition would not adversely affect product manufacturers given there are viable alternatives to the use of NP and NPEs in product formulations. However, we are requesting comment on the potential impacts of modifying existing products to meet this new requirement, including cost.

Alternatively, EPA considered a broader prohibition from listing and from authorizing the use of chemical or biological agents formulated with any endocrine disrupting compounds (EDC). EDCs impact exposed organisms by altering the hormonal and homeostatic systems that allow them to interact with and respond to their environment. The group of molecules identified as potential endocrine disruptors is highly varied and may be present in chemicals used as industrial solvents or surfactants that can be found in dispersants and surface washing agents. Because of the common properties of these compounds and the similarities of the receptors and enzymes involved in the synthesis, release, and degradation of hormones, no endocrine system is immune to endocrine disrupting compounds.[7]

The 1996 Food Quality Protection Act directed EPA to develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have hormonal effects in humans; the 1996 amendments to the Safe Drinking Water Act authorized EPA to screen substances that may be found in sources of drinking water for endocrine disruption potential. In response to these mandates, the Agency's Endocrine Disruptor Screening Program is developing requirements for the screening and testing of pesticides, commercial chemicals, and

[6] For more information, refer to *http://www.epa.gov/oppt/existingchemicals/pubs/actionplans/np-npe.html.*

[7] For more background information on endocrine disrupting compounds and their human health and environmental effects, please see *http://www.epa.gov/endo/pubs/edspoverview/index.htm.*

environmental contaminants for their potential to disrupt the endocrine system.[8] The science related to measuring and demonstrating endocrine disruption is relatively new and validated testing methods are still being developed. When complete, EPA will use these validated methods or assays to identify and characterize the endocrine activity of pesticides, commercial chemicals, and environmental contaminants, specifically in relation to estrogen, androgen, and thyroid hormones. EPA plans to use the assays in a two-tiered screening and testing process: Tier 1 will serve to identify chemicals that have the potential to interact with the endocrine system; and Tier 2 will determine the endocrine-related effects caused by each chemical and obtain information about effects at various doses. With this two-tiered approach, the Agency will gather information needed to identify endocrine-active substances and take appropriate action, as mandated by Congress. To date, EPA has developed and validated 11 assays that comprise the Tier 1 Screening Battery. EPA's validation effort continues with the current focus on Tier 2 tests and potential replacement assays for Tier 1.[9] When all tests are finalized and chemical information begins to be collected, EPA may further consider how to incorporate that information into the Subpart J NCP requirements, including additional testing and listing requirements. Because validated testing methods are still being developed, the agency is not proposing any test requirements with respect to endocrine disruption for products to be listed on the Schedule.

However, because NP and the NPE are extremely or highly toxic to aquatic organisms, the Agency is proposing a prohibition specific to Subpart J products containing NP and NPE as components. The Agency is not proposing to include a broad prohibition on Subpart J products that contain substances in which at least part of the toxic mode of action may include disruption of the organism's endocrine system, but is requesting comments on prohibiting similar substances that may be found in products/agents that could be listed on the Schedule, or to what the criteria should be in order to make that determination. As an alternative, the Agency could require that all product components be tested for a toxic mode of action that includes disruption of the organism's endocrine system and is requesting comment on this approach as well.

The Agency believes the proposed prohibitions are appropriate in all cases, notwithstanding the proposed provisions for case-by-case use authorization of burning agents under §300.910(c), or for authorization of use of any chemical or biological agent when it is necessary to prevent or substantially reduce an immediate threat to human life under §300.910(d). There are chemical and biological agent alternatives to sinking agents and to agents containing NP or NPE, as well as mechanical methods for responding to oil discharges, including those situations that pose extreme threats or are time critical.

(f) Storage and Use of Agents

Section 300.915 currently requires that information be provided on recommended conditions of storage and use for each product at the time an application for listing a product under Subpart J is submitted to the Agency. This information is summarized in EPA's *NCP Product Schedule Technical Notebook*. Specifically, this information includes: Special handling and worker precautions for storage and field application; maximum and minimum storage temperatures (optimum ranges and temperatures that will cause phase separations, chemical changes, or other alterations to product efficacy); shelf life of the product; recommended application rates and procedures, concentrations and conditions (considering water temperatures, salinity, types and ages of the oil); and any other application restrictions.

The Agency proposes to add a new paragraph (f) to this section that complements the existing information requirements for the person or entity submitting a product for listing ("submitter"). The proposed requirements focus on the use of this information by the responder and the OSC. Specifically, the revised provisions require the OSC to only authorize for use those products that are stored under the conditions specified by the submitter of the product for listing, including the maximum, minimum and optimum temperatures, humidity and any other relevant conditions. Additionally, the Agency proposes to require that the OSC only authorize for use those products whose date of use does not exceed the expiration date listed on the container's label at the time of an incident, unless the responsible party[10] provides the OSC documentation, developed in consultation with the submitter of the product for listing, prior to product use, affirming the product has maintained its integrity, including no changes in the composition, storage conditions, efficacy, and toxicity of any product. In such cases where the product expiration date has passed but the owner or operator of the facility/vessel storing the product still wants to be able to use the product inventory, EPA proposes that samples of the expired product lot representative of all storage conditions at any end user location be tested following the applicable testing protocols in Appendix C. The testing documentation is to include laboratory information (*i.e.,* contacts, accreditations) and all test data and calculations (*i.e.,* raw data and replicates, notes and observations, calculated means and standard deviations, stock solution preparations, source and preparations of test organisms, test conditions, chain of custody forms, and summary reports). Only if the owner or operator can demonstrate that the product has maintained its integrity is the OSC allowed to authorize the use of that product inventory. The owner or operator of the facility/vessel, or their representative, must re-label the tested product lots and maintain documentation of the test results until those lots are used or discarded, and must retest the expired product lot representative of the product at least once every 5 years to ensure efficacy and to allow an OSC to authorize the product for use. The intent of these proposed revisions is to ensure that products being authorized and used have maintained their efficacy, even though storage beyond the products' original shelf life may have occurred. The Agency believes it is the owner or operator's responsibility to ensure that any product it stockpiles for future use has maintained its efficacy and has not changed from its listed composition, including the possibility of degrading into more toxic byproducts, once the manufacturer's expiration date has passed. This retesting provision is supported by the proposed requirements that the submitter of the product for listing provide not only the recommended storage conditions, but the anticipated shelf life of a product at those conditions, and that the product label include both the manufacture and expiration dates, and conditions for storage. The flexibility in this proposed

[8] *http://www.epa.gov/endo/pubs/edspoverview/background.htm.*

[9] *http://www.epa.gov/endo/pubs/assayvalidation/index.htm.*

[10] Responsible party is defined in the NCP under 40 CFR 300.5.

revision allows for the use of available product inventory that may still be viable. To alleviate concerns that applications are submitted that establish an extended or indefinite shelf life for a product, the Agency is requesting comments on whether any additional data or information requirements should be included for product listing determinations specific to a product's shelf life, or whether alternative approaches, such as limiting the shelf life for product categories to a given timeframe, should be considered.

(g) Supplemental Testing, Monitoring, and Information

This proposal maintains RRT authority in that they may require supplementary toxicity and efficacy testing, or available data or information that addresses site, area, or ecosystem specific concerns relative to the use of a product for both planning and authorization of use. While parallel to the current provisions, the proposed revisions provide added flexibility. The current provisions allow RRTs to require supplementary toxicity and efficacy testing of products, in addition to those specified as technical requirements following the test methods described in Appendix C to part 300, due to existing site-specific or area-specific concerns when developing preauthorization plans. EPA proposes to remove the qualifier "When developing preauthorization plans" to provide greater flexibility for RRTs to require supplementary toxicity and efficacy testing, or available data or information that addresses site, area, or ecosystem specific concerns relative to the use of a product for situations even when preauthorization plans are not being developed. For example, RRTs may need additional testing or information for situations that fall under paragraph (b) of this section, including when developing an expedited decision making plan. The proposed revision also allows for RRTs to require supplementary product toxicity and efficacy testing, or available data or information for both planning and authorization of use situations, and based on that information may consider establishing limitations for the use of products in certain areas. The Agency is including "available data and information" to compliment the supplementary toxicity and efficacy testing provision recognizing that existing data or information that addresses site, area, or ecosystem specific concerns relative to the use of a product may be available. The Agency is also proposing to include "ecosystem" with area and site specific concerns, as RRTs may want to gather additional information on the use of certain products when assessing the use of a product relative to the biological communities specific to their area. The proposal removes "in addition to the test methods specified in §300.915 and described in appendix C to part 300." While RRTs may want to use the efficacy and toxicity testing protocols specified in Appendix C of the NCP for comparative purposes, the proposed revisions clearly establish the RRTs authority to require tests using parameters beyond those specified in Appendix C. Furthermore, there may be supplementary toxicity and efficacy testing information based on recognized standard testing methods already available that RRTs may want to consider when addressing site, area, or ecosystem specific concerns.

This proposal also provides the RRT authority to request that the OSC require a responsible party to conduct additional monitoring associated with the use of a product during a discharge incident. The proposed revision compliments the proposed monitoring requirements for dispersant use, but also including other chemical or biological agents, or other testing endpoints. The Agency believes the RRT must be afforded the ability to request that the OSC direct the responsible party to conduct additional monitoring under Subpart J for the use of a product in the environment. The RRT may request that the OSC consider additional monitoring during an oil discharge response to support operational decisions on dispersant use. For example, the RRT may want to monitor the exposure of marine mammals to oil constituents, including dispersed oil, or to monitor toxicity in the water column using biological assays. The Agency requests comments on these proposed revisions.

(h) Recovery of Agents From the Environment

The proposal identifies certain agent categories and substances intended to be removed from the environment following their use: Solidifiers, sorbents and surface washing agents. For those categories, the Agency expects the agents to be recovered from the environment to minimize any potential adverse impact. The proposal adds a new requirement that charges the responsible party, under OSC oversight, to recover these products from the environment. Recovery activities after the use of these agents would include containment of the agents in the water, collection of the agents mixed with oil or any residual agent, storage of the oil-agent waste prior to disposal, and disposal of that oil-agent waste. The Agency also recognizes there may be situations where the safety of response personnel is threatened, or where additional harm to the environment could occur during recovery operations. Consequently, we are proposing these factors be considered when initiating recovery actions. While it is appropriate to have the OSC ensure these activities take place, it is ultimately the responsibility of the responsible party to conduct these activities and ensure that the agents are recovered from the environment. The Agency requests comments on this proposed requirement.

(i) Reporting of Agent Use

The proposal includes a new requirement for the OSC to provide to the RRT certain information after the use of a chemical or biological agent within 30 days of completion of agent operations. The information required in this report includes the information on any chemical or biological agent used, including product name, the quantity and concentration of the agent used during the response, the duration of use, the locations where the agent was used, and any data collected and analysis of efficacy or environmental effects. The proposal allows this information to be provided in the OSC report to the NRT or RRT as required under section 300.165 of the NCP, if such a report has been requested. While other existing notification requirements serve to activate an immediate response to an event, the proposed requirement gathers information that will be useful in specifically evaluating the use of chemical or biological agents in the response. It will also inform the review of preauthorization plans and provide a basis for any necessary changes to improve environmental protection. Given that response and removal actions can greatly vary depending on the discharge situation, the Agency requests comments on whether it would be appropriate to allow the timeframe for submitting the report to be agreed upon by the RRT, rather than establishing a set timeframe. The Agency is requesting comments on this proposed new requirement, including on the proposed timeframe.

3. Monitoring the Use of Dispersants

The goal of establishing a Schedule under the NCP is to protect the environment from possible damage related to spill mitigating products used in response to oil discharges. The proposal establishes a regulatory approach under Subpart J that includes test data and information requirements

for certain chemical and biological agents, procedures for authorizing the use of those agents, and monitoring requirements for certain discharge situations.

Each oil discharge represents a unique situation with distinct conditions, which may require various response methods. When dispersants are applied to an oil discharge, field monitoring can be used to inform operational decisions by gathering site-specific information on the overall effectiveness, including the transport and environmental effects of the dispersant and the dispersed oil. The revisions to product test data and information requirements are intended to provide OSCs, RRTs, and ACs with the best information available when selecting products for use on an oil discharge. While laboratory test protocols allow for comparison between different products under standardized laboratory conditions and may be useful during the monitoring and assessment of a discharge event and/or for selection of the agents used in the response, they do not necessarily reflect field conditions. Monitoring of agents in the field informs the OSC and other agencies on the overall effectiveness of dispersant use, including the environmental effects and transport of dispersed oil.

The Agency believes that comprehensive monitoring in certain discharge situations is necessary to determine the overall effectiveness of dispersants and should transcend from the initial dispersant application to include the transport and environmental effects of the dispersant and dispersed oil in the water column. Monitoring the overall effectiveness of dispersant use in the field provides those Agencies with responsibility for authorizing the use of dispersant product information for decision-making during subsurface or prolonged surface dispersant applications. Adverse effects on ecological receptors from exposures to dispersed oil depend on the length of time and concentration of the exposure, which in turn is dependent on the transport of dispersed oil. Because these exposures may vary depending on the discharge situation, the Agency believes comprehensive monitoring is important for certain discharge situations. This monitoring data will enhance the information needed for an effective response without delaying the use of agents under these conditions.

Equipment is being contemplated to inject dispersants subsurface, directly into the oil near the source of the discharge.[11] This type of application is intended to minimize dispersant dilution in the water before the dispersant has had an opportunity to interact with the oil.[12] This application approach that is closer to the source is expected to reduce potential adverse environmental consequences from the use of excessive quantities of dispersants.[13] However, applying dispersant to an oil discharge does not result in the physical recovery of oil from the environment. Instead, dispersing oil increases the potential exposure of aquatic organisms to the dispersant-oil mixture, at least transiently, and subsurface application has the potential to more immediately and effectively increase these exposures near the discharge. The Agency believes this new subsurface application approach requires new environmental monitoring capabilities to support operational decision-making. These new monitoring capabilities must be able to meet the operational conditions (*e.g.,* water depths, temperatures) and be supported by knowledgeable personnel familiar with them.

The proposed rule adds §300.913 establishing requirements for the responsible party to monitor any subsurface use of dispersant in response to an oil discharge, surface use of dispersants in response to oil discharges of more than 100,000 U.S. gallons occurring within 24 hours, and surface use of dispersants for more than 96 hours in response to an oil discharge, as directed by the OSC. The purpose of monitoring subsurface application is to characterize the dispersed oil, follow the plume integrity and transport with the underwater current, and identify and assess the potential adverse effects from the dispersed oil. The proposal requires the responsible party to implement monitoring for any subsurface dispersant use in response to an oil discharge upon initiation and for the duration of subsurface dispersant use. The Agency believes monitoring subsurface use of dispersants is critical to inform response actions to minimize potential environmental effects.

While surface application of dispersants is not a new approach and understood for small, short duration discharges, the Agency believes it is appropriate to require comprehensive monitoring for situations where dispersants are used for an extended period of time or in cases of major oil discharges. The Agency chose 100,000 U.S. gallons as a threshold criterion for a major oil discharge because the NCP classifies a discharge of more than this quantity to coastal waters as major. In addition, EPA is proposing to this quantity monitoring of dispersant use in response to major oil discharges occurring within 24 hours since a larger quantity of dispersant may be required in a short time frame for an incident of this scale. The Agency also believes comprehensive monitoring should be required when surface dispersant is used for more than 96 hours because of potentially longer exposures of biological aquatic resources to dispersant and dispersed oil. Further, many acute toxicity studies use 96-hour exposure durations, including the acute toxicity tests using *Menidia beryllina* described in this proposal. While other toxicity tests have shorter exposure durations (*e.g.,* 24, 48, or 72 hours), the Agency believes 96 hours is a reasonable threshold given its commonality of use in the toxicology field. Therefore, the proposal requires a responsible party to implement monitoring for surface dispersant use in response to an oil discharge under these discharge conditions and for the duration of dispersant use. The proposal also requires the submission of a Quality Assurance Project Plan for approval to the OSC covering the collection of all environmental data to ensure and maximize its quality, objectivity, utility, and integrity. The Agency welcomes comments on the proposed monitoring requirements, including the thresholds. Specifically, the Agency requests comments on whether it should also consider a threshold for surface use of dispersants that is based on the quantity of dispersant used within a given timeframe. Alternative thresholds must include a rationale in order for the EPA to consider them for final action.

(a) Dispersant Application

The proposal requires the responsible party to document the characteristics of the source oil; best estimate of the oil discharge flow rate, periodically reevaluated as conditions dictate, including a description of the method, associated uncertainties, and materials; dispersant(s) product used, rationale for dispersant choice(s), including the results of any efficacy and toxicity tests, recommended dispersant-to-oil ratio; and the application method and procedures, including a description of the equipment to be used, hourly application rates, capacities, and total amount of dispersant needed. For subsurface discharges, the proposal also

[11] *http://marinewellcontainment.com/expanded_system.php.*

[12] NRC, (2005), Oil Spill Dispersants: Efficacy and Effects, The National Academies Press, pp. 138, *http://www.nap.edu/catalog.php?record_id=11283.*

[13] Ibid.

requires the responsible party to document the best estimate of the discharge flow rate of any associated volatile petroleum hydrocarbons, periodically reevaluated as conditions dictate, including as description of the method, associated uncertainties, and materials. This would provide the OSC with the necessary information for operational decision-making and coordination of the dispersant application monitoring.

Because of the many factors that influence the selection of a dispersant product (*e.g.,* its availability, the type of oil it will be used on, the prevalent weather conditions, and the particular discharge situations) the Agency proposes that the responsible party document its product choice and selection rationale. Documenting the characteristics of the source oil provides specific chemical data to identify the oil associated with discharge and to inform decisions on dispersant use. Documenting application methods and procedures ensures that dispersant use is consistent with the dispersant manufacturer's recommended concentrations, and conditions provided as part of the data requirements for listing the product on the Schedule. Documenting results of any additional efficacy and toxicity testing, or available data or information specific to the area or site conditions will assist the OSC in establishing the appropriateness of the dispersant choice. Documentation and clear understanding of the estimated daily oil discharge flow rate and the recommended dispersant-to-oil ratio (DOR) allows a dispersant rate to be established that would aid the OSC in determining the adequate dispersant usage. The OSC can compare the recommended DOR to the manufacturer's recommended application concentration to ensure it is within the recommended range or to provide an opportunity for the responsible party to explain any deviations that may arise due to operational considerations.

The biodegradation of petroleum constituents may result in a reduction in the dissolved oxygen concentration in the water column that could lead to hypoxia, which could be detrimental to marine organisms. The best estimate of the oil discharge flow rate, periodically reevaluated as conditions dictate, is an important consideration for monitoring dispersant use since the estimated amount of oil discharged may be used to provide insight into the potential oxygen demand exerted on the water column and to characterize the potential oil distribution. The proposal would also require the responsible party to document the best estimate of the discharge flow rate of any associated volatile petroleum hydrocarbons for subsurface applications. Volatile petroleum hydrocarbons (*e.g.,* methane, and low molecular weight alkanes such as ethane, propane, and butane) associated with the discharge could contribute to a reduction in dissolved oxygen as a result of microbial degradation. The Agency is not proposing this requirement for surface applications given these compounds are expected to readily evaporate, making it unlikely they would be incorporated into the water column in quantities that would result in hypoxia.

The proposal requires the documentation of the dispersant application method and procedures to include a description of the equipment to be used, including hourly application rates, capacities, and total amount of dispersant needed. Dispersant and equipment should be available to maintain the hourly dispersant application rate without significant deviation from the set rate. The Agency believes this information will assist in ensuring a consistent dispersant application rate. Dispersant application rates outside of established parameters may result in their over or under use, altering their effectiveness. Significant fluctuations in dispersant application rates could also indicate equipment malfunctions, requiring a reassessment of the response technique. EPA welcomes comment on the proposed requirements for dispersant application. Alternatives must include a rationale in order for the Agency to consider them for final action.

(b) Water Column Sampling

The proposal requires the responsible party to collect representative background samples from the water column at the closest safe distance determined by the OSC from the discharge, and in any direction of likely transport. The sampling should consider surface and subsurface currents and oil properties. Establishing background information prior to oil contamination from the discharge source provides reference data to compare against the results from water samples taken during the response. In establishing the background information, the responsible party should consider other potential sources of petroleum hydrocarbons (*e.g.,* natural seeps) in the water column. The background samples would be collected in areas not affected by the discharge of oil throughout the water column upon initiation of dispersant use, at the closest safe distance as determined by the OSC from the oil discharge. Sampling would be conducted in any direction of likely transport considering surface and subsurface currents and the oil properties in those areas. The responsible party should consider using trajectory models that incorporate relevant factors such as oil type to determine the likely direction of the dispersed oil to inform where the water samples should be collected.

The Agency is requiring that sample collection follow established standard operating and quality assurance procedures that are reliable and defensible. An accurate assessment of environmental data depends on the reliability, timeliness, and integrity of the data collected. Standard operating procedures should describe the appropriateness of the sampling method; the equipment needed for sample collection; a description of potential interferences, problems that may be encountered and corrective actions that would be taken; the sample collection procedure, including the preparation steps, representative sampling considerations, and sampling steps for each sampling device used; sample preservation, containers, handling, and storage; decontamination of sampling equipment; and record keeping and quality control procedures. These elements are generally described in various guidance documents on standard operating and quality assurance procedures for environmental sampling.[14] [15]

In addition to the background water sample collection, the proposal requires the responsible party to collect water column samples daily in the dispersed oil plume at such depths and locations where dispersed oil is likely to be present. EPA believes the dispersed oil plume captures the presence of oil from either the subsurface or the surface application of dispersant, including where oil could be rising to the surface from a subsurface discharge or the dispersed oil plume at the water surface as a result of surface dispersant application. The responsible party also would need to analyze for:

• *In-situ* oil droplet size distribution analysis, including the mass or volume mean diameters between droplet sizes ranging from 2.5 to 2000 mm, with the majority of data collected between the 2.5 and 100 mm sizes. Droplet size distribution, which is an important component to understanding the chemical and hydrodynamic effectiveness of dispersants, can be measured with devices such as the Laser

[14] *http://www.epa.gov/quality/qa_docs.html.*

[15] *http://www2.epa.gov/region8/environmental-sampling.*

In-Situ Scattering and Transmissometry (LISST) instrument or other similar instruments. Small oil droplets (*e.g.,* diameters in the tens of mm range) have such low rise velocities that they tend to remain suspended in the water column (neutrally buoyant), where they can become widely dispersed by advective forces, turbulent motion, and to a lesser extent diffusion. These small droplets are more readily amenable to biodegradation due to their higher surface area to volume ratio. In contrast, large oil droplets (*e.g.,* diameters >100 mm) will tend to recoalesce and rise faster to the surface.[16] Effective application of dispersants is expected to generate a larger number of small droplets, reducing the average droplet size.[17] This will change the trajectory of rising oil that is subjected to stratified horizontal subsurface currents. Near the water surface, hydrodynamic and other environmental factors also influence the oil droplet size distribution. Mixing energy, especially from breaking waves, dictates the breakup of large oil droplets into smaller droplets and the depth of submergence of the droplets. Plunging breaking waves under experimental conditions produced the smallest oil droplets.[13 14] Additionally, studies indicate that the type of chemical dispersant used is an important factor in controlling the dispersed oil droplet size distributions. Further, wave tank studies have shown that the chemically dispersed oil may result in a droplet size distribution that has not been observed without the use of dispersants (*i.e.,* physically dispersed oil), both in terms of a smaller volumetric mean diameter and a bimodal distribution for droplet size diameters below 100 mm.[18] EPA recognizes that the aforementioned studies were conducted under conditions characteristic of surface waters. However, the effect of the high velocities of oil that may occur from a discharge from a subsurface oil well on the oil droplet size distribution requires further scientific investigation to understand the contribution of physically dispersed oil relative to chemically dispersed oil. In either event, it is clear droplet size distribution analysis provides important data useful in optimizing operational guidelines and decision making, modeling transport and fate, and potentially evaluating biological effects of chemically dispersed oil. The proposal also includes larger oil droplet sizes of up to 2000 mm, intended to provide information that can inform the oil distribution using trajectory modeling analysis.

• *In-situ* fluorometry. When exposed to ultraviolet (UV) light, certain compounds absorb energy that can elevate electrons into an excited higher energy level. The wavelength that excites electrons into a higher energy state is typically referred to as the excitation wavelength. Electrons unstable at the higher energy state return to a lower energy state, emitting energy at longer emission wavelengths resulting in fluorescence.[19] Many organic compounds fluoresce at specific excitation and emission wavelengths that allow for identifying many of the components of dissolved organic matter and other compounds (*e.g.,* crude oil) in seawater.[20] For crude petroleum oils, the aromatic fraction is responsible for the fluorescence property of petroleum, and these compounds are used to determine the specific excitation and emission wavelengths for monitoring. When subject to excitation at certain wavelengths in the near ultraviolet spectrum, polycyclic aromatic hydrocarbons (PAH) fluoresce over a range of higher wavelengths, depending on the number of aromatic rings in the structure.[21] Fluorometers can be targeted to the type of oil discharged, and the excitation and emission wavelengths chosen should match the aromatic properties of the oil discharged. Although this measure does not include all oil constituents, fluorescence is a valuable screening tool deployed during a response,[22] providing a rapid indication of potential dispersed oil in the water column, as well as an indicator of dispersion effectiveness as discussed in the following section. The Agency request comments on alternative spectroscopy techniques such as absorption measurements that may be used to rapidly estimate oil concentrations in the water during a discharge event.

• Fluorescence signatures. In addition to *in-situ* fluorometry, the proposal requires the responsible party to conduct a fluorescence intensity analyses on water samples collected to determine fluorescence signatures of the dispersed oil. EPA proposes to use fluorescence signatures as a relatively simple and rapid means to assess dispersion effectiveness. This includes but is not limited to, identifying the peak wavelength position, magnitude and ratios of fluorescence intensity of the dispersed oil. For example, measuring the reduction in an intensity ratio after applying dispersant to the oil in seawater may provide an estimate of the effect of chemical dispersion.[23 24] Ratios of fluorescence intensity are derived from the three dimensional excitation/emission matrix spectra (EEMS) acquired by combining multiple emission spectra obtained from oil exposed to individual excitation wavelengths typically using a scanning spectrofluorometer. If the optimal excitation and emission bands are known, then these ratios may be derived from measuring the fluorescence intensity at two different fixed emission wavelengths at a single fixed excitation wavelength. The ratios are calculated by dividing the fluorescence intensity of the shorter emission band by the fluorescence intensity of the longer band. Studies have shown that adding dispersants to crude petroleum oil increases the fluorescence intensity of the longer emission band, driving down the value of the ratio. Although individual petroleum oils may have common spectral characteristics, the ideal three-dimensional EEMS for the discharged oil should be obtained from scanning the source oil. Therefore, the monitoring should include procedures to obtain the EEMS using the source oil to determine the optimal excitation-emission wavelengths. The Agency

[16] Li, M. and Garrett, C., (1998) The Relationship Between Oil Droplet Size and Upper Ocean Turbulence, Mar. Poll. Bull., Vol. 36, pp. 961–970.

[17] Li, Z., Lee, K., King, T., Boufadel, M.C., Venosa, A.D., (2008) Assessment of Chemical Dispersant Effectiveness in a Wave Tank under Regular Non-Breaking and Breaking Wave Conditions, Mar Pollut Bull, Vol. 56, pp. 903–912.

[18] Li, Z., Lee, K., King, T., Boufadel, M.C., Venosa, A.D., (2009) Evaluating Chemical Dispersant Efficacy in an Experimental Wave Tank: 2—Significant Factors Determining In Situ Oil Droplet Size Distribution, Env Eng Scien, Vol. 26, pp 1407–1418.

[19] Henry, C.B., Roberts, P.O., Overton, E.B., A Primer on In Situ Fluorometry to Monitor Dispersed Oil, IOSC 1999;246.

[20] Coble, P.G., (2007) Marine Optical Biogeochemistry: The Chemistry of Ocean Color, Chem. Rev., Vol. 107, pp. 402–418.

[21] Bugden, J.B.C., Yeung, C.W., Kepkay, P.E., Lee, K. (2008) Application of Ultraviolet Fluorometry and Excitation-Emission Matrix Spectroscopy (EEMS) to Fingerprint Oil and Chemically Dispersed Oil in Seawater, Mar Pollut Bull, Vol. 56, pp. 677–685.

[22] Conmy, R.N., Coble, P.G., Farr, J., Wood, A.M., Lee, K., Pegau, W.S, Walsh, I.D., Koch, C.R., Abercrombie, M.I., Miles, M.S, Lewis, M.R., Ryan, S.A., Robinson, B.J., King, T.L., Kelble, C.R., and Lacoste, J. (2014) Submersible Optical Sensors Exposed to Chemically Dispersed Crude Oil: Wave Tank Simulations for Improved Oil Spill Monitoring, Environ Sci Technol, Vol. 48, pp. 1803–1810.

[23] Bugden, J.B.C., Yeung, C.W., Kepkay, P.E., Lee, K. (2008) Application of Ultraviolet Fluorometry and Excitation-Emission Matrix Spectroscopy (EEMS) to Fingerprint Oil and Chemically Dispersed Oil in Seawater, Mar Pollut Bull, Vol. 56, pp. 677–685.

[24] Kepkay, P.E., Yeung, C.W., Bugden, J.C.B., Li, Z., and Lee, K. (2008) Ultraviolet Fluorescence Spectroscopy (UVFS): A New Means of Determining the Effect of Chemical Dispersants on Oil Spills, IOSC, pp. 639–644. *http://ioscproceedings.org/doi/abs/10.7901/2169-3358-2008-1-639.*

request comments on alternative techniques or measurements that may be used to rapidly assess dispersion effectiveness during a discharge event.

• Dissolved oxygen (DO). Dissolved oxygen is an important variable to monitor in the application of dispersants, particularity in subsurface waters that may inform operational decisions. For surface dispersant application, dissolved oxygen is expected to be higher in the mixed layer in the surface water. Dissolved and/or dispersed oil represents a readily available carbon source for microbial oxidation, and this metabolic activity can lead to a decrease in the DO content in the water column. The oil degrading community in the water is an important DO sink for the mass transfer of hydrocarbons in the subsurface, and advantage can be taken of this fact during a response. The decline in DO due to hydrocarbon biodegradation could approach or exceed levels of concern (*i.e.,* hypoxia) and thus be detrimental to surrounding organisms (*e.g.,* invertebrates and vertebrates). Dissolved oxygen, which relies on the diffusion of molecular oxygen through a membrane in the sensor, is commonly measured during oceanographic survey cruises using an *in-situ* DO instrument that provides real-time results. However, a DO sensor instrument that passes through a dissolved and/or dispersed oil layer is subject to contamination by the oil and could lead to questionable measurements if such fouling of the electrodes occurs, particularly at depths where the dissolved and/or dispersed plume depth are expected to coincide with depressions in dissolved oxygen. Therefore, the Agency is concerned that relying solely on measurements from *in-situ* oxygen instruments may lead to an erroneous interpretation of oxygen data. Thus, the Agency believes that *ex-situ* confirmatory DO measurements should also be conducted using Winkler titrations to confirm *in-situ* dissolved oxygen measurements.

• Total petroleum hydrocarbons, individual resolvable constituents, including volatile organic compounds, aliphatic hydrocarbons, monocyclic, polycyclic, and other aromatic hydrocarbons, including alkylated homologs, and hopane and sterane biomarker compounds. The Agency is proposing that the responsible party analyze each water sample collected for total petroleum hydrocarbons (TPHs), individual resolvable constituents, including volatile petroleum hydrocarbons, and branched and normal aliphatic hydrocarbons. The analysis would also include monocyclic, polycyclic and other aromatic hydrocarbons (*e.g.,* heterocyclic aromatic hydrocarbons), including their alkylated homologs, and hopanes and steranes biomarker compounds. TPHs are best measured using gas chromatograph (GC) equipped with a flame ionization detector (FID). TPH analysis measures total alkanes and aromatics, can be obtained relatively quickly, and provides a good measurement for initial screening. Given that PAHs and biomarkers are typically measured using GC/MS, the proposal includes GC/MS analysis to provide the alkanes, PAHs, and biomarker (*e.g.,* hopane) specificity important in identifying certain oil constituents that may be present in the oil discharge. Identifying the concentrations of PAHs and biomarkers assists responders in making informed operational decisions regarding the dispersant application because these compounds can be compared against the composition of the source oil, which serves as a reference to determine the degree of weathering for oil.

• Carbon Dioxide (subsurface only). The aerobic biodegradation of oil constituents not only consumes dissolved oxygen, but would also produce carbon dioxide. Increases in the concentration of carbon dioxide that coincide with decreases in the concentration of dissolved oxygen would provide credible evidence that biodegradation of oil is occurring. Thus, the Agency believes that measuring the *in-situ* carbon dioxide for subsurface dispersant applications would be a good indicator of microbial oxidation and inform the OSC on the potential fate.

• Methane if present (subsurface only). The microbial degradation of methane may contribute to a reduction in dissolved oxygen. Thus, the Agency believes that, when present, subsurface *in-situ* methane measurements are an important factor to consider in evaluating dissolved oxygen levels.

• Heavy metals analysis, including nickel and vanadium. Crude petroleum oil may contain certain heavy metals, including nickel and vanadium.[25 26 27] Dispersing oil may increase the bioavailability of certain associated heavy metals to marine organisms. Therefore, the Agency is proposing to include heavy metal analysis as part of the sampling analyses to be conducted.

• Turbidity. Turbidity is a general measure of water clarity. Turbidity is determined by measuring how much material suspended in water decreases the passage of light through the water.[28] Suspended materials may include soil particles (clay, silt, and sand), algae, plankton, microbes, and other substances. This measure provides a quick assessment of suspended materials in receiving waters from other water bodies and is useful in determining the presence of materials that could interfere with oil particle size determinations. Alternatively, the Agency is requesting comments on whether there are other physical measurements in the water that would provide similar timely information, or that can serve as validation for turbidity values collected in situ.

• Water temperature. Water temperature typically is measured using an electronic thermometer on the conductivity-temperature-depth (CTD) instrument. When combined with salinity and pressure, temperature measurements are used to calculate water density, which may change along the vertical profile. Along with the horizontal subsurface currents and the rise velocity of the dispersed oil, the ambient density gradient is an important factor in determining the behavior of dispersed oil in the water column. Water temperature is also an important variable that may influence the effectiveness of dispersant applications. For example, cold temperatures may, among other environmental factors, impact the effectiveness of dispersants as it affects certain oil properties (*e.g.,* viscosity). Colder temperatures also may affect the degree of oil weathering (*e.g.,* evaporation), and the amount of dispersant/oil mixing energy (wave action) needed to effectively disperse oil relative to warmer temperatures.

• pH. The pH is a simple standard measurement used to analyze water quality that can affect chemical or biological processes in water. pH also determines the solubility and biological availability of chemical constituents such as heavy metals.

• Conductivity. Conductivity is measured by passing a current through the water in a CTD instrument, which is then compared against known salinities to obtain the water salinity. When combined with temperature, and pressure, salinity measurements are

[25] Barwise, A. J. G. and Whitehead, E. V. (1980). Separation and Structure of Petroporphyrins. In: *Advances in Organic Geochemistry 1979.* (A. G. Douglas and J. R. Maxwell, eds.), Pergamon, New York, pp. 181–92.

[26] Barwise, A. J. G. (1990). Role of Nickel and Vanadium in Petroleum Classification. *Energy & Fuels,* 4, 647–52.

[27] Ali, M.F., Bukharl, A., and Saleem, M., (1983) Trace Metals in Crude Oils from Saudi Arabia, Ind. Eng. Chem. Prod. Res. Dev. 1983, Vol 22, pp. 691–694.

[28] *http://water.epa.gov/type/rsl/monitoring/vms55.cfm.*

used to calculate water density, which may change significantly along the vertical profile. Along with the horizontal subsurface currents and the rise velocity of the dispersed oil, the ambient density gradient is an important factor in determining the behavior of dispersed oil in the water column.

The Agency requests comment on the proposed approach to water column sampling. Alternatives must include a rationale in order for the Agency to consider them for final action.

(c) Oil Distribution Analyses

The proposal requires that the responsible party, in consultation with the OSC and using best available technologies, characterize the dispersant effectiveness and oil distribution, considering the condition of the oil, dispersant, and dispersed oil components from the discharge location. EPA believes this is necessary to inform sampling locations by assessing the horizontal and vertical boundaries of the dispersed oil plume and the direction of likely transport. The majority of the sampling can then be focused on known or suspected locations and depths of the dispersed oil. Based on the initial water sampling results to characterize the boundaries of the dispersed oil, the sampling design can be tailored to optimize sampling. The Agency proposes that the responsible party characterize the dispersant effectiveness to determine the changes in the condition of the oil due to weathering, which can include changes in oil viscosity due to water uptake (*e.g.,* mousse). Weathered oil that mixes with water may result in significant emulsification (*e.g.,* water-in-oil emulsions), which in turn may decrease the dispersant's chemical effectiveness and diminish biodegradability. Therefore, the characterization of dispersant effectiveness should provide a reasonable estimate to the extent (*e.g.,* distance from the discharge source) in which the dispersant can be applied to the oil and still be effective. The Agency believes this will result in better dispersant applications and minimize excessive dispersant use. The Agency requests comment on this approach. Alternatives posed must include a rationale for the Agency to consider them for final action.

(d) Ecological Characterization

The Agency proposes that the responsible party characterize the ecological receptors (*e.g.* aquatic species, wildlife, and/or other biological resources), their habitats, and exposure pathways that may be present in the discharge area, in consultation with the OSC. Exposure pathways, such as ingestion, refer to the way in which ecological receptors may come into contact with discharged oil and result in exposure that would be detrimental to ecological receptors. The Agency believes that the ecological characterization should include those species that may be in sensitive life stages, transient or migratory species, breeding or breeding-related activities (*e.g.,* embryo and larvae development), and threatened and/or endangered species that may be exposed to oil, dispersed oil, and dispersant, which in turn will assist the OSC in managing response actions, including those actions that were determined not to be needed. In developing the characterization of ecological receptors, the responsible party may refer to relevant sources of information such as applicable expedited decision-making plans, environmental assessments or statements, Federal and state environmental databases (*e.g.,* ACP—Fish and Wildlife and Sensitive Environments Annex; NOAA—Environmental Sensitivity Indices; NOAA—NMFS Southeast Area Monitoring and Assessment Program; EPA—National Coastal Assessment [29]) or through studies conducted by universities or other research-oriented institutions.

The proposal requires the responsible party to consult with the OSC to determine an acute toxicity level of concern for the dispersed oil using available dose/response information relevant to potentially exposed species. The envisioned approach would be to monitor acute toxicity in the water column concurrently with dispersed oil sampling for fluorometry, particle size, and water quality (*e.g.,* dissolved oxygen). The TPH concentrations in water samples taken throughout the water column could then be compared to TPH-based ecotoxicity benchmarks (EBs). Water samples collected for comparison of aqueous TPH concentrations to EBs would be analyzed and reported within the timeframe necessary to make operational decisions (*e.g.,* within 24 hours of collection). Sampling could also be performed in areas without dispersant application to distinguish toxicity associated with physically dispersed oil from that of chemically dispersed oil.

While EBs may have already been established during the development of contingency plans, they can also be derived at the time of an incident. One approach is to use a species sensitivity distribution (SSD),[30] which allows for species relevant to the location of the discharge to be considered. An SSD is a probability distribution of the sensitivity of a group of species to a toxicant.[31] SSDs could be developed for representative oils (*e.g.,* crude oils) using existing acute toxicity values for mortality or immobility (*e.g.,* 48 and 96 hr LC_{50}) where sufficient species diversity are available (*e.g.,* toxicity data for 10 or more species). For example, the EBs could be computed from the fifth percentile of the SSD as the hazard concentration 5 percent (HC5), as they are considered protective of 95% of species, have been used by EPA for developing ambient water quality criteria, and are generally accepted by the international community.[32] Chronic toxicity benchmarks may be derived applying safety factors to the acute toxicity EBs. The Agency requests comments on the proposed approach to ecotoxicity assessment, and whether it should consider making SSDs a requirement for deriving EBs. If alternative approaches are suggested, the commenter must include an appropriate rationale in order for the Agency to consider them for final action.

(e) Immediate Reporting

The proposal requires the responsible party to immediately report to the OSC any deviation of more than 10 percent of the mean hourly subsurface dispersant use rate for the total dispersant volume authorized for that 24 hour use, and the reason for the deviation (*e.g.,* equipment malfunction). The Agency believes that inconsistent dispersant application rates confound data sampling interpretation due to variations in the DOR. The proposal, however, provides a margin for variation within 10 percent of the mean hourly subsurface dispersant application rate to account for equipment performance. The Agency believes this margin to adequately account for variations in dispersant

[29] *http://www.epa.gov/emap/nca/.*

[30] Barron, M.G., Hemmer, M.J., and Jackson, C.R., (2013) Development of Aquatic Toxicity Benchmarks for Oil Products Using Species Sensitivity Distributions, Integr Environ Assess Manag, Vol. 9, pp. 610–615.

[31] Aldenberg, T. and Jaworska, J.S., (2002) Uncertainty of the Hazardous Concentration and Fraction Affected for Normal Species Sensitivity Distributions, Ecotox and Environ Safety, Vol. 46, pp. 1–18.

[32] Barron, M.G., and Wharton, S., (2005) Survey of Methodologies for Developing Media Screening Values for Ecological Risk Assessment, Integrated Environmental Assessment and Management, Vol. 1, pp. 320–332.

injection equipment without being overly restrictive. EPA requests comments on whether it should consider a similar requirement for dispersant surface application.

The Agency also is proposing to require the immediate reporting of ecological receptors, including any threatened or endangered species that may be exposed to dispersed oil based on trajectory modeling and the estimated acute toxicity level of concern. Results from daily sampling (*e.g.,* droplet size distribution, TPH) would provide input data to refine predictions of the likely dispersed oil direction using trajectory modeling. Further, results may also inform decisions to alter dispersant application in order to minimize effects on biological resources.

(f) Daily Reporting

The proposal requires daily reporting of sampling and data analyses collected within the timeframe necessary to make operational decisions (*e.g.,* within 24 hours of collection), including documented observations, photographs, video, and any other information related to dispersant use, unless an alternate timeframe is authorized by the OSC. Daily reporting would also include the total amount of dispersant used for the previous reporting period. Additionally, the proposal would require a schedule for any data analyses that require time beyond 24 hours due to analytical methods; this schedule is not to exceed 120 hours (*i.e.,* 5 days) unless authorized by the OSC. Timely sample analyses afford the OSC and other responders with multiple relevant data that can be analyzed together to inform situational awareness of dispersant operations and adjust dispersant application if necessary. The Agency believes that a 120-hour window for analyses requiring additional time provides an adequate opportunity to conduct all requested analyses in a timely manner without being overly restrictive. Finally, the proposal requires the responsible party to report the estimated daily transport of dispersed and non-dispersed oil, and associated volatile petroleum hydrocarbons if applicable, and dispersants, using the best available trajectory modeling. This information is intended to assist response planners to coordinate response activities and to schedule monitoring activities based on the expected transport of oil and dispersed oil.

4. Data and Information Requirements for Product Schedule Listing

The Agency is proposing to revise the data and information requirements for listing products on the Schedule in §300.915 of Subpart J. The proposed amendments revise the efficacy and toxicity testing protocols and listing criteria for all chemical and biological agents on the Schedule. Additionally, the Agency proposes revisions to the requirements for general product information, Confidential Business Information (CBI) claims, submission package contents, EPA review and listing procedures, requests for decision review, changes to products, transitioning products from the current Schedule to the new Schedule, mandatory product disclaimer, and removal of products from the Schedule.

(a) General Product Information

The Agency is proposing to consolidate the general submission requirements applicable to all types of agents that may be listed on the Schedule in paragraph (a) of §300.915. Subsequent regulatory paragraphs in this section are proposed to include requirements specific to each product category. Additionally, the Agency is proposing to require some additional information, as applicable, regarding products that are submitted for Schedule listing consideration. The proposed general information requirements for all products are proposed to be revised as follow:

- Submitter: Name, physical address, email, telephone number, identity of submitter (*i.e.,* manufacturer, vendor, importer, distributor, designated agent for the manufacturer), and documentation of such identity.
- General Product: All name(s), brand(s), and/or trademark(s) under which the product is to be sold; Safety Data Sheet (SDS); sample product labels for all name(s), brand(s), and/or trademark(s); chemical or biological agent categories under which the product is submitted for listing, including information on the specific process(es) through which the product affects the oil, and the specific environment (waters and/or adjoining shorelines) on which it is intended to be used.
- Supplier: Names, physical addresses, emails and telephone numbers of the primary distributors, vendors, importers, and/or designated agent acting on behalf of the manufacturer.
- Product Storage: Maximum, minimum and optimum temperature, humidity and other relevant conditions for product storage; impact on product performance if the product is not stored within recommended limits; and anticipated shelf life at the recommended storage conditions.
- Product Use: Recommended procedures, including product concentrations, application ratios, types of application equipment, conditions for use, and any application restrictions. The procedures must address variables such as weather, water salinity, water temperature, types and weathering states of oils or other pollutants, and product and oil containment, collection, recovery and disposal, and include supporting documentation and standard methods used to determine them.
- Environmental Fate: Known measured data and supporting documentation on the persistence, bioconcentration factor, bioaccumulation factor, and biodegradability of the product and all of its components.
- Physical/Chemical Properties and Formulation: Physical state and appearance; vapor pressure; flash point; pour point; viscosity; specific gravity; particle size for solid components; pH; citation of standard methods used to determine the physical/chemical properties; identity of all components in the product, including each specific component name; corresponding Chemical Abstract Service (CAS) Registry Number; the maximum, minimum, and average weight percent of each product component; and the intended function of each component (*e.g.,* solvent, surfactant).
- For products that contain microorganisms, enzymes and/or nutrients: All microorganisms and weight percent by current genus and species, including any reclassifications; all enzymes and their International Union of Biochemistry (I.U.B.) number(s); Enzyme Classification (EC) code numbers; the source of each enzyme, units, and specific oil-degrading activity; name(s) and maximum, minimum, and average weight percent of the nutrients contained in the product; citation or description of the methodology used to determine product components; certification, including data, methodology, and supporting documentation, indicating that the product does not contain levels that exceed the National Ambient Water Quality Criteria lowest density value for the following: Bacterial, fungal, or viral pathogens or opportunistic pathogens, including, but not limited to, enteric bacteria such as Salmonella, fecal coliforms, Shigella, Coagulase positive

Staphylococci, and Beta Hemolytic Streptococci or enterococci.

• National Water Quality Standard Contaminants: Certification, including data, methodology, and supporting documentation, indicating that the product does not contain levels that exceed the National Water Quality Standards lowest aquatic life acute value for the following contaminants: Metals reasonably expected to be in the product including arsenic, cadmium, chromium, copper, lead, mercury, nickel, vanadium, zinc; cyanide; chlorinated hydrocarbons; pesticides; polychlorinated biphenyls (PCBs); and polynuclear aromatic hydrocarbons (PAHs).

• Certification, including data, methodology, and supporting documentation, indicating that the product does not contain prohibited agents (sinking agents, nonylphenol, or nonylphenol ethoxylates).

• Testing Laboratory Information: Name, address, contact name, email, phone number; national or international accreditations.

• Laboratory Testing Data and Reports: All applicable information, data and analysis specified in the testing protocols, including raw test data and replicates, notes and observations, calculated mean values and standard deviations, summary of stock solution preparation, source and preparation of test organisms, test conditions, and chain of custody forms.

• Production capacity: Estimated annual production volume; average and maximum volume per day; time needed to reach that maximum production rate (days).

• Performance Capabilities/Benefits: Recognition received from, if applicable; national and/or international product testing or use data, recognitions (*e.g.,* EPA's Design for the Environment), and/or certifications, informing the performance capabilities or environmental benefits of the product.

The proposed revisions group together and simplify the general submission requirements applicable to all product types. EPA believes reorganizing the general requirements in a central location will clarify which requirements are applicable to all submissions, and which are specific to each product type by including them in separate sections. While most of the information listed above is currently required to be submitted under Subpart J, the Agency is proposing revisions to several of the existing general requirements and several new data and information requirements. The Agency believes the proposed revisions and added new requirements will better characterize the nature of the product and will assist EPA in product listing decisions. The information will also assist the RRTs in their area planning activities, and the OSCs in authorizing the appropriate use of chemical and biological agents. Details on the proposed additions and revisions are as follow:

(1) *Identification and documentation by the submitter of its status in relationship to the product as the manufacturer, vendor, importer, distributor, or other designated agent of the manufacturer.* This proposed requirement is intended to clearly establish the point of contact responsible for the submission, and to avoid any conflicts or claims from unauthorized entities on products listed or submitted for consideration.

(2) *Chemical or biological agent categories under which the product is submitted for listing, including information on the specific process(es) through which the product affects the oil, and the specific environment(s) (water and/or adjoining shorelines) on which it is intended to be used.* Currently, when a product meets the definitions of two or more product categories, a submitter must provide technical product data for each of those product categories. After review of the submitted technical product data, EPA makes a determination on whether and under which category the "mixed product" should be listed on the Schedule. It has been Agency policy to list products only under one category to avoid confusion in the field during a response, and because the process(es) through which the products affect the oil for different product categories do not generally overlap or allow for multiple uses of one product. However, EPA has received comments from responders, vendors, and manufacturers who believe that it is appropriate for certain products to be listed under more than one category. The Agency is proposing to revise the current limitations for mixed products. The proposal allows for products to be considered under multiple categories provided they meet all of the listing requirements for each. To this end, the Agency is also requiring that information be provided identifying which environments the product is intended to be used for, in the water and/or on the shoreline. EPA is soliciting comments on the issue of multiple category product listing.

(3) *Copy of the Safety Data Sheet (SDS) required by the Occupational Safety and Health Administration (OSHA) under their Hazard Communication Standard (HAZCOM) for the product.* EPA recognizes that chemical and biological agents may contain substances that could cause harm to oil spill responders who, if unaware of the product's formula, may not wear the proper personal protective equipment. SDSs describe the hazards that may be involved with the product and recommend safety measures that would minimize or avoid adverse consequences that may result from exposures. The Agency believes SDS information will be useful to both OSCs and responders when authorizing and using the product respectively, and that adding this new requirement for a product SDS is appropriate. While the Agency believes that the relevant SDS information should be that of the product, we request comment on the value for responders of requiring an SDS for each individual component. The Agency requests comments on this new requirement, as well as whether the submitter should provide any additional information on potential adverse human health effects based on the product's formula and application methods not captured in the SDS, and how to best communicate this information to product end users.

(4) *Sample product label for all names, brands, or trademarked versions of the product that include the manufacture and expiration dates of the product, as well as the conditions for storage.* The Agency would be allowing flexibility in complying with this requirement by specifying that the submitter does not need to affix new labels to comply with this section if existing labels already convey the required information. This proposed requirement is not intended in any way to supersede any other federal labeling requirement in place (*e.g.,* OSHA's HAZCOM). The proposed requirement is intended to assist the OSC in ensuring that the product used to respond to an incident is still viable and effective, and the oil spill response organizations (OSROs) or any other responder that is storing the product to ensure that their stockpile is viable and available to be authorized for use.

(5) *Recommended product use procedures.* The Agency is proposing to revise the requirement for providing information on the recommended application procedures. While the proposal is maintaining the specific elements included in the current requirement, the supporting documentation and information on the standard methods the product manufacturer used to establish the procedures is requested. EPA believes that providing detailed information on the recommended product use

procedures is necessary to inform the OSC when authorizing these products. This supporting documentation and specific information on the methods and standards used to establish them will inform OSCs and other response personnel in selecting products that can be effectively used under the operating conditions encountered for any given incident. The Agency requests comment on the revised data requirement, and whether there are other elements that should also be included to ensure the proper use and application of the products.

(6) *Environmental fate information.* The Agency is proposing to request any known and available measured data and supporting documentation on the persistence, bioconcentration factor, bioaccumulation factor, and biodegradability of the product and all of its components. The Agency currently has no restriction on use of persistent bio-accumulative products. The Agency considered requirements using existing testing protocols and modeling approaches to establish thresholds for listing based on bioconcentration factors (BCF) or bioaccumulation factors (BAF). These factors offer ratios of the concentration of a particular chemical in a biological tissue per concentration of that chemical in water surrounding that tissue, or in the environment respectively. The Agency considered a tiered testing approach, where the submitter would provide known BCF/BAF information for listing purposes, but would also be required to provide testing based on application rates authorized for use. That is, the lower application rates would only require existing information or the use of existing EPA models, while higher rates would require additional testing of fish, bivalves, and earthworms, as needed.

Similarly, there are no restrictions on the use of environmentally persistent products. The Agency considered requiring the submitter to use estimation techniques/models, such as the EPA model EPI Suite™, which estimates environmental fate properties (breakdown in water or air, etc.) that can indicate where a chemical will go in the environment and how long it will stay there. A tiered testing approach for larger quantity/duration spills as discussed above for bioconcentration and bioaccumulation was also considered.

EPA believes environmental fate information is necessary to inform the OSCs when authorizing these products for use, given the potential for their extended use in significant quantities. However, given that the Agency can estimate these factors, it is only proposing to require that available information or data be submitted on the product rather than specific product testing, as specific product testing for these factors can add significantly to the testing cost for each product. The Agency requests comment on whether testing for products' bioconcentration, bioaccumulation and biodegradation should be required for listing purposes. Additionally, the Agency requests comment on whether thresholds for BCF and BAF should be established for listing a product on the Schedule.

(7) *New physical/chemical properties and removal of the incorporation by reference of the measurement standards.* The Agency is proposing to add three new elements to the physical/chemical property requirements: Physical state and appearance; vapor pressure; and particle size for solid components. The Agency believes these basic data requirements will provide added context when evaluating the products for listing determinations. These, in combination with the other general product information requirements, will assist the Agency in evaluating the expected product behavior, and the process through which it would affect the oil when used in the intended water and/or shoreline environment. Additionally, the Agency is proposing to remove the current incorporation by reference of specific standards to determine physical/chemical properties, and replacing this with a requirement for a citation of the standard methodology used to determine these values. EPA believes that citing the standard methodology used to determine the required values is sufficient in lieu of specifying commonly recognized standard methodologies. The Agency believes it is appropriate to make this change given the new requirements for accredited laboratories to conduct testing.

(8) *Chemical Abstract Service (CAS) Registry Numbers for each component in a product.* The CAS Registry is a collection of information covering a wide variety of substances identified from the scientific literature since 1957. CAS Numbers serve as an international resource for substance identifiers and are used by scientists, industry, and regulatory bodies because it can be validated quickly and reliably. This will assist the Agency in better characterizing a product's components and assessing its specific process for affecting the oil prior to listing on the Schedule.

(9) *Certification that bioremediation agents do not contain, at levels that exceed the National Ambient Water Quality Criteria lowest density value, bacterial, fungal, viral or opportunistic pathogens.* While providing information on these product contaminants is currently required for bioremediation agents, there are no threshold levels for product listing; a positive result for any of the above pathogens may raise concern, but would not prevent the product from being listed on the Schedule. The proposed requirement that these contaminants not exceed the National Ambient Water Quality Criteria lowest density value is intended to provide information for listing decisions that ensure the use of bioremediation agents that will not result in exceeding established water safety levels. The Agency believes that this information is necessary to determine if a product is suitable for listing, particularly for bioremediation agents, which could potentially be used at recreational beaches. The Agency requests comments on whether it should establish listing thresholds for products based on this criteria, and whether the levels selected for certification are appropriate for this application.

(10) *Certification that the product does not contain levels that exceed the National Water Quality Standards lowest acute value for aquatic life of the following contaminants: Arsenic, cadmium, chromium, copper, lead, mercury, nickel, zinc, or any other heavy metal reasonably expected to be in the product; cyanide; chlorinated hydrocarbons; pesticides; polychlorinated biphenyls (PCBs); and polynuclear aromatic hydrocarbons (PAHs).* Providing information (*i.e.,* upper limit/concentration, detailed analytical methods, and sample preparation) on most of these contaminants is currently required for all products, with no established threshold levels for product listing. The Agency will continue to require information on the methodology and the data and supporting documentation used to determine the levels of these contaminants in a product. The Agency, however, will not specify what analytical testing method the submitter should use to make these determinations, as it currently does for chlorinated hydrocarbons, allowing the submitter flexibility in testing their product. Additionally, the Agency is proposing to require data on several new contaminants: Pesticides, PCBs, and PAHs. The Agency's concern with pesticides as contaminants is mostly due to their potential use on organic sorbents (*e.g.,* peat moss, corn cobs, and cellulose fibers). The concern for PCBs is for their toxicity and classification as

persistent organic pollutants, having toxic effects such as endocrine disruption. PAHs are potent atmospheric pollutants, of concern because some compounds have been identified as carcinogenic, mutagenic, and teratogenic. The proposed threshold requirements for all of these contaminants is intended to provide information for listing decisions that ensure the use of any product will not result in exceeding established water safety levels. The Agency requests comments on whether it should establish a listing threshold for products based on these criteria, and whether the levels selected for certification are the appropriate levels for this application. The Agency also requests comments on whether there are any other contaminants that should be included for evaluation of a product prior to listing.

(11) *Removal of the requirement for laboratories performing the efficacy and toxicity testing for products to have prior experience specific to the required methodology in lieu of recognized national or international accreditations.* Currently, laboratories performing testing for products to be submitted for listing consideration are required to document previous experience with the specific testing methodologies in Appendix C to part 300. The Agency believes it is more appropriate to require that laboratories be nationally or internationally accredited. Accredited laboratories are expected to be capable of following a prescribed testing protocol and good general practices, providing assurance that the test results will be reliable. Therefore, the Agency is proposing to remove the requirement for laboratories to have previous experience specific to the methodologies in Appendix C, and instead require that the laboratories hold accreditations from recognized national or international organizations. National and international accreditation organizations include, for example, the International Organization for Standardization (ISO), and the Laboratory Accreditation Bureau (recognized in the US through the National Cooperation for Laboratory Accreditation (NACLA) and the International Laboratory Accreditation Cooperation (ILAC)). The ISO 17025, Laboratory Competence standard, identifies high technical competence and management system requirements to guarantee laboratory test results and calibrations are consistently accurate. NACLA is a national voluntary organization of regulators, accreditation bodies and laboratories cooperatively working towards standardization of laboratory accreditations throughout the industry; ILAC is the international counterpart collaboration of laboratory and inspection accreditation bodies. Established to ensure that laboratories are subject to oversight by an authoritative body, accreditation organizations have been evaluated by peers as competent and have signed arrangements to enhance the acceptance of products and services. Because interested parties can have confidence in the test results and certifications provided by accredited laboratories, the Agency is proposing to amend the laboratory requirement and believes that having no prior experience with a specific methodology should not disqualify a laboratory that has been accredited by an appropriate authoritative body. However, the Agency reserves the right to not accept particular lab data should EPA find cause to doubt the quality and integrity of the work. EPA also reserves the right to conduct its own testing of any product.

(12) *Estimated annual product production volume, average and maximum amounts that could be produced per day, and time frame needed to reach maximum production rate (days).* While there is currently no requirement for production capability information, the Agency believes it is important for the OSCs and responders to have this information. The availability of a product may impact decisions of authorization of use, depending on inventory or production capabilities. This would prove to be of key importance, for example, in the event of a major environmental disaster (*e.g.,* a SONS event).

(13) *Recognition received from EPA's Design for the Environment (DfE).* EPA's Design for the Environment (DfE)[33] works in partnership with industry, environmental groups, and academia to reduce risk to people and the environment by finding ways to prevent pollution. DfE evaluates human health and environmental concerns associated with traditional and alternative chemicals and processes in a range of industries in order to select safer chemicals and technologies. DfE focuses its review of formulation ingredients on key environmental and human health characteristics of concern within functional classes. This allows formulators to use those ingredients with the lowest hazard in their functional class, while still formulating high-performing products. The DfE label means that EPA scientists have evaluated every ingredient in the product to ensure it meets DfE's stringent criteria. Products that meet the DfE criteria are safer chemical choices. In an effort to encourage the development and use of safer technologies, the Agency is proposing a requirement for the submitter to identify products that have met and can be labeled DfE certified as part of the general information, and would include this information in the NCP Product Schedule Technical Notebook.[34] This Technical Notebook presents manufacturer's summary information on the conditions under which each product is recommended to be used, and is a source of information for the OSC in the event of a response.

(14) *International product certifications, testing or use data informing the performance capabilities or environmental benefits of the product.* The Agency believes that any additional data available from other countries may help identify the benefits or concerns for the listing and/or the authorization of use of a product. The Agency, however, is not proposing any specific listing criterion or threshold associated with this broad information request, as some products (particularly new formulations) may not have any of this additional data available.

(b) Dispersant Testing and Listing Requirements

The Agency is proposing revisions to the efficacy and toxicity testing protocols, as well as establishing new thresholds for listing dispersants on the Schedule in §300.915(b). The Agency proposes to define dispersants as typically mixtures of solvents, surfactants, and additives that promote the formation of small droplets of oil in the water column by reducing the oil-water interfacial tension. These droplets are driven into the water column by wave action. Emergency response personnel need to know whether a dispersant or any other type of chemical or biological agent on the Schedule could have negative environmental impacts relative to the oil before decisions are made about its use in a particular oil discharge situation. Consequently, it is essential to consider comparative information about the efficacy and the toxicity of these products. The proposed revisions are in response to concerns not only for an increase in the frequency of planning for the use of these agents, but also for their potential use in large quantities, such as when responding to oil discharges from

[33] *http://www.epa.gov/dfe/.*

[34] *http://www.epa.gov/oem/docs/oil/ncp/notebook.pdf.*

oil tanker accidents and offshore well blowouts, as evidenced during the Deepwater Horizon incident in 2010.

(1) Dispersant Efficacy

Current Requirements: The current NCP Subpart J requires dispersants to be tested for saltwater efficacy in order to determine listing eligibility on the Schedule. Dispersant efficacy is tested following the Swirling Flask Test (SFT) listed in Appendix C to part 300 of the NCP. This protocol was developed by Environment Canada to provide a relatively rapid and simple testing procedure for evaluating dispersant efficacy (*i.e.,* the percentage of oil that is dispersed). The procedure places seawater, oil, and a dispersant into an Erlenmeyer flask, which is then placed on a shaker table for a specified period of time. The flask is modified by the addition of a side spout attached to the bottom of the flask, which allows the analyst to pour off dispersed oil from the bottom into a collection vessel for extraction and measurement. After 20 minutes, a sample of water is poured off and chemically analyzed for dispersed oil. For products to be listed, they must attain an efficacy value of 45 percent or greater average dispersion efficacy of two different crude oils (South Louisiana Crude (SLC) and Prudhoe Bay Crude (PBC)) at room temperature (20–23 degrees Celsius (°C)).

Proposed Revisions: The Agency proposes to change the testing protocol for measuring efficacy and adopt the Baffled Flask Test (BFT) as the testing protocol for dispersant efficacy. The new BFT procedure incorporates a redesign of the testing flask by eliminating the side arm, incorporating baffles in the wall of the flask, and adding a stopcock at the bottom, which improves reproducibility in the hands of different operators. This protocol has undergone extensive peer review [35] and has been tested in several laboratories, providing reproducible and repeatable results. The Agency also proposes revisions to the efficacy listing criteria for dispersants to be listed. Specifically, the dispersant must demonstrate that the lower 95% confidence level (LCL_{95}) of six replicate flasks meets the new proposed efficacy listing criteria at two test temperatures. New test oils representing a wider range of characteristics are also proposed for this protocol: Alaska North Slope (ANS) and Intermediate Fuel Oil (IFO–120) were tested for this proposal. Both oils are to be tested at two temperatures: 5 °C and 25 °C. Finally, based on the ANS and IFO–120 testing, the Agency proposes that dispersants considered for listing must demonstrate that they attain all of the following dispersant effectiveness (DE) values:

- ANS at 5 °C: $DE_{LCL95} \geq 70\%$.
- ANS at 25 °C: $DE_{LCL95} \geq 75\%$.
- IFO–120 at 5 °C: $DE_{LCL95} \geq 55\%$.
- IFO–120 at 25 °C: $DE_{LCL95} \geq 65\%$.

The Agency tested eight of the dispersants currently listed on the Schedule. Three of the eight dispersants clearly differentiated themselves from the other five dispersants for having the best DE_{LCL95} efficacy results; they would meet all the proposed thresholds. For the next best dispersant tested, the results were substantially lower than those in the top group and would not meet all the thresholds proposed today. This natural break in the results provided the Agency with the basis for the proposed threshold criteria. The Agency is also proposing to replace the SLC and PBC test oils currently used for efficacy testing with two other oils that represent a wider range of characteristics. The proposal is based on the results of tests using ANS and IFO–120 oils with the new protocol. In proposing two new oils for efficacy testing, the Agency is seeking to ensure that the efficacy testing uses oils that represent a wider range of oil gravities throughout the different geographic locations in the country where dispersants might be used. The oils selected should exhibit sufficiently different characteristics to produce results that have statistically significant variation between them. The proposal is to replace the SLC and PBC oils, both of which were light oils, with a range of light-to-medium, and heavier oils. The light-to-medium gravity ANS pipeline blend and the heavier refined product, IFO–120 were selected for testing the protocol in this proposal. The current protocol, testing 4 replicates of both the SLC and the PBC oils and combining their results, provided one threshold criterion after determining that each of the oils produced statistically similar results. In contrast, the efficacy test results between the ANS and the IFO–120 showed statistically significant differences at each temperature. The Agency believes the use of ANS and IFO–120, or similar oils that represent a wider range of oil gravities, will provide better information on the efficacy of the products when used on different types of oils.

Additionally, the Agency is proposing that efficacy testing be conducted at two different temperatures, 5 °C and 25 °C, rather than at an ambient temperature range of 20–23 °C as currently required. The Agency recognizes the current and future interest in arctic and deepwater drilling, and the continued oil production in the southern, more tropical areas of the country. Given the potential range of locations where dispersants may be used, the Agency believes it is appropriate to have products tested at temperatures that would reflect that range. These temperatures are intended to capture dispersant use scenarios in a wide range of geographic locations and under different temperatures that may occur in the same geographical location (such as, for example, the deep sea and surface water in the Gulf of Mexico, where the temperatures are typically 5 and 25 °C, respectively).

The Agency is also proposing to replace the current SFT with the BFT, which is designed to be more representative of moderately turbulent sea conditions where dispersants are more likely to be successful when used. The revised testing protocol improves test repeatability and reproducibility within and between laboratories, as well as greatly reduces both the inherent error of the method and the human error associated with the current SFT, as discussed below. In addition, reporting the test results in terms of the product's LCL_{95} accounts for between- and within-laboratory error variability and the inherent error of the method. Only one number is reported compared to a mean and standard deviation, as the variation has already been subtracted in the reported number. Specifically:

- The new baffled trypsinizing flask design, fitted with a glass stopcock positioned at the bottom side, promotes less manipulation that could result in erroneous re-suspension of non-dispersed oil, as in the SFT. The re-suspension of oil when using the SFT test protocol was a major source of error. In the SFT, the flask has a side arm spout, requiring the analyst to pick up the flask and pour the contents out manually. Through that action, re-suspension of the dispersed oil can easily take place, leading to potentially erroneous results. In contrast, in the BFT, the flask contains a stopcock at the bottom, so the analyst need only open the stopcock to drain the contents from the flask without the potential of remixing or re-suspending the dispersed oil.
- The BFT provides more turbulent mixing and better enables more reproducible and repeatable dispersant

[35] Venosa, Albert D., National Risk Management Research Laboratory, US EPA; Sorial, George A., Department of Civil & Environmental Engineering, University of Cincinnati; King, Dennis W., Statking Consulting; *Round-Robin Testing of a New EPA Dispersant Effectiveness Protocol,* International Oil Spill Conference, 2001.

action than the SFT. The mixing energy within the flask is higher and, as a result of this increased mixing energy, better dispersion is possible. Turbulence is needed for the proper mixing of dispersant and oil and for producing the sheer forces needed to create the small droplet sizes with high surface area-to-volume ratio that promote the effective dispersion of the oil into the water column. The BFT provides such mixing and better enables more repeatable and reproducible dispersant effectiveness than the SFT. The BFT was tested extensively in a nine-participant round-robin inter-laboratory calibration test on six commercial dispersant products.[36] In addition, based on the mixing energy that can be achieved using the new baffled flask, the Agency believes a higher efficacy threshold is warranted. As a result of this increased mixing energy, better dispersion is realized under conditions more realistic of wave action in the sea.

• The variability both between analysts and within analysts is substantially lower for the BFT compared to the SFT, as measured by the coefficient of variation (CV). The CV is defined as the standard deviation divided by the mean and is expressed in terms of percent; the higher the CV, the greater the variability.[37 38]

The Agency considered establishing listing thresholds for efficacy based on the types of oil. The Schedule could list sub-categories of dispersants based on gravities of the oil (*i.e.,* light-medium or heavy), so that a dispersant would only be authorized for use on oils that fall within the range for which it tested effective. Similarly, the Schedule could list dispersants based on temperature, allowing a dispersant to be authorized for use only at the temperature for which it tested effective. However, determining ranges or definitions for when oil changes from light to medium or heavy, is challenging because they may be affected by a number of external factors, including temperature. Likewise, listing dispersants based on temperature would require an additional degree of complexity to the authorization for use, as the temperatures may change drastically in some geographical regions affected by the same oil discharge. Even within more stable geographical regions, there may be variations at the time of an oil discharge that could affect the decision to use a dispersant stockpiled for a different temperature range. While these more specific listing approaches would provide more information on effective product choices, the Agency is concerned about how such specific listing approaches may contribute to OSC confusion in an emergency, the cost of stockpiling multiple types of dispersants, or the immediate availability of the appropriate dispersant in an emergency. Furthermore, the Agency is proposing revisions to Subpart J to authorize RRTs to request supplementary testing and information to provide greater flexibility in tailoring testing conditions to address area- and site-specific concerns. Given these factors, the Agency believes that it is appropriate that only those dispersants that achieve efficacy for both types of oils and at both temperatures be listed. Listing only the products that achieve efficacy for both types of oils at both temperatures would demonstrate a wide range of efficacy for the product, and a single listing would facilitate authorizing their use during a response action. The Agency requests comments on this approach.

(2) Dispersant Toxicity

Current Requirements: The current NCP Subpart J requires dispersants to be tested for toxicity to saltwater species. This information is available to the OSC for their consideration in authorizing dispersant use. Dispersant toxicity is tested following the protocols in Appendix C to part 300 of the NCP. The standard toxicity test for dispersants involves exposing two saltwater species, the inland silverside fish (*Menidia beryllina or M. beryllina*) and the mysid shrimp (*Americamysis bahia or A. bahia*), to five concentrations of the test product and No. 2 fuel oil, alone and in a 1:10 mixture of product to oil. Reference toxicity tests are conducted using dodecyl sodium sulfate (DSS) as a reference toxicant. The test length is 96 hours for *M. beryllina* and 48 hours for *A. bahia.* The concentration of test product causing 50% lethality to the test organisms (LC_{50}) is calculated at the end of the exposure period.

Proposed Revisions: The Agency proposes to revise the toxicity testing requirements for dispersants, including the testing protocol and the use of the test results. The proposal would require acute toxicity testing for the dispersant alone, and the dispersant mixed with both oils used for efficacy testing; the proposal is based on the ANS and IFO–120 oils. While the Agency currently provides the toxicity testing results to the OSC to assist in authorization of use determinations, it is proposing to use these testing results to determine eligibility for listing on the Schedule.

Specifically, the Agency is proposing to require evaluations of dispersant toxicity using acute toxicity tests following existing USEPA effluent testing guidelines,[39 40] modified for use with dispersant product or dispersant product/oil mixtures, as detailed in Appendix C to part 300. Measured toxicity values for dispersant product and dispersant product/oil mixtures are proposed to include static acute toxicity tests using *A. bahia* and *M. beryllina.* Developmental toxicity using a sea urchin assay, and sub-chronic effects using *A. bahia* and *M. beryllina* are also proposed to be conducted, but with dispersant product only. The Agency is proposing the following toxicity thresholds for listing dispersants on the Schedule:

• Lethal concentration for 50% of the test species (LC_{50}) at the lower 95% confidence interval for all acute toxicity tests greater than 10 ppm;

• Inhibition concentration for 50% of the test species (IC_{50}) at the lower 95% confidence interval greater than 10 ppm;

• No Observed Effect Concentration (NOEC) equal to or greater than 1 ppm for sub-chronic toxicity.

Test Oils and Oil Acute Toxicity Values. The Agency proposes to replace the No. 2 fuel oil currently used for toxicity testing with two test oils that better represent a wider range of characteristics and that may be encountered during an incident. The proposal is based on ANS and IFO–120, as previously discussed. In addition to making the test oils consistent with the dispersant efficacy tests, testing oils of different gravities also provides a better estimation of dispersant/oil toxicity associated with differing oil constituencies, and ultimately a better representation of the potential overall toxicity of a product.

The Agency proposes to conduct the oil-only acute toxicity tests for the two reference oils, with both *A. bahia* and

[36] Venosa, A. D., D. W. King, and G. A. Sorial. 2002. The Baffled Flask Test for Dispersant Effectiveness: A Round Robin Evaluation of Reproducibility and Repeatability. Spill Sci. & Technol. Bulletin 7(5–6):299–308.

[37] Sorial, G. A., A. D. Venosa, K. M. Miller, E. Holder, and D. W. King. 2004a. Oil Spill Dispersant Effectiveness Protocol—Part I Impact of Operational Variables. ASCE. J. Env. Eng. Div., 130(10):1073–1084.

[38] Sorial, G. A., A. D. Venosa, K. M. Miller, E. Holder, and D. W. King. 2004b. Oil Spill Dispersant Effectiveness Protocol—Part II Performance of the Revised Protocol. ASCE ASCE. J. Env. Eng. Div., 130(10):1085–1093.

[39] USEPA 2002a Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth edition. (Acute Methods Manual), Office of Water, Cincinnati, OH. EPA–821–R–02–012.

[40] USEPA 2002b. Short-term Methods for Estimating the Chronic toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition. (Saltwater Chronic Methods manual). Office of Water, Cincinnati, OH. EPA–821–R–02–014.

M. beryllina, and provide this data for comparisons to dispersant and dispersant-oil mixture acute toxicity tests. EPA will make all of the reference oil toxicity test results available to the public on its Web site, including raw data and calculated median LC_{50} values. By providing this information, the Agency is reducing the number of required toxicity tests the submitter would need to conduct. Alternatively, the Agency considered requiring submitters to conduct the oil acute toxicity testing as it would provide an opportunity to detect anomalies in the submitted data. The Agency is requesting comments on whether the submitter should be required to conduct the oil-only acute toxicity testing for the two test oils.

Dispersant and Dispersant-Oil Acute Toxicity Threshold. Using the EPA toxicity classification scheme,[41] LC_{50} values ranging from 10 ppm to 100 ppm are classified as slightly toxic and above 100 ppm substances are considered acutely nontoxic to aquatic organisms. For both *M. beryllina* and *A. bahia,* the Agency is proposing as the threshold value the lower bound of the LC_{50} 95% confidence interval (CI) greater than or equal to 10 ppm for all toxicity tests to qualify a dispersant to be listed on the Schedule. The rationale for using the lower bound of the CI is that the CI should not contain any values less than or equal to 10 ppm since theoretically, the LC_{50} can fall anywhere within the CI. By using the lower CI, the Agency is providing a conservative decision criterion for acute toxicity, and by proposing a greater than or equal to 10 ppm threshold level, it is establishing an adequate safety margin without being overly restrictive.

Dispersant-Oil Mixture Acute Toxicity Protocol. The Agency is proposing to substitute the existing preparation procedure for the dispersant-oil mixture (DOM) in Appendix C to Part 300, Section 3 method for the preparation procedure for dispersant-oil mixture using a modified protocol[42] first described by Baron and Ka'aihue[43] and fully described by Hemmer et al.[44] These new modified procedures for preparation of the chemically enhanced water accommodated fractions (CEWAFs) of dispersant-oil mixtures are used to make exposure solutions. Evaluations of the dispersant-oil mixture toxicity using acute toxicity tests follow existing EPA effluent testing guidelines,[45] [46] modified for use with these exposure solutions and are detailed in Appendix C to part 300. EPA proposes to modify the variable loading preparation procedure described in Chemical Response to Oil Spills: Ecological Research Forum (CROSERF)[47] to a variable dilution procedure to standardize the oil-to-water ratio and provide a consistent chemical concentration in a test series. Additionally, the modified preparation procedure provides economies in analytical costs by reducing the need to analyze the composition of every tested concentration. Chemical analysis for the total petroleum hydrocarbon (TPH) concentration of the CEWAF stock solution is required.

Sea Urchin Developmental Assay. The Agency is proposing to require a sea urchin developmental assay (also referred to as an embryogenesis assay) to assess the potential for a dispersant product to cause adverse effects on the developmental process. During the Deep Water Horizon spill event, the possible adverse effects of oil dispersants on the developmental processes of fish and invertebrate species were identified as critical issues of concern. By incorporating the sea urchin developmental assay, the Agency is using it as a sensitive surrogate test for pelagic early life stages. This assay would employ an existing EPA test protocol[48] routinely used in effluent testing. As suggested for the dispersant acute toxicity test criteria with *A. bahia* and *M. beryllina,* the Agency is proposing as the threshold value the lower bound of the developmental IC_{50} 95% confidence interval greater than or equal to 10 ppm. For this test, the IC_{50} is defined as the concentration of dispersant that inhibits the development of 50% of exposed embryos. As described above for acute toxicity levels, the Agency is adopting a similar rationale for IC such that IC_{50} values ranging from 10 ppm to 100 ppm are considered to cause slight adverse effects on the developmental process, and above 100 ppm substances are considered to cause no adverse effects. By using the lower CI the Agency is providing a conservative decision criterion for developmental toxicity, and by proposing a greater than or equal to 10 ppm threshold level it is establishing an adequate safety margin without being overly restrictive.

Sublethal/Subchronic Studies. The Agency has limited information concerning the possible sublethal effects of dispersants currently listed on the Product Schedule. The Agency is proposing requirements for subchronic assays (duration approximately 7 days) to be performed with *A. bahia* and *M. beryllina* following established EPA short-term methods for estimating chronic toxicity of effluents.[49] While the Agency considered requiring longer-term tests, it believes the 7-day subchronic tests are reasonable alternatives for estimating chronic toxicity. This information would also be of value as guidance to regional responders and OSC's on possible adverse effects on survival and growth of larval fish and invertebrates caused by longer-term exposure to dispersants. The Agency is proposing a NOEC equal to or greater than 1 ppm listing threshold level for sub-chronic effects. This threshold level provides a tenfold safety factor from the proposed acute toxicity threshold values. The tenfold safety factor is a common practice in ecological risk assessment and thus the Agency believes it provides an adequate safety margin without being overly restrictive. The Agency is requesting comment on whether it should consider longer-term tests (*e.g.,* early life stage tests), which may be more relevant to sublethal effects caused by longer

[41] *http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm#Ecotox.*

[42] The modified protocol is based on a variable dilution modification of the Chemical Response to Oil Spills: Ecological Research Forum (CROSERF) testing protocol; Aurand, D. and G. Coelho (Editors). 2005. Cooperative Aquatic Toxicity Testing of Dispersed Oil and the "Chemical Response to Oil Spills: Ecological Effects Research Forum (CROSERF)." Ecosystem Management & Associates, Inc. Lusby, MD. Technical Report 07–03.

[43] Barron, MG, Ka'aihue L. 2003. Critical evaluation of CROSERF test methods for oil dispersant toxicity testing under subarctic conditions. Mar Pollut Bull 46:1191–1199.

[44] Hemmer, MJ, Barron, MG, Greene, R. 2011. Comparative toxicity of eight oil dispersants, Louisiana sweet crude oil (LSC) and chemically dispersed LSC to two aquatic species. Environ Toxicol Chem, 30: 2244–2252.

[45] USEPA 2002a Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth edition. (Acute Methods Manual), Office of Water, Cincinnati, OH. EPA–821–R–02–012.

[46] USEPA 2002b. Short-term Methods for Estimating the Chronic toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition. (Saltwater Chronic Methods manual). Office of Water, Cincinnati, OH. EPA–821–R–02–014.

[47] CROSERF was established as a working group of industry, government, and university scientists to coordinate and disseminate research on chemical oil spill dispersants. CROSERF participants developed aquatic toxicity testing protocols during 1994 to 2000 with the foremost objective of standardizing test methods and reducing inter-laboratory variability.

[48] USEPA 1996. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms. EPA/600/R–95/136.

[49] USEPA 2002b. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition. (Saltwater Chronic Methods manual). Office of Water, Cincinnati, OH. EPA–821–R–02–014.

duration exposure to low concentrations of a dispersant product.

Alternative Options

In addition to the criteria discussed above, the Agency considered calculating a toxicity threshold based on the ratio of the LC_{50} of oil-alone to the LC_{50} of the dispersant-oil mixture for *M. beryllina* and *A. bahia* to assist in comparing the relative toxicity of crude oil to chemically dispersed oil. A ratio of less than or equal to 1 would indicate the toxicity of the dispersant-oil mixture is less than or equal to the toxicity of the oil-alone. The Agency considered whether this ratio should be used as a criterion for inclusion of dispersants on the Schedule or as guidance to emergency responders. Using a less than or equal to 1 ratio as a guideline would afford an adequate safety margin without being overly restrictive. While the ratio would provide a simple, interpretable, and easily justifiable value for determining acceptable toxicity levels of dispersant-oil mixtures, it would not be specific to oils in particular discharge situations. The Agency is requesting comment as to whether such a value should be calculated, and if so, whether it should be an additional listing criterion, or provided as information for the responders to consider in authorizing dispersant use.

The Agency also considered including geographically or ecologically representative species in the testing protocol. The Agency believes, however, that this issue is addressed by the proposed revisions to the supplemental testing and information requirements in section 300.910(g), with the decisions of testing geographically and ecologically representative species left to the discretion of the RRTs. While inclusion of species from different phyla and habitats would provide useful and important information on possible adverse effects of dispersant products and dispersed oil, the proposed testing protocols would need to be modified and validated. Further, the Agency is concerned about balancing the time and cost associated with the development of these tests on the part of the submitter rather than on the end users.

The Schedule currently requires no specific toxicity or efficacy tests for subsea dispersant listing or authorization of use. While the differences in toxicity values and efficacy may be affected by application in subsea environments, the Agency believes that the proposed requirements establish an adequate baseline for listing dispersants on the Schedule and for authorizing their use by responders in case of an incident. The Agency is addressing these concerns by proposing new provisions for dispersant monitoring for all subsea use, and requests comments on alternative testing and listing approaches to specifically address subsea concerns.

(3) Limitations of Use

The Agency is proposing a conditional use listing for dispersants. The proposal specifies that dispersants may only be used in saltwater environments. Dispersants are typically designed and traditionally used for responding to oil discharges in saltwater. In general, the effectiveness of dispersants decreases as the salinity of the water decreases, given the same hydrophilic-lipophilic balance, and the effectiveness is minimal in freshwater environments. Surfactants, the main active ingredient in dispersants, sustain oil droplets in water by orienting the lipophilic side of its molecule in the oil and the hydrophilic side in the water. The hydrophilic side of the surfactant is generally more soluble in waters with higher salinity values, causing the oil droplets to be more stable in saline water environments. In waters with no salinity, many dispersants have shown a very low effectiveness or are sometimes completely ineffective.[50]

The Agency is also concerned with using dispersants in freshwater environments because of the limited dilution typically available as compared with the open sea and because of the existence of water intakes in rivers, streams, and lakes for use in drinking water supplies. Using dispersants in freshwater has the potential for compounding the impacts caused by already discharged petroleum products, particularly near potable and non-potable subsurface water intakes. The Agency does recognize, however, that as a last resort, dispersants may have some limited applicability in freshwater. The provision for authorization of use under §300.910(d) would allow for exceptions to the prohibition on the use of dispersants in freshwater in cases where there is an immediate threat to human life. The Agency is requesting comment on this proposed listing limitation.

(c) Surface Washing Agent Testing and Listing Requirements

The Agency is proposing to define Surface washing agents (SWA) as substances that separate oil from solid surfaces, such as beaches, rocks, or concrete, through a detergency mechanism that lifts and floats oil for collection and recovery from the environment with minimal dissolution, dispersion, or transfer of oil into the water column. For this agent category, the Agency is proposing to revise the toxicity testing protocols, to establish efficacy testing protocols, and to establish both toxicity and efficacy listing thresholds in §300.915(c). The proposed revisions respond to concerns regarding their frequent use and the potential for residual impacts after their use.

(1) Surface Washing Agent Efficacy

Current Requirements: There are currently no efficacy testing requirements in the NCP Subpart J for surface washing agents to determine listing eligibility on the Schedule.

Proposed Revisions: The Agency is developing a laboratory testing protocol to evaluate the efficacy of SWAs in removing crude oil from a solid substrate.[51] Currently available efficacy testing protocols for these agents are concerned with assessing the detergency, or cleaning power, of the agents by quantifying the physical removal of oil from non-vegetative substrates as a result of their use. The protocols typically involve the application of oil to a solid substrate (or the use of pre-oiled substrate), weathering of the oil on the substrate, application of the agent to the oiled substrate, a contact period for SWA penetration, and washing of the substrate with water. The fractions of oil removed in the wash water and/or remaining on the substrate are quantified. Oil removal efficiencies for surface washing treatments are typically compared to the washing efficiency of water without surface washing agents (untreated controls). Disadvantages or potential sources of error have been identified in the existing protocols that may not reflect field performance. Of concern are, for example, the use of substrates (*e.g.,* stainless steel, porcelain) with different wetting and adhesion properties than natural substrates such as sand or gravel; short weathering times insufficient for oil attachment; and the absence of mixing energy during the washing step, which may lead to incomplete detachment of partially released oil.

The Agency's goal is to develop and evaluate a new bench-scale testing protocol that would provide a standard for EPA to use in SWA product

[50] Fingas, M., (Ed.), 2011, *Oil Spill Science and Technology*, Gulf Professional Publishing, pp. 513–518.

[51] Koran, K.M., Venosa, A.D., Luedeker, C.C., Dunnigan, K., Sorial, G.A., 2009. Development and Testing of a New Protocol for Evaluating the Effectiveness of Oil Spill Surface Washing Agents. Mar. Pollut. Bull., 58: 1903–1908.

evaluation prior to listing on the Schedule. Changes over established protocols the Agency considered include: Using natural substrates to reflect the most commonly impacted shoreline material; establishing sufficient weathering time to permit strong attachment of the oil to the substrate surface; fully quantifiable approaches with known volumes and precise measurements to allow mass balance; and a standardized and reproducible methodology to minimize operator error. PBC oil was used as the representative standard reference oil in developing the protocol since it had previously been selected as one of two standard oils for the Agency's published protocols for dispersant and bioremediation agent testing. However, the Agency is proposing revisions to the dispersant and bioremediation testing protocols, including replacing the PBC reference oil. The protocol being developed has not been tested: With the IFO–120 or the ANS oils that are proposed to be used in the revised dispersant efficacy testing protocol; at multiple temperatures; or to include freshwater systems. The Agency expects to develop final recommendations for the testing protocol following round robin testing after adjusting for the new variables. The Agency would propose this protocol in the **Federal Register** through notice and comment before adopting it as part of Subpart J requirements.

Consequently, because of the additional work needed to finalize its protocol, the Agency is proposing to allow the use of standard recognized efficacy testing methodologies for surface washing agents. An example of such a standard recognized methodology is the American Society for Testing and Materials (ASTM) Standard Test Method for Evaluating the Effectiveness of Cleaning Agents.[52] Another methodology is Environment Canada's Test Method.[53] The capability of a particular surface washing agent depends upon the application procedures and the characteristics of the surface being cleaned, such as size, shape, and material. The ASTM test method in particular covers a procedure for evaluating the capability of the agents, providing a relatively rough surface to which the oil can adhere. The Environment Canada method uses a stainless-steel 'trough' which is placed at a specified angle. The target oil is placed on an area on the trough. The treating agent is then applied in droplets to the surface of the oil and after 10 minutes at 5-minute intervals, rinses of water are applied to the trough. After drying, the trough is weighed and the removal calculated from the weight loss. Repeatability is within 5%. The Agency is requesting comment on available methodologies and its published protocol to incorporate as the testing protocol for these agents. The Agency also requests comment on whether the rule should identify the specific methodologies to be used until EPA develops and adopts a new testing protocol for SWA efficacy testing.

The Agency is also proposing an efficacy threshold of 30% in either fresh or salt water or both depending on the intended product use. This is based on the efficacy data published in "Surface-washing Agents or Beach Cleaners." [48] Numerous surface washing agent products, including several from the Schedule were tested using the Environment Canada methodology. The average efficacy of the surface washing agents tested is approximately 30%. The Agency requests comments on this approach and other efficacy test data available.

(2) Surface Washing Agent Toxicity

Current Requirements: The current NCP Subpart J requires surface washing agents to be tested for saltwater species toxicity. Surface washing agent toxicity is tested following the dispersant toxicity testing protocols in Appendix C to part 300 of the NCP.

Proposed Revisions: The Agency is proposing revisions to the toxicity testing requirements for surface washing agents, including the testing protocol. While the Agency currently provides the toxicity testing results to the OSC to assist in authorization of use determinations, it is proposing to use these toxicity testing results to determine listing eligibility on the Schedule.

The proposed revisions to the testing protocols for surface washing agents are detailed in Appendix C to part 300. The proposed acute toxicity test protocol for surface washing agents is based on EPA's protocol, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters for Freshwater and Marine Organisms.*[54] The Agency proposes to require surface washing agents be tested for acute toxicity using fresh water species *Ceriodaphnia dubia* and *Pimephales promelas,* or saltwater species *Americamysis bahia* and *Menidia beryllina,* or both, depending on the intended product use. The concentration of test product causing 50% lethality to the test organisms (LC_{50}) and lower and upper 95% confidence intervals (LCI_{95} and $ULCI_{95}$) are calculated at the end of the exposure period. To be listed on the Schedule, surface washing agents must demonstrate an acute lethal concentration for 50% of the test species (LC_{50}) at the lower 95% confidence interval greater than 10 ppm in either fresh or salt water, consistent with the acute toxicity thresholds proposed for dispersants. EPA's toxicity classification scheme[55] classifies LC_{50} values ranging from 10 ppm to 100 ppm as slightly toxic and values above 100 ppm substances are considered practically nontoxic to aquatic organisms. By proposing this threshold level, the Agency is establishing an adequate safety margin without being overly restrictive. The Agency requests comments on the proposed acute toxicity methods (or any others available) and thresholds for surface washing agents.

The Agency is proposing provisions for conducting acute toxicity tests with dispersant-oil mixtures, but not for any other agent, including SWAs. EPA believes that non-dispersant products are not likely to be used in the same quantities or durations as dispersants and some may be recovered making a requirement to conduct toxicity tests of product/oil mixtures unnecessary. There may be concerns, however, for increased product toxicity because of their interaction with the oil. Thus, the Agency is requesting comment on the need for acute toxicity tests conducted with surface washing agents-reference oil mixtures. A protocol for preparation of product/oil mixtures for toxicity testing is available for review in the docket.

(3) Limitations

The Agency is proposing a conditional use listing for SWAs. The proposal specifies that these products may only be used in those water environments for which the product was tested and for which it met the listing threshold criteria. The Agency recognizes that products may yield effective results in certain environments and not in others. Products that may be

[52] ASTM Standard Test Method for Evaluating the Effectiveness of Cleaning Agents. Designation: G122—96 (Reapproved 2008). ASTM International, 100 Barr Harbour Dr., P.O. Box C–700 West Conshohocken, Pennsylvania 19428–2959, United States

[53] Fingas, Merv and Fieldhouse, Ben; "Surface-washing Agents or Beach Cleaners" (2010). Chapter 21 Surface-Washing Agents or Beach Cleaners. In Oil Spill Science and Technology (p716). London: Gulf Professional Publishing.

[54] *http://water.epa.gov/scitech/methods/cwa/wet/upload/2007_07_10_methods_wet_disk2_atx1-6.pdf.*

[55] USEPA 2010. *http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysiseco.htm#Ecotox.*

effective in freshwater environments may not necessarily be so in saltwater environments, and vice versa. The Agency is proposing this limitation to allow product manufacturers the flexibility to select which environment the product is to be tested and authorized for use, either saltwater, fresh water, or both environments. Therefore, the product would be listed and could only be authorized for use in those water environments for which it was tested and for which it met both the efficacy and toxicity listing criteria.

(d) Bioremediation Testing and Listing Requirements

The Agency is proposing to define bioremediation agents as biological agents and/or nutrient additives deliberately introduced into a contaminated environment to increase the rate of biodegradation and mitigate any deleterious effects caused by the contaminant constituents. Bioremediation agents include microorganisms, enzymes, and nutrient additives such as fertilizers containing bioavailable forms of nitrogen, phosphorus and potassium. For this agent category, the Agency is proposing to revise the efficacy testing protocols, to establish toxicity testing protocols, and to establish both efficacy and toxicity listing thresholds in §300.915(d).

(1) Bioremediation Agent Efficacy

Current Requirements: Bioremediation agent efficacy is currently tested following the protocols in Appendix C to part 300 of the NCP. The current bioremediation agent efficacy testing protocol is designed to determine a product's ability to biodegrade oil by quantifying changes in the oil composition resulting from biodegradation using natural seawater as the test water. The protocol tests the bioremediation agent for microbial activity and quantifies the disappearance of saturated hydrocarbons and PAHs in weathered oil; for purposes of the proposal the Agency tested the protocol using ANS 521.[56] The sample preparation procedure extracts the oil phase into dichloromethane (DCM), with a subsequent solvent exchange into hexane. The analytical technique uses a high-resolution gas chromatograph/mass spectrometer (GC/MS); GC/MS provides for a high degree of chemical separation and spectral resolution. Concurrently with the chemical analysis, a microbiological analysis is also performed to determine and monitor the viability and growth of the microbial cultures being studied. For commercial products that are strictly microbial agents not containing their own nutrients, a mineral nutrient solution is provided if requested by the submitter; no further nutrients are added to commercial products containing their own nutrients. To be listed on the Schedule, bioremediation agents must demonstrate a statistically significant difference between the average degradation of both total alkanes and total aromatics in the oil achieved by the product after 28 days and the average degradation for the same compounds achieved by the non-nutrient control in the same time period.

Proposed Revisions: The proposal reflects a series of changes to the current efficacy testing protocol for bioremediation agents. The protocol includes freshwater testing in addition to the current saltwater-based test, and uses artificial water for both fresh and saltwater testing, replacing the natural seawater currently used. The proposed protocol also eliminates several gravimetric and microbiological analyses and testing endpoints not used in the proposed listing determinations. Additionally, the proposed protocol limits the levels at which external nutrients may be added, which allows the addition for product formulations without nutrients, or for product formulations that have nutrient concentrations at insufficient levels for the experimental setup. Finally, the methodology streamlines the statistical analysis. The proposed revisions address concerns with the existing methodology as discussed below, expanding its application to include freshwater environments, improving the consistency and comparability of the test results, and generally streamlining the protocol.

Protocol. The proposed efficacy testing protocol is designed to determine a bioremediation agent's ability to biodegrade weathered crude oil in the exposure water (saltwater or freshwater) by quantifying changes in the oil composition resulting from biodegradation. It consists of a series of six product and control flasks containing artificial seawater or artificial freshwater and weathered crude oil in which biodegradation of the crude oil hydrocarbons is monitored for 28 days. Product flasks at day 28 are compared to product flasks at day 0 to determine if a specified reduction both in total resolved alkanes and total resolved aromatics was achieved in that time period. Product flasks contain exposure water, weathered oil, and product in concentrations specified by the manufacturer. Positive control flasks must contain exposure water, oil, nutrients, and the standard culture supplied by the Agency. A negative control, consisting of artificial exposure water, product, weathered crude oil, nutrients, and a sterilant is also used to ensure that the observed degradation of hydrocarbons was not caused by abiotic losses or interaction with the product. The Agency is requesting comment on whether an additional protocol specific to products containing enzymes only would be appropriate; this would consist only of exposure water, weathered oil and the enzymatic product in the concentrations specified by the manufacturer. The proposed protocol quantifies the degradation (or disappearance) of alkane hydrocarbons and aromatic hydrocarbons. The sample preparation procedure extracts the oil phase into the solvent dichloromethane (DCM; also known as methylene chloride) with a subsequent solvent exchange into hexane. The test method targets the relatively easy to degrade normal alkanes and the more resistant and toxic aromatics. The analytical technique uses a high resolution GC/MS because of its high degree of chemical separation and spectral resolution. GC/MS has long been used to study the weathering and fate of oil spilled into the environment. It gives unambiguous results for determining biodegradation efficacy. For quantitative analyses, the instrument is operated in the selected ion monitoring mode (SIM) of detection at a scan rate of > 5 scans per second to maximize the linear quantitative range and precision of the instrument.

Fresh and Saltwater Environments. The existing bioremediation test is exclusively for saline environments with no equivalent test for freshwater environments. Further, the existing test uses natural seawater and has been found to give variable results due to the compositional variability of seawater both chemically and microbiologically. The Agency proposes to replace the natural seawater with a standardized artificial saltwater formula called GP2, whose components and concentrations are generally recognized, and which is easily made. The use of artificial saltwater allows for better test reproducibility. Additionally, the Agency proposes to expand the requirement for efficacy testing to include freshwater, which allows for a better evaluation of the use of these agents in this environment. Similarly to

[56] Artificially distilled Alaska North Slope crude oil at 521 °F (272°C) to remove the low molecular weight hydrocarbons to approximate natural weathering processes that occur after a spill.

the artificial saltwater, artificial minimal salts freshwater would be used with known ingredients (Bushnell and Haas; Journal of Bacteriology, Vol. 41: 653, 1941). Because the Agency is adding a freshwater test, it is also allowing the submitter to test a product for freshwater only, saltwater only, or both. The Schedule listing would specify in which environment the product was tested and authorized for use. Products could be listed for use on saltwater only, on freshwater only, or for use on both environments, giving the option to the submitter to market its products accordingly.

GC/MS Testing Endpoints. GC/MS has long been used to study the weathering and fate of oil spilled into the environment. However, GC/MS being the primary tool used in the analysis makes this test expensive. The current protocol includes several measured variables in both the product flasks and the non-nutrient control flasks, including the gravimetric residual oil weight analysis, a Most Probable Number microbiological analysis, and the GC/MS analysis at 3 different time points, days 0, 7, and 28. The Agency is proposing to eliminate all of these analyses except the day 0 and 28 GC/MS analysis of both the non-nutrient control and the product flasks. The Agency believes this is appropriate because the other measurements are not used in determining whether a product meets the listing criterion. Additionally, the statistical analysis has been greatly simplified, and a new decision rule is proposed for listing determinations rather than relying on a statistical significance test.

• *Gravimetric Weight Analysis:* Originally, the gravimetric weight analysis was used as a preliminary test to avoid having to perform a GC/MS analysis. Products that failed to significantly reduce the gravimetric weight of the oil within 28 days were considered not effective, thereby eliminating the need to conduct the more expensive GC/MS analysis. However, numerous compounds can give rise to positive interferences with the gravimetric weight analysis, such as biomass lipids and other biogenic materials, thereby rendering the gravimetric analysis suspect. Furthermore, numerous factors can confound the interpretation of gravimetric weight changes in oil over time. This has led to confusion as some manufacturers have mistakenly concluded their product passed the testing protocol simply by complying with the gravimetric weight loss measurement. By eliminating the gravimetric analysis altogether, EPA is streamlining the testing process, resulting in less confusion and a modest analytical cost savings for the manufacturer.

• *Most Probable Number Enumeration (Microbiological Analysis), Nutrient Control, and Day-Seven Sampling Event:* Results of these test endpoints, while currently required, would not be used in determining whether a product meets the proposed listing requirements. Therefore, the Agency is proposing to eliminate these test requirements from the proposed protocol.

• *28 day Sampling Event:* Generally, results of biodegradation may occur in less than 28 days, particularly for alkanes for which biodegradation often takes place in a much shorter time period. However, 28 days are needed to make sure that the aromatic components, which biodegrade more slowly, have been reduced significantly; testing protocols that accommodate aromatics are of particular importance because of their potential ecological toxicity. While the Agency could propose different time frames in determining whether the product met the reduction criterion for alkanes and aromatics, it believes that such an approach would be confusing. Therefore, the 28 day time period is being proposed for both fractions for simplicity and to provide sufficient time for degradation of the aromatics.

Protocol Verification. The Agency proposes to provide a standard culture for performing laboratories to use as a positive control benchmark. A positive control is needed as an indication that the test was performed properly. The Agency is proposing to use as the standard culture an oil-degrading bacteria isolated from Disk Island in Prince William Sound in 1990. This culture is an excellent degrader of alkanes and aromatics in saltwater and freshwater, although it performs better in saltwater, especially in degrading the aromatics. This culture has the ability to degrade ANS 521 oil, with known efficacy values for both fresh and saltwater. The proposed positive control consists of triplicate flasks containing sterile artificial seawater or freshwater, nutrients (salts of nitrogen and phosphorus), the weathered ANS 521 oil, and the standard culture. The performing laboratory must achieve the known reduction in alkane and aromatic fractions in these positive control flasks in order to certify they successfully conducted the test procedure. Additionally, data from the positive control are required to be submitted with the product test results. The Agency is not proposing, however, that the positive control be repeated every time a product test is performed. Rather, the certifying laboratory would be required to report the results of the positive control tests conducted within a year prior to the product testing, as part of the product submission package. Additionally, this positive control culture would be used as a standard microbial inoculum to test abiotic products, such as nutrients and enzymes.

Added Nutrients. The current protocol allows the manufacturer to request the addition of nutrients to support a culture's ability to degrade the crude oil hydrocarbons. This addition is currently allowed for commercial products that are strictly microbial agents and do not contain any nutrients; the addition of nutrients is not allowed for commercial products containing its own nutrients. A modified salt solution of nitrogen and phosphorus is used as the mineral nutrient. All microbial cultures need nutrients to survive and grow. In the natural environment, biodegradation is not completely limited by nutrient availability given that a low level flow of nutrients is continuous. To maximize biodegradation rates, the addition of nutrients is typically recommended in the field with a bioremediation product unless nutrients are already high (*i.e.,* >5 mg/L). However, in a closed laboratory flask, the amount of nitrogen and phosphorus supplied by a typical product might easily become limiting quickly as biodegradation takes place because they are supplied only once at the beginning of the test. If nutrient limitation occurs, further reduction of hydrocarbons would likely be inhibited, and this may cause the product to fail the listing criteria. To address this problem, the Agency proposes modifying the protocol by allowing the addition of nutrients to any product containing living microbes that does not contain said nutrients in sufficient quantities to allow a fair evaluation. However, the additional quantities allowed are limited to total concentrations of nitrogen and phosphorus that do not to exceed the equivalent amounts used in the positive control. Capping the amount of nutrients that can be added to all products will yield more consistent results and more effective comparisons among products.

Efficacy Thresholds. The Agency is proposing to revise the listing criteria by replacing the current "statistically significant difference" test with defined values for the percent reduction of total alkane and aromatic concentrations. For a bioremediation agent to be listed on

the Schedule, the Agency proposes the percent reduction of the total alkane fraction (sum of all alkane concentrations determined by GC/MS) to be greater than 95% at day 28, and that the percent reduction of the total aromatic fraction (sum of all aromatic concentrations determined by GC/MS) must be greater than 70% at day 28, both based on the one-tailed Upper 95% Confidence Limit (UCL_{95}) for salt water. For freshwater, the Agency proposes the percent reduction of the total alkane fraction (sum of all alkane concentrations determined by GC/MS) to be greater than 95% at day 28, and that the percent reduction of the total aromatic fraction (sum of all aromatic concentrations determined by GC/MS) must be greater than 40% at day 28, both based on the one-tailed Upper 95% Confidence Limit (UCL_{95}).

Basis of Efficacy Thresholds. According to Haines *et al.* (2005),[57] the positive control culture was able to biodegrade total alkanes in saltwater by 98.9% and total aromatics by 79.8% in 28 days, based on the UCL_{90}. In freshwater, the percent reduction values were 97.9% and 37.8%, respectively, again based on the UCL_{90}. Thus, the proposed listing thresholds for the percent reduction of total alkanes and total aromatics from the GC/MS analysis are based on the findings of Haines *et al.* (95% for alkanes vs. 98.9% in saltwater and 97.9% in freshwater, and 70% for aromatics in saltwater and 40% in freshwater vs. 79.8% in saltwater and 37.8% in freshwater). As for the positive control, the Agency believes a reasonable target in saltwater would be 95% for total alkanes in both types of exposure waters. For aromatics reduction, 70% is considered reasonable in saltwater and 40% in freshwater, based on Haines *et al.* EPA is using UCL_{95} values rather than the UCL_{90} values from Haines because EPA's latest research uses 6 replicates for the test protocol which increases precision. The proposed criteria demonstrate that the product can cause a substantial degradation of the alkane and aromatic fractions of weathered crude oil compared to a control, as determined solely by GC/MS analysis.

Of note, these proposed thresholds for listing bioremediation products on the Schedule are similar to those established for Canadian efficacy testing, which are reductions of 30% for total aliphatics and 10% for total aromatics. The 30% reduction in total aliphatics (including all resolvable and non-resolvable but GC-detectable aliphatics) is approximately equivalent to an 80% reduction in total GC/MS-resolvable target n-alkanes, based on Canadian data. The 10% reduction in total aromatics is approximately equivalent to a 50% reduction of the 5-aromatic homologue group consisting of naphthalene, fluorene, dibenzothiophene, phenanthrene, and chrysene and their alkylated homologs. The U.S. aromatic series includes 2 other 4-ring aromatics in addition to those 5 aromatic series considered by Blenkinsopp et al.[58] Thus, the U.S. and Canadian protocols are similar in terms of decision criteria. The Agency requests comments on all the proposed changes and listing thresholds.

(2) Bioremediation Agent Toxicity

Current Requirements: The Agency currently has no bioremediation agent toxicity testing requirements for purposes of listing these agents on the Schedule. Section 5 of Appendix C is reserved for this purpose. The Agency has, however, on a case-by-case basis, requested manufacturers to test bioremediation agents for toxicity if the product contains surfactants or other ingredients that may be harmful to the environment.

Proposed Revisions: The Agency is proposing an acute toxicity testing protocol for bioremediation agents to include both fresh and saltwater. The Agency will use these testing results to determine listing eligibility on the Schedule. The proposed testing protocols for bioremediation agents are detailed in Appendix C. The proposed acute toxicity test protocol for bioremediation agents is based on EPA's protocol, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters for Freshwater and Marine Organisms.* The Agency proposes to require bioremediation agents be tested for acute toxicity for the product alone using fresh water species *Ceriodaphnia dubia* and *Pimephales promelas,* and saltwater species *Americamysis bahia* and *Menidia beryllina.* The concentration of test product causing 50% lethality to the test organisms (LC_{50}) lower and upper 95% confidence intervals (LCI_{95} and $ULCI_{95}$) are calculated at the end of the exposure period. To be listed on the Schedule, bioremediation agents must demonstrate an acute lethal concentration for 50% of the test species (LC_{50}) at the lower 95% confidence interval greater than 10 ppm in either fresh or salt water, consistent with the acute toxicity thresholds proposed for dispersants. EPA's toxicity classification scheme[59] classifies LC_{50} values ranging from 10 ppm to 100 ppm as slightly toxic, and values above 100 ppm substances are considered practically nontoxic to aquatic organisms. By proposing this threshold level the Agency is establishing an adequate safety margin without being overly restrictive. The Agency requests comments on the proposed acute toxicity methods (or any others available) and thresholds for bioremediation agents.

Similarly to surface washing agents, the Agency is not proposing provisions for conducting acute toxicity tests with bioremediation agent-oil mixtures. The Agency is requesting comment on the need for acute toxicity tests conducted with bioremediation agents-reference oil mixtures. A protocol for preparation of product/oil mixtures for toxicity testing is available for review in the docket.

(3) Listing of Non-Proprietary Nutrients

The Agency recognizes there may be oil discharge situations where it is determined that the addition of nutrients in the form of salts of nitrogen, phosphorus and potassium (*i.e.,* fertilizers) to stimulate or enhance bioremediation may be the most effective and environmentally favorable mitigation method. However, non-proprietary commercially available formulations of nutrients are not specifically listed on the Schedule, even though as nutrient additives they are subject to Subpart J requirements. Currently, an OSC may only authorize the use of an agent not listed on the Schedule when its use is necessary to prevent or substantially reduce a hazard to human life. Further, RRTs are not currently able to preauthorize the use of nutrients unless they are listed on the Schedule. To address these concerns, the Agency proposes to include on the Schedule a specific list of the following generic non-proprietary nutrients: Ammonium nitrate, ammonium phosphate, sodium nitrate, potassium nitrate, urea, sodium triphosphate (or tripolyphosphate), sodium phosphate, potassium phosphate (mono- or dibasic), triple super phosphate, or any combination thereof. For these non-proprietary commercial nutrients, the Agency believes there is no need for submission of readily available

[57] Haines, J.R., E.J. Kleiner, K.A. McClellan, K.M. Koran, E.L. Holder, D.W. King, and A.D. Venosa. 2005. "Laboratory evaluation of oil spill bioremediation products in salt and freshwater systems." J. Ind. Micorbiol. Biotechnol. 32:171–185.

[58] Blenkinsopp, S, G Sergy, Z Wang, MF Fingas, J Foght and DWS Westlake. 1995. Oil spill bioremediation agents-Canadian efficacy test protocols. Proceedings of the 1995 International Oil Spill Conference, Feb 27–March 2, Long Beach, CA. American Petroleum Institute, Washington, DC. pp. 91–96.

[59] USEPA 2010. *http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysiseco.htm#Ecotox.*

information. Thus, the proposal requires no technical product data or further action on the part of a manufacturer prior to authorizing their use in response to a discharge event. However, this listing will be limited to products commonly formulated entirely of those mineral nutrients and synthetically-derived urea listed. For products that may contain additional proprietary components or have unique proprietary formulations, the requirement for the submitter to provide the toxicity and efficacy data under the bioremediation agent category will apply.

The Agency considered proposing a definition of ''fertilizer'' to specifically capture the listed bioremediation agents proposed to be exempted. However, fertilizer is a term commonly used to describe organic nutrients, which may contain substances that do not play a role in mitigating oil discharges, and that may have deleterious effects on the environment. Some non-proprietary fertilizers, for example, comprised of activated sewage sludge or manure, could contain unwanted pathogens and trace metals that could further stress the environment if used in response to an oil discharge. Thus, instead of an exemption based on a fertilizer definition, EPA proposes to restrict the exemption under the bioremediation category only to those nutrients listed above in order to avoid any confusion. The Agency requests comments on this approach.

(e) Solidifier Testing and Listing Requirements

The proposal defines solidifiers as substances that cause oil to become a coherent mass, preventing oil from dissolving or dispersing into the water column, and which are collected and recovered from the environment. For this agent category, the Agency proposes to revise the toxicity testing protocol and to establish a listing threshold for toxicity in §300.915(e). Although solidifiers are intended to be removed from the environment, the proposed revisions and new toxicity listing threshold respond to concerns regarding the general increase in the use of chemical and biological agents as tools available for oil discharge responses.

(1) Solidifier Efficacy

There are currently no efficacy testing requirements in the NCP Subpart J for solidifiers to determine listing eligibility on the Schedule. These agents would have been captured by the current MOSCA agent category, which currently has no efficacy testing requirements, and which the Agency is proposing to eliminate. While the Agency is aware of existing protocols to determine the effectiveness of solidifiers, its focus has been on reviewing the protocols for dispersants and bioremediation agents, given that their specific process for affecting the oil allows them to be left in the environment, whereas solidifiers are intended for removal from the environment. Therefore, the Agency is not proposing an efficacy testing methodology for solidifiers, but is requesting comment on available methodologies and/or performance criteria (*e.g.,* buoyancy) it can consider.

(2) Solidifier Toxicity

Current Requirements: The current NCP Subpart J requires solidifiers to be tested for saltwater species toxicity. Solidifier toxicity is tested following the dispersant toxicity testing protocols in Appendix C to part 300 of the NCP.

Proposed Revisions: The proposal revises the toxicity testing requirements for solidifiers, including the testing protocol. While the Agency currently provides the toxicity testing results to the OSC to assist in authorization of use determinations, it is proposing to use these results to determine listing eligibility on the Schedule.

The proposed revisions to the testing protocols for solidifiers are detailed in Appendix C to part 300. The proposed acute toxicity test protocol for solidifiers is based on EPA's protocol, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters for Freshwater and Marine Organisms.* The Agency proposes to require solidifiers be tested for acute toxicity for the product alone using fresh water species *Ceriodaphnia dubia* and *Pimephales promelas,* or saltwater species *Americamysis bahia* and *Menidia beryllina,* or both, depending on the intended product use. The concentration of test product causing 50% lethality to the test organisms (LC_{50}) and lower and upper 95% confidence intervals (LCI_{95} and $ULCI_{95}$) are calculated at the end of the exposure period. To be listed on the Schedule, solidifiers must demonstrate an acute lethal concentration for 50% of the test species (LC_{50}) at the lower 95% confidence interval greater than 10 ppm in either fresh or salt water, consistent with the acute toxicity thresholds proposed for dispersants. EPA's toxicity classification scheme[60] considers LC_{50} values ranging from 10 ppm to 100 ppm as slightly toxic and values above 100 ppm substances practically nontoxic to aquatic organisms. By proposing this threshold level, the Agency is establishing an adequate safety margin without being overly restrictive. The Agency requests comments on the proposed acute toxicity methods (or any others available) and thresholds for solidifiers.

Similarly to surface washing agents, the Agency is not proposing provisions for conducting acute toxicity tests with solidifiers-oil mixtures. The Agency is requesting comment on the need for acute toxicity tests conducted with solidifiers-reference oil mixtures. A protocol for preparation of product/oil mixtures for toxicity testing is available for review in the docket.

(3) Limitations

The Agency is proposing a conditional use listing for solidifiers. The proposal specifies that the listing of these products is limited to use only in those water environments for which the product was tested and for which it met the listing threshold criteria. The Agency recognizes that products may yield effective results in certain environments and not in others. Products that may be effective in freshwater may not necessarily be so in saltwater, and vice versa. The Agency is proposing this limitation to allow product manufacturers the flexibility to select which environment the product is to be tested and could be authorized for use, either saltwater, fresh water, or both. Therefore, the product would be listed and may only be authorized for use in those water environments for which it was tested and for which it met the listing criteria.

(f) Herding Agent Testing and Listing Requirements

The proposal defines herding agents as substances that are used to control the spreading of oil across the water surface. For this agent category, the Agency proposes to revise the toxicity testing protocol and to establish a listing threshold for toxicity in §300.915(f). While these agents are intended to be removed from the environment, the proposed revisions and new toxicity listing threshold respond to concerns regarding the general increase in the use of chemical and biological agents as tools available for oil discharge responses.

Currently, there is a test requirement for distinguishing surface collecting agents from other chemical agents (§300.915(c)(9), *Test to Distinguish Between Surface Collecting Agents and Other Chemical Agents*). Because the proposal eliminates surface collecting agents as a category and redefines herding agents to better reflect its specific process for affecting the oil, and

[60] USEPA 2010. *http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm#Ecotox.*

given that the agents will need to be identified in order for the required testing to be submitted, the Agency is eliminating this requirement.

(1) Herding Agent Efficacy

There are currently no efficacy testing requirements in the NCP Subpart J for herding agents to determine listing eligibility on the Schedule. These agents would have been captured by the current surface collecting agent category, which currently has no efficacy testing requirements, and which the proposal eliminates. The Agency is not proposing an efficacy testing methodology for herding agents, but is requesting comment on available methodologies and/or performance criteria it can consider.

(2) Herding Agent Toxicity

Current Requirements: The current NCP Subpart J requires herding agents to be tested for saltwater species toxicity. Herding agent toxicity is tested following the dispersant toxicity testing protocols in Appendix C to part 300 of the NCP.

Proposed Revisions: The proposal revises the toxicity testing requirements for herding agents, including the testing protocol. While the Agency currently provides the toxicity testing results to the OSC to assist in authorization of use determinations, we are proposing to use these results to determine listing eligibility on the Schedule.

The proposed revisions to the testing protocols for herding agents follow the same procedures as described for surface washing agents and are detailed in Appendix C to part 300. The acute toxicity test protocol for herding agents is based on EPA's protocol, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters for Freshwater and Marine Organisms.* The Agency proposes to require herding agents to be tested for acute toxicity for the product alone using fresh water species *Ceriodaphnia dubia* and *Pimephales promelas,* or saltwater species *Americamysis bahia* and *Menidia beryllina,* or both, depending on the intended product use. The concentration of test product causing 50% lethality to the test organisms (LC50) and lower and upper 95% confidence intervals (LCI95 and ULCI95) are calculated at the end of the exposure period. To be listed on the Schedule, herding agents must demonstrate an acute lethal concentration for 50% of the test species (LC_{50}) at the lower 95% confidence interval greater than 10 ppm in either fresh or salt water, consistent with the acute toxicity thresholds proposed for dispersants. EPA's toxicity classification scheme[61] considers LC_{50} values ranging from 10 ppm to 100 ppm as slightly toxic and values above 100 ppm substances practically nontoxic to aquatic organisms. By proposing this threshold level, the Agency is establishing an adequate safety margin without being overly restrictive. The Agency requests comments on the proposed acute toxicity methods (or any others available) and thresholds for herding agents.

Herding agents form a monolayer of surfactants on the water surface that result in the contraction of the oil slick.[62] [63] Herding agent composition may include hydrocarbons, fluorosurfactants and/or silicone-based surfactants, which suggests that a stock solution prepared using a WAF procedure similar to solidifiers may be appropriate. The Agency is requesting comment on the procedure for preparing the stock solution for herding agents for the acute toxicity tests proposed in Appendix C. Any alternative procedure for preparing the stock solution must include an appropriate rationale in order for the Agency to be able to consider it for final action.

Similarly to surface washing agents, the Agency is not proposing provisions for conducting acute toxicity tests with herding agents-oil mixtures. The Agency is requesting comment on the need for acute toxicity tests conducted with herding agents-reference oil mixtures. A protocol for preparation of product/oil mixtures for toxicity testing is available for review in the docket.

(3) Limitations

The Agency is proposing a conditional use listing for herding agents. The proposal specifies that these products may only be used in those water environments for which the product was tested and for which it met the listing threshold criteria. The Agency recognizes that products may yield effective results in certain environments and not in others. Products that may be effective in freshwater may not necessarily be so in saltwater, and vice versa. The Agency is proposing this limitation to allow product manufacturers the flexibility to select which environment the product is to be tested and could be authorized for use, either saltwater, fresh water, or both. Therefore, the product would be listed and may only be authorized for use in those water environments for which it was tested and for which it met the listing criteria.

(g) Sorbent Requirements

The proposal defines sorbents as inert, insoluble substances that readily absorb and/or adsorb oil or hazardous substances. Sorbents would exclude those contaminated with substances that would interact with the environment beyond their absorption/adsorption capabilities (*e.g.,* an invasive species). The proposed definition states that sorbents are generally collected and removed from the environment and may be used in their natural bulk form, or as manufactured products in particulate form, sheets, rolls, pillows, or booms. The list of sorbent materials in the proposed definition includes: Natural organic substances (*e.g.,* feathers, cork, peat moss, and cellulose fibers such as bagasse, corncobs, and straw); (2) inorganic/mineral compounds (*e.g.,* volcanic ash, perlite, vermiculite, zeolite, clay); and (3) synthetic compounds (*e.g.,* polypropylene, polyethylene, polyurethane, polyester). The Agency proposes to identify a list of known sorbent materials, and make it publicly available so that emergency responders can be aware and make use of such sorbents on oil discharges. The Agency is also proposing a process for submitters to request to include other products as sorbents if they can certify they meet the inert, insoluble criteria.

Current Requirements: Sorbents are currently not listed on the Schedule, but rather a list characterizing these materials is included in section 300.915(g). The current rule allows the OSC to request written certification from the manufacturers that their particular sorbent product is comprised solely of those materials identified in the rule. When a sorbent consists of materials that are not specifically listed in the current rule, the Agency issues written certifications, and sometimes requires testing and evaluation for possible listing on the Schedule.

Proposed Revisions: The Agency proposes to provide a Sorbent Product List that includes certain publicly available non-proprietary sorbent materials. The Agency also proposes to allow submitters to request that other products be included in this list as sorbents if they provide the technical information required, including data to support the claim that their product meets the sorbent definition. The Agency recognizes that a sorbent

[61] USEPA 2010. *http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm#Ecotox.*

[62] Buist, I., Potter, S., Nedwed, T., (2011) Herding Agents to Thicken Oil Spills in Drift Ice for In Situ Burning: New Developments, IOSC, *http://ioscproceedings.org/doi/abs/10.7901/2169-3358-2011-1-230.*

[63] Buist, I., Nedwed, T., (2011) Using Herders for Rapid *In Situ* Burning Of Oil Spills on Open Water, IOSC, *http://ioscproceedings.org/doi/abs/10.7901/2169-3358-2011-1-231.*

material may consist of one or more substances not specifically identified in the proposed non-proprietary listing. For products consisting of one or more substances not specifically identified in the proposed listing, the Agency proposes submission requirements that include general and product characterization information required under paragraphs (a)(1) through (8), and paragraph (a)(13) of proposed §300.915. These include name and contact information, identification as manufacturer or designated agent, product name/brand/trademark(s), contact information for primary distributors or importers, product SDS, conditions for product storage, product shelf life, and product label samples. The proposal would additionally require the certification statements required under paragraphs (a)(14)(iv), (a)(15), and (a)(16) of proposed §300.915. The submitter would be required to certify that the product does not contain specified bacterial, fungal, or viral pathogens or opportunistic pathogens above National Ambient Water Quality Criteria lowest density values; that the product does not contain specified contaminants above National Water Quality Standards levels; and that the product does not contain any of the prohibited agents. Finally, the submitter would be required to include information to support the claim that the product meets the sorbent definition, including data such as its relative solubility and non-reactivity in fresh and/or salt water. The Agency will review the submission and make a determination to include the product as a sorbent on the Sorbent Product List. The Agency requests comments on this approach.

Testing Options

The current Subpart J has no toxicity or efficacy testing requirements for sorbents. There are two ASTM standards for performance testing that could be applicable to sorbents: The ASTM F716–09 *Standard Test Methods for Sorbent Performance of Absorbents,* and the ASTM F726–12 *Standard Test Method for Sorbent Performance of Adsorbents.* These methods include laboratory tests that describe the performance of these products in removing oils that are not emulsified and other floating, immiscible liquids from the surface of water. While these methods are to be individually used as a basis for comparison of either absorbents or adsorbents in a consistent manner, they are not useful for a cross comparison of absorbents with adsorbents, even though according to ASTM F716–09 all absorbents exhibit adsorbent properties. Further, these agents are comprised of inert and insoluble materials that are generally removed from the environment after use, minimizing their potential harm. Thus, the Agency is not proposing to include efficacy or toxicity testing requirements for these agents, but is requesting comments on whether testing information should be included as part of the submission requirements for other materials or products to be included as part of the Sorbent Product List.

5. Submission of Confidential Business Information (CBI)

Currently, manufacturers may assert a claim of confidential business information (CBI) for any information in their product package submissions to EPA. Typically, manufacturers claim as CBI the chemical identity (*e.g.,* chemical name and chemical abstracts number [CAS]) and concentration (weight percent) of each chemical component in the product along with information about the concentrations of those components in the product. The manufacturer may also claim certain other information in the technical product data, including technical data for sorbents, as CBI. EPA safeguards CBI information under the requirements in 40 CFR part 2, subpart B. The proposal addresses the CBI provisions for product submission under Subpart J in §300.950.

Due to the amount of dispersant used during the Deepwater Horizon incident, and the need for the public's right-to-know about chemicals intentionally discharged into the environment, EPA wanted to make public information about the chemicals in the dispersant used, the results of air and water monitoring for these chemicals, and the concerns for human and environmental impact. A number of stakeholders wanted to know the chemical makeup of not only the dispersant used during the incident, but also of all other dispersants on the Schedule. This resulted in both a number of Freedom of Information Act (FOIA) requests and a Notice of Intent to Sue for Failure to Perform Nondiscretionary Duties under the CWA, requesting that the Agency release CBI for all dispersants on the Schedule.

EPA worked with the manufacturer for the dispersant used on the Gulf in response to the Deepwater Horizon incident to make the product chemical information publicly available (see *http://www.epa.gov/bpspill/*). The Agency also compiled a comprehensive aggregate list of the chemicals used in all listed dispersants. During this process, some manufacturers recognized the need to make information available to the public, but also argued the need to protect proprietary business information from competitors. Companies provided information on the magnitude of resources expended to develop a product and test it for listing on the Schedule; however, they argued that disclosing the chemical components, CAS numbers, and/or concentrations for their product would allow domestic and international competitors to freely duplicate or reverse engineer their product and potentially drive them out of the market. EPA is aware that over 90% of businesses on the current Schedule are small, and that for some companies the product they developed for the Schedule is their only business. While companies could protect their product via the U.S. patent process, they would be required to release components and concentrations information, which would be made public. Manufacturers voiced concern that not only others may use that information to tailor competitive products, but that the U.S. patent process would offer no protection against international competitors.

EPA believes that when chemical agents are used on oil discharges, it is critically important for the public and all other stakeholders to have information regarding the chemicals being added to the environment, along with information about their toxicity and fate. This is particularly true for major discharge events where larger quantities of chemical or biological agents may be authorized for use. Prompt and accurate information will allow the public to evaluate and understand the potential human and environmental effects of these chemical agents. While EPA understands it is essential for companies to protect their investment in developing unique products, it is proposing limitations to what submitters are allowed to claim as CBI in an effort to balance public access to information with proprietary business needs. The proposal provides that if a company submits a product for listing on the Schedule, then it will only be allowed to claim CBI for the concentrations of all chemical components, microbiological cultures, enzymes, or nutrients; all other information submitted to EPA for listing a product on the Schedule will not be considered CBI and will be made public. While providing confidential treatment for the concentrations of product components, the proposal allows public access to the identity of chemical components and relevant health and environmental effects

information. All other information required for a product submission (*e.g.,* company data, distributors, general product properties, recommended use procedures, the product category, contaminants, production capacities, product testing data) would already be publicly available for commercial products, and would not constitute proprietary business information or provide a business advantage. The Agency requests comments on whether this approach safeguards against duplication or reverse engineering of products by competitors and whether other information in Subpart J should be considered as CBI.

Alternatively, the Agency considered maintaining the current approach of allowing CBI claims for any information in their submission. When the incident arises where affected stakeholders or the public wants access to specific information, the Agency would request that the company substantiate its claims and make a determination whether to honor the claim or release the information to the public as provided in 40 CFR part 2, subpart B. The Agency also considered modifying the current approach by making the component identity and concentration information public without further notice or action for major discharge events or SONS, or for events where a given amount of chemical or biological agent would be allowed for use. Finally, another option for modifying the current approach would be to allow manufacturers to waive CBI claims only for certain chemical components for monitoring purposes (*e.g.* manufacturer identifies a ''marker'' component as a condition for listing) was also considered. The Agency is rejecting the current approach because it does not believe that, even with the modifications considered, it offers the appropriate balance between the public interest and business needs.

EPA also considered developing an aggregate list of components used in categories of chemical and biological agents for public disclosure. For example, a list of all the chemicals used in listed dispersants, a separate list for those substances used in surface washing agents, etc. This would allow information to be disassociated with specific products and protected from reverse engineering or duplication of products by competitors, while providing public access. The concern with this approach is how to update the aggregate list for new products without potentially revealing the components added to the list for that new product. Further, in the event of a major discharge or SONS, the interest will be for information and monitoring data specific to the product being used. Consequently, EPA is not adopting this approach, but requests comments on these and other options to handle CBI while balancing all interests.

6. Addition of a Product to the Schedule

The proposal establishes the requirements for submitters to request a product be listed on the Schedule in §300.955. It provides administrative information, such as the address where to submit the package, as well as details of the requirements for a complete submission package. Additionally, it addresses how a submitter may request a listing determination review and the requirements when there are changes in a listed product. Finally, the proposal addresses the process the Agency will follow to review all new submissions, requests for review of decisions and product changes, as well as how it will transition from the current Schedule to a new one that reflects the new and amended testing and data requirement.

Submission. The proposal updates the address where the package is to be submitted.

Package contents. The proposal specifies a complete package must include, as follows:

- A company letter certifying all testing was conducted on representative product samples at a nationally or internationally accredited laboratory, that it was conducted in accordance with all technical rule requirements, and that all test results and product technical data and information reported are true and accurate;
- A numbered Table of Contents showing all required information and data submitted;
- All required data and information (both general and product category specific) in the order the requirements appear in the rule; and
- A separate inner envelope labeled: ''CONFIDENTIAL BUSINESS INFORMATION—TO BE OPENED BY THE PRODUCT SCHEDULE MANAGER ONLY'', if applicable.

Because of their intended function in responding to oil discharges, products listed on the Schedule will certainly impact the environment. It is important that the information provided by the submitter is true and accurate, as it serves as the basis for evaluating those potential environmental impacts. The Agency believes that it is appropriate for the submitter to be held accountable for the technical data and information provided to make these listing determinations. Thus, the proposal requires the submitter to certify the accuracy of the information submitted, and will reject any submission that is determined to be incomplete or non-compliant, misleading, or inaccurate.

The requirements for a Table of Contents and for the information to be organized as it appears in the regulation are intended to make the Agency review process as efficient as possible. These requirements will assist the Agency in conducting a quick and accurate review, both during the transition period, as well as for future submissions, by generally simplifying the review process.

While the Agency needs to process packages containing information claimed to be CBI with additional safeguards, it is the responsibility of the submitter to ensure that this information comes to the Agency clearly identified as such. Therefore, the proposal requires a separate and clearly marked envelope for CBI to ensure proper handling.

EPA Review. The proposal maintains most of the existing Agency process for reviewing product submissions. A revision to the current process increases the number of days allowed for the Agency to complete its product review from the current 60 days to 90 days from the date of receipt. This proposed change considers the additional technical data and information proposed to be required, as well as the Agency's past experience with submission packages based on the current requirements.

EPA will first review the package for completeness and compliance with all data and information requirements and will contact the submitter to verify information, or to request clarification or additional information, including a product sample, as necessary. The Agency will make product listing determinations based on a technical evaluation of all data and information submitted, any relevant information on impacts or potential impacts of the product or any of its components on human health or the environment, and on the intended use of the product. The Agency reserves the right to make a determination on whether the product will be listed, and under which category. For products that may meet more than one category (*e.g.,* a product that meets both the bioremediation and dispersant agent criteria), the Agency requests comments on whether there should be any product listing limitations beyond those identified for each individual product category. Within the 90-day timeframe, the Agency will notify the submitter, in writing, of its decision to either list the product on the Schedule and under which category or categories, or of its decision and supporting rationale to reject the submission. Submitters may

revise submission packages to address test results, data, or information deficiencies and resubmit them. Because the Agency will need a complete set of data and technical information to make a listing determination, the 90-day review time period will start anew once a complete package is resubmitted.

Request for review of decision. The proposal is not substantively changing the process for a submitter to request that the Agency review its determination on a product. If the Agency rejects a product for listing on the Schedule, the proposal continues to allow for a submitter to appeal to the EPA Administrator to review its determination to reject the product listing. The proposal maintains the requirement that such a request be in writing, within 30 days of receipt of the written notification of EPA's decision not to list the product on the Schedule. The request to review the Agency's determination must include a clear and concise statement with supporting facts and technical analysis that demonstrates why the submitter believes the Agency's assessment of the product was incorrect. The proposal allows the Administrator to request additional information or a meeting opportunity. Within 60 days of receipt of any such request, or within 60 days of receipt of any requested additional information, the proposal requires the Administrator or her designee to notify the submitter in writing of the review decision, maintaining the current timeframe.

Changes to a product listing. The Agency proposes to revise the provisions for notification of changes to a product listing. Submitters must notify EPA in writing within 30 days of any changes to the general product information submitted for listing on the Schedule so the OSCs have timely updated information. The proposal revises the notification requirement to include details of the specific changes to information submitted under §300.915(a)(1) through (8) and §300.915(a)(19) through (21) for a product on the Schedule, including the reasons for such changes and the supporting data and information, and maintains the provision allowing EPA to request additional information and clarification regarding these changes. For any changes to the chemical components and/or their concentrations, the proposed revisions would require retesting of the product according to the requirements for the product category, and the resubmission of a complete new package for a new review and consideration for a listing determination by the Agency of the reformulated product. While the Agency currently has and is retaining the option of requiring additional testing, it believes that when the chemical components or concentrations of a product change, an automatic retesting requirement is merited. The Agency believes this requirement is appropriate when the identity of the product itself changes; the only way of evaluating the potential effects of these changes on the efficacy and toxicity of a new product formulation is to retest it. The Agency considered whether it was necessary to explicitly provide the flexibility to waive this requirement under extraordinary circumstances (*i.e.,* a SONS event). However, OSCs already have broad authority to use agents in areas impacted or threatened by a release or discharge, whether the agent is identified or not on the Schedule, to prevent or substantially reduce an immediate threat to human life. In addition, the Agency considered whether there is a chemical concentration threshold that could accommodate minor adjustments to a product. For example, a producer may make a slight variation in a product formula to account for a food grade or technical grade chemical component. Such a threshold might be that if concentration changes vary by no more than 1%, no retesting is necessary. However, the Agency has no basis for such a threshold and requests comment on this approach with appropriate technical details. Because of this, and the concern for the potential impact reformulated products may have on the environment, the Agency is proposing to require retesting whenever the chemical components or concentrations of a product change. However, the Agency is requesting comments on whether it should require the retesting of all products that have a change in composition, even if the only change is of chemical components that may considered inert.

Transitioning Listed Products from the Current Schedule to the New Schedule. The Agency believes it important that products on the current Schedule continue to be available during the transition period to a new Schedule that reflects the amended requirements. During this transition period, all products on the current Schedule will remain conditionally listed and the Agency will rely on it for planning and response activities. Because of the proposed revisions to test protocols and listing criteria, and because of the additional test requirements, the Agency proposes that all products currently on the Schedule be retested, and that the new data and information be submitted to the Agency for reevaluation of the current listings within 24 months of the effective date of a final rule. The Agency believes this provides adequate time for submitters to prepare and submit new packages to EPA and for the Agency to review and make decisions on these products. For a product to be transitioned from the current Schedule to the new Schedule, manufacturers would be required to submit a new, complete package according to the amended test and listing criteria, and EPA would need to make a favorable finding to list the product on the new Schedule, either as currently listed or with modifications. Products on the current Schedule for which a new submission is not received, or that upon review of their submissions do not meet the revised listing criteria, would be removed from the Schedule at the end of the 24-month transition period. While the Agency is reviewing the new submission packages, planning and response authorities are encouraged to consider those products that based on existing data would meet the revised listing criteria. While the existing data may be limited and likely developed with different protocols, considering the new requirements in selecting products for planning and response activities would provide an increased level of environmental protection. The Agency notes, however, that this is not a requirement, and that the proposal would allow any product on the current Schedule to be authorized for use following the current protocols, until the 24-month transition period is over. The Agency is requesting comments on the practicability of this transition process, and whether the 24-month period allows sufficient time for retesting of products on the current Schedule, and for EPA to review and make listing determinations on the submissions for the new Schedule. The Agency also requests comments on potential R&D costs of modifying existing products to meet the new requirements that could be incurred during this transition.

7. Mandatory Product Disclaimer

The current requirements provide that the listing of a product on the Schedule does not constitute approval or endorsement of the product. To avoid possible misinterpretation or misrepresentation, the Agency currently requires that any label, advertisement, or technical literature that refers to the placement of the product on the Schedule must either reproduce in its entirety EPA's written notification that it will add the product to the Schedule

currently in §300.920(a)(2) or (b)(2), or include the disclaimer currently set forth in §300.920(e). It remains the Agency's position that listing a product on the Schedule does not constitute approval or endorsement of that product, nor a recommendation of its use. The Agency continues to believe that it is important to avoid any possible misinterpretation or misrepresentation of this policy. Thus, the requirement for a disclaimer to be included on any label, advertisement, or technical literature for the product is maintained. However, the proposal removes the alternative to reproduce in its entirety EPA's written notification that it will add the product to the Schedule currently in §300.920(a)(2) or (b)(2). The Agency believes it will be able to update the Schedule list within a reasonable timeframe given the advances in information technology, and thus the option of producing the EPA letter of notification for a product listing should no longer be necessary. The Agency is proposing to modify the disclaimer language as follows:

[PRODUCT NAME] is listed on the National Contingency Plan (NCP) Product Schedule. This listing does NOT mean that EPA approves, recommends, licenses, or certifies the use of [PRODUCT NAME] on an oil discharge. This listing means only that data have been submitted to EPA as required by Subpart J of the NCP. Only a Federal On-Scene Coordinator (OSC) may authorize use of this product according to the NCP.

The proposed revisions set forth in §300.965 are intended to clarify that the use of these products is conditional to OSC authorization following the requirements set forth under the NCP regulations. The disclaimer language must continue to be conspicuously displayed in its entirety, and must be fully reproduced on all product literatures, labels, and electronic media, including Web site pages. As discussed in the next section, a product can be removed from the Schedule if the submitter does not comply with the disclaimer requirements, or makes any improper attempt to demonstrate the approval or endorsement of a product. The Agency requests comments on the proposed revisions.

8. Removal of a Product From the Schedule

Products that are not properly used in the field may cause harm to human health and the environment, and may constitute violations of the CWA, and other federal, state, or local laws. Misleading, inaccurate, or incorrect statements within a product submittal package or within language that refers to the listing of a product on the Schedule may result in their improper or incorrect use. Falsification of federal documents, unsupported toxicity or efficacy claims, submission of incorrect product composition or use information, or withholding technical product data are some examples of these acts. To minimize potential misuse of listed products, the Agency believes it is appropriate to further clarify the criteria for the removal of a product from the Schedule. In §300.970 the proposal specifically includes, but does not limit, as causes for removal from the Schedule: Any misleading, inaccurate, or incorrect statements within the product submission to EPA or to any person or private or public entity regarding the composition or use of the product to remove or control oil discharges, including on labels, advertisements, or technical literature; any alterations to the chemical components, concentrations, or use conditions of the product without proper notification to EPA as required by §300.955(e); the failure to print the disclaimer provided in §300.965 on all labels, advertisements, or technical literature; or any new or previously unknown relevant information concerning the impacts or potential impacts of the product to human health or the environment. It also establishes a process for removal if the Agency obtains evidence of cause for removal. EPA would notify the submitter in writing, at the address of record, of its reasons for removal of the product from the Schedule. The proposal would allow for an appeals process similar to the one set forth for listing determinations. Appeals must be received within 30 days of receipt of EPA's removal notification and must contain a clear and concise statement with supporting facts and technical analysis demonstrating why the submitter believes EPA's decision was incorrect. Written notification from the Administrator will be sent to the submitter within 60 days of any appeal, or within 60 days of receipt of any requested additional information. However, if no appeal is received within the 30 days of receipt of EPA's removal notification, the product would be delisted without further notice. The Agency requests comments on the proposed clarification of criteria for removal of products from the Schedule, and on the associated appeals process.

9. Appendix C to Part 300

The Agency is proposing to revise the current Appendix C—*Swirling Flask Dispersant Efficacy Test, Revised Standard Dispersant Toxicity Test, and Bioremediation Agent Efficacy Test* as Appendix C—*Requirements for Product Testing Protocols and Summary Test Data: Dispersant Baffled Flask Efficacy and Toxicity Tests; Standard Acute Toxicity Test for Bioremediation Agents, Surface Washing Agents, Herding Agents, and Solidifiers; and Bioremediation Agent Efficacy Test.* The proposed revisions reflect the proposed new and revised testing protocols for listing agents on the Schedule. The details of the technical changes and rationale are discussed for each agent in section V.C.4 of this preamble—Data and Information Requirements for Product Schedule Listing. The appendix reflects the proposed technical considerations and listing requirements. The Agency is requesting comment on the protocols and their technical rationale. The Agency is also requesting comment on its organization and ease of use.

10. Appendix E to Part 300

The 1994 revisions to the NCP established Appendix E, *Oil Spill Response,* which separates the oil spill response requirements of the NCP from the hazardous substance release requirements (59 FR 47414). The purpose of creating this appendix was to compile general oil discharge response requirements into one document to aid responsible parties and responders with their duties under the national response system. The Agency's intent was to provide guidance, and not to alter in any way the meaning or policy stated in other sections or subparts of the NCP. However, some minor variations between the Appendix E provisions and the analogous provisions of the NCP rule language were necessary to ensure that the appendix addressed only oil discharges; hazardous substance releases continue to be addressed in the NCP rule but are not addressed in Appendix E.

The Agency proposes to remove Appendix E. While having all of the information pertaining to oil discharges compiled in one location may offer useful guidance, it is not necessary that this guidance be codified as a regulatory appendix to the NCP. The provisions in the appendix do not alter any NCP requirement; however, they do contain variations from the main NCP requirements in order to have the appendix be relevant solely to oil discharges. While EPA carefully reviewed the appendix and the relevant sections of the NCP at the time it was established to ensure consistency in policy, instructions, guidance and requirements, there were intentional minor variations. These may result in having responses to oil discharges

subject to two sets of what appear to be potentially conflicting requirements, causing unnecessary confusion.

Because all requirements in Appendix E are part of the NCP, any revisions to the NCP necessitate revisions to this appendix. This adds burden not only for the Agency in revising and ensuring consistency, but also for the regulated community in reviewing redundant and duplicative requirements. While it may be a useful tool to have all of the oil discharge specific requirements in one location, the Agency has reconsidered its position and believes that this is more appropriately achieved through a separate guidance document, one that does not codify duplicative regulatory requirements. The Agency requests comments on the proposal to remove Appendix E from the NCP regulation, and whether it should continue to offer similar guidance through other formats.

VI. Summary of Proposed Rule Provisions

This section summarizes the proposed changes to 40 CFR parts 110 and 300. Subpart J has been renumbered to include new, consolidated, and revised sections. Some of the rule sections have been retained, removed, or moved in their entirety. The Table below provides an overview of the existing rule and proposed rule citations for a quick reference of the proposed changes.

Section 110.4, Dispersants, would be revised to link the rule with the new and amended regulatory definitions for Subpart J product categories.

Section 300.5, Definitions, would be revised to include new, amended, and deleted definitions.

Subpart J—Use of Dispersants, Chemicals, and Bioremediations Agents, heading would be revised to reflect new and amended regulatory definitions for product categories.

Section 300.900, General, paragraphs (a) and (c) would be revised to reflect new and amended regulatory definitions for product categories.

Section 300.905, NCP Product Schedule, would be removed.

Section 300.910, Authorization of Use, would be revised and new paragraphs added to clarify planning and preauthorization responsibilities.

- Paragraph (a) would be revised to clarify the requirements, including process, responsibilities, and factors to consider for preauthorization; and add new requirements for preauthorization plan review, concurrence, and withdrawal procedures.
- Paragraph (b) would be revised to clarify the requirements for using a listed product not addressed by a preauthorization plan and add new parameters for use considerations.
- Paragraph (c) would be revised to clarify the requirements for authorizing the use of burning agents by an OSC for authorized in-situ burns.
- Paragraph (d) would be revised to clarify the exception requirements and add specific time frames for notification of continued agent use.
- Paragraph (e) would be revised to expand the prohibition to include nonylphenol (NP) or nonylphenol ethoxylates (NPEs) as components of chemical or biological agents.
- Paragraph (f) would be revised to add new regulatory requirements for agent storage and use. Existing paragraph (f) requirements would be moved to new paragraph (g), Supplemental Testing, Monitoring, and Information.
- New paragraph (g), Supplemental Testing, Monitoring, and Information, would revise the regulatory text to clarify the requirements for supplemental testing, monitoring and information.
- New paragraph (h), Recovery of Oil and Agents from the Environment, would add regulatory requirements for recovery of oil and agents from the environment.
- New paragraph (i), Reporting of Agent Use, would add regulatory requirements for notification of agent use on an oil discharge.

New section 300.913, Monitoring the Use of Dispersants, would add regulatory requirements for monitoring certain prolonged surface and subsurface use of dispersants.

Section 300.915, Data Requirements, would be revised to consolidate general submission requirements applicable to all product categories. The section would be restructured to include new testing and listing requirements for specific product categories.

- Paragraph (a), Dispersants, would be revised to consolidate general testing and listing requirements from existing paragraphs (a), (b), (d), and (f). The paragraph would include revisions and new requirements for the identification of and testing for all product categories designated for listing. Existing paragraph (a) requirements specific to dispersants would be moved to new section 300.915(b), Dispersant Testing and Listing Requirements. The paragraph would also be revised to add new toxicity and efficacy testing requirements, limitations for use, and new criteria for listing a dispersant to the Schedule. Existing paragraph (b) would be moved to new paragraph (c), Surface Washing Agent Testing and Listing Requirements.
- Paragraph (b), Surface Washing Agents, would be moved to new paragraph (c), Surface Washing Agent Testing and Listing Requirements. The paragraph would be revised to add new toxicity and efficacy testing requirements, limitations for use, and new criteria for listing a surface washing agent to the Schedule. Existing paragraph (c), Surface Collecting Agents, would be deleted.
- Paragraph (d), Bioremediation Agents, would be revised to add new toxicity and efficacy testing requirements, limitations for use, and new criteria for listing a bioremediation agent to the Schedule. Existing paragraphs (d)(9) and (10) were moved to new paragraph (a), General Product Information.
- Paragraph (e) would be revised to add new regulatory requirements for submission and listing of a solidifier. Existing paragraph (e), Burning Agents, would be deleted.
- Paragraph (f) would be revised to add new toxicity testing requirements, limitations of use, and criteria for listing a herding agent on the Schedule. Existing paragraph (f), Miscellaneous Oil Spill Control Agents, would be deleted.
- Paragraph (g), Sorbents, would be revised to add new exceptions for listing a sorbent to the Schedule.

Section 300.920, Addition of Products to Schedule, would be moved to new section 300.955, Addition of a Product to the Schedule.

- Paragraph (a) would be revised to include submission instructions for all product categories. Existing paragraphs (a)(1) through (3) regulatory text specific to dispersant applications would be moved to new §§300.915(b) and 300.955(c) and (d).
- Paragraph (b) would be revised to add new regulatory text for preparation of complete submission packages. Existing paragraph (b) regulatory text would be moved to new §300.955(c) and (d).
- Paragraph (c) would be revised to add regulatory text for EPA's review of submission packages and decision criteria for listing. Existing paragraph (c) would be moved to new §300.950, Submission of Confidential Business Information (CBI).
- Paragraph (d) would be revised to add regulatory text for requesting a listing decision review. Existing paragraph (d) would be moved to new §300.955(e), Changes to a Listed Product.
- Paragraph (e) would be revised to add new regulatory text for notification of changes to a listed product. Existing paragraph (e) would be moved to new

§300.965, Mandatory Product Disclaimer.

• New paragraph (f) would add new regulatory requirements for transitioning products on the current Schedule to the new Schedule.

New §300.950, Confidential Business Information (CBI), would revise and clarify the allowable CBI claims in a submission package.

New §300.965, Mandatory Product Disclaimer, would clarify the regulatory text for including a disclaimer statement on all product labels and literature.

New §300.970, Removal of a Product from the Schedule, would add basis for removal of products from the Schedule, EPA notification of decision, and appeals process.

Revised Appendix C to Part 300—Requirements for Product Testing Protocols and Summary Test Data: Dispersant Baffled Flask Efficacy and Toxicity Tests; Standard Acute Toxicity Test for Bioremediation Agents, Surface Washing Agents, Herding Agents, and Solidifiers; and Bioremediation Agent Efficacy Test.

Removed Appendix E to Part 300—Oil Spill Response.

40 CFR PART 100 DISCHARGE OF OIL—DISTRIBUTION TABLE

Current citation	Proposed rule citation
110.4 Dispersants	110.4 Chemical and Biological Agents.

40 CFR PART 300—NATIONAL OIL AND HAZARDOUS SUBSTANCES POLLUTION CONTINGENCY PLAN—DISTRIBUTION TABLE

Current citations	Proposed rule citations
§300.5 Definitions	§300.5 Definitions.
Subpart J—Use of Dispersants and Other Chemicals	Subpart J—Use of Dispersants, and Other Chemical and Biological Agents.
§300.900 General	§300.900 General.
§300.900(a)	§300.900(a)
§300.900(c)	§300.900(c)
§300.905 NCP Product Schedule	Deleted.
§300.910 Authorization of Use	§300.910 Authorization for Agent Use.
§300.910(a)	§300.910(a) Use of Agents Identified on the Schedule on Oil Discharges Addressed by a Preauthorization Plan.
§300.910(b)	§300.910(b) Use of Agents Identified on the Schedule on Oil Discharges Not Addressed by a Preauthorization Plan.
§300.910(c)	§300.910(c) Burning Agents.
§300.910(d)	§300.910(d) Exceptions.
§300.910(e)	§300.910(e) Prohibited Agents.
§300.910(f)	§300.910(g) Supplemental Testing, Monitoring, and Information.
	§300.910(f) Storage and Use of Agents.
	§300.910(h) Recovery of Oil and Agents from the Environment.
	§300.910(i) Reporting of Agent Use.
	§300.913 Monitoring the Use of Dispersants.
	§300.913(a).
	§300.913(b).
	§300.913(c).
	§300.913(d).
	§300.913(e).
	§300.913(f).
§300.915 Data Requirements	§300.915 Data and Information Requirements for Product Schedule Listing.
§300.915(a)(1) through (12) Dispersants	§300.915(a)(1) through (21) General Product Information.
	§300.915(b) Dispersant Testing and Listing Requirements.
§300.915(b) Surface Washing Agents	§300.915(a)(1) through (21) General Product Information.
	§300.915(c) Surface Washing Agent Testing and Listing Requirements.
§300.915(c) Surface Collecting Agents	Deleted.
$300.915(d) Bioremediation Agents	§300.915(a)(1) through (21) General Product Information.
	§300.915(d) Bioremediation Agent Testing and Listing Requirements.
§300.915(e) Burning Agents	Deleted.
§300.915(f) Miscellaneous Oil Spill Control Agents	Deleted.
§300.915(g) Sorbents	§300.915(g) Sorbent Listing Requirements.
§300.915(h) Mixed Products	Deleted.
	§300.915(e) Solidifier Testing and Listing Requirements, including §300.915(a)(1) through (21) General Product Information.
	§300.915(f) Herding Agent Testing and Listing Requirements, including §300.915(a)(1) through (21) General Product Information.
§300.920 Addition of Products to Schedule	§300.955 Addition of a Product to the Schedule.
§300.920(a)(1) Dispersants	§300.955(a) Submission.
	§300.915(b) Dispersant Testing and Listing Requirements.
§300.920(a)(2)	§300.955(c) EPA Review.
§300.920(a)(3)	§300.955(d) Request for Review of Decision.
	§300.955(b) Package Contents.
§300.920(b)(1) Surface Washing Agents, Surface Collecting Agents, Bioremediation Agents, and Miscellaneous Oil Spill Control Agents.	§300.955(a) Submission.

40 CFR PART 300—NATIONAL OIL AND HAZARDOUS SUBSTANCES POLLUTION CONTINGENCY PLAN—DISTRIBUTION TABLE—Continued

Current citations	Proposed rule citations
§300.920(b)(2)	§300.955(c) EPA Review.
§300.920(c)	§300.950 Submission of Confidential Business Information (CBI).
§300.920(d)	§300.955(e) Changes to a Listed Product.
	§300.955(f) Transitioning Listed Products from the Current Schedule to the New Schedule.
§300.920(e)	§300.965 Mandatory Product Disclaimer.
	§300.970 Removal of a Product from the Schedule.

VII. Statutory and Executive Order Reviews

A. Executive Order 12866: Regulatory Planning and Review and Executive Order 13563: Improving Regulation and Regulatory Review

Under Executive Order 12866 (58 FR 51735, October 4, 1993), this action is a ''significant regulatory action.'' This action raises novel legal or policy issues arising out of legal mandates, the President's priorities, or the principles set forth in the Executive Order. Accordingly, EPA submitted this action to the Office of Management and Budget (OMB) for review under Executive Orders 12866 and 13563 (76 FR 3821, January 21, 2011) and any changes made in response to OMB recommendations have been documented in the docket for this action.

In addition, EPA prepared an analysis of the potential costs and benefits associated with this action. This analysis is contained in the *Regulatory Impact Analysis for Proposed Revisions to the National Oil and Hazardous Substances Pollution Contingency Plan Regulations.* A copy of the analysis is available in the docket for this action and the analysis is briefly summarized here.

The Agency expects the proposed rule would not cause a significant economic impact on a substantial number of small businesses. The total incremental costs are estimated as $667,610 to $694,343 annually at 3% and 7% ($2011) annualization rates, respectively. The benefits of this action are assessed qualitatively and include, for example, greater clarity of regulatory requirements, as well as less toxic products. The resulting ratio of compliance cost to annual sales revenue for the proposed rule for existing and new product manufacturers would be less than one percent in most instances.

B. Paperwork Reduction Act

The information collection requirements in this proposed rule have been submitted for approval to the Office of Management and Budget (OMB) under the *Paperwork Reduction Act,* 44 U.S.C. 3501 *et seq.* The Information Collection Request (ICR) document prepared by EPA has been assigned EPA ICR number 1664.10. You can find a copy of the ICR in the docket for this rule, and it is briefly summarized here. The ICR supporting this proposed rule is largely self-implementing. The information collection is to ensure that: (1) The Agency has the necessary information to make Schedule listing determinations specific to the different product categories; (2) product use by owners or operators of facilities or vessels, or response personnel, in response to oil discharges is performed in accordance with all applicable requirements; and (3) the Agency can verify compliance as needed. Section 300.950 of the NCP contains provisions for confidentiality.

EPA has carefully considered the burden imposed upon the regulated community by the proposed regulations. EPA believes that the activities required are necessary and, to the extent possible, has attempted to minimize the burden imposed. The minimum requirements specified in the proposed rule are intended to ensure that, when needed, products are used properly in the field to respond to an oil discharge in a manner protective of human health and the environment.

Respondents/affected entities: Manufacturers of oil spill mitigating agents (products)/Oil spill responsible parties.

Respondent's obligation to respond: Mandatory (40 CFR Part 300, Subpart J).

Estimated number of respondents: 65.

Frequency of response: Initially.

Total estimated burden: 721 hours (per year). Burden is defined at 5 CFR 1320.3(b).

Total estimated cost: $584,504 (per year), includes $575,400 operation & maintenance costs.

An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number. The OMB control numbers for EPA's regulations in 40 CFR are listed in 40 CFR part 9.

Submit your comments on the Agency's need for this information, the accuracy of the provided burden estimates, and any suggested methods for minimizing respondent burden to the EPA using the docket identified at the beginning of this rule. You may also send your ICR-related comments to OMB's Office of Information and Regulatory Affairs via email to *oria_submisssions@omb.eop.gov*, Attention: Desk Officer for EPA. Since OMB is required to make a decision concerning the ICR between 30 and 60 days after receipt, OMB must receive comments no later than February 23, 2015. The EPA will respond to any ICR-related comments in the final rule.

C. Regulatory Flexibility Act (RFA)

The Regulatory Flexibility Act (RFA) generally requires an agency to prepare a regulatory flexibility analysis of any rule subject to notice and comment rulemaking requirements under the Administrative Procedure Act or any other statute unless the agency certifies that the rule will not have a significant economic impact on a substantial number of small entities. Small entities include small businesses, small organizations, and small governmental jurisdictions.

For purposes of assessing the impacts of the proposed rule on small entities, small entity is defined as: (1) As defined by the Small Business Administration's (SBA) regulations at 13 CFR 121.201; (2) a small governmental jurisdiction that is a government of a city, county, town, school district or special district with a population of less than 50,000; or (3) a small organization that is any not-for-profit enterprise that is independently owned and operated and is not dominant in its field.

After considering the economic impacts of this proposed rule on small entities, I certify that this action will not have a significant economic impact on a substantial number of small entities. The small entities directly regulated by this proposed rule are product

manufacturers and laboratories and state and local governments that are involved in product development, testing and use for oil discharge response. EPA conducted a small business analysis consistent with the Agency's 2006 small business guidance. The Agency's analysis indicates that about 95 percent of manufacturers are small businesses. In conducting the small business analysis, the agency compared the incremental annualized compliance costs to the annual sales revenue for the smallest entities. The results indicate that 90 percent of the smallest manufacturers have annualized compliance costs that are less than 1 percent of their annual sales revenue, and that no manufacturers are expected to have incremental costs that exceed 3 percent of annual sales. The small business analysis is available for review in the Regulatory Impact Analysis (RIA). Therefore, we have determined that this proposed rule does not have a significant impact on a substantial number of small entities.

Nonetheless, EPA has tried to reduce the impact of this rule on small entities in developing the regulatory requirements that balance the costs and burden, while addressing the environmental protection concerns. We continue to be interested in the potential impacts of this proposed rule on small business entities and welcome comments on the issues related to such impacts.

D. Unfunded Mandates Reform Act

This action contains no Federal mandates under the provisions of Title II of the Unfunded Mandates Reform Act of 1995 (UMRA), 2 U.S.C. 1531–1538 for State, local, or tribal governments or the private sector. This proposed rule imposes no new enforceable duty on any state, local or tribal governments or the private sector. UMRA excludes from the definition of "Federal intergovernmental mandate" and "Federal private sector mandate" duties that arise from conditions of Federal assistance. UMRA generally excludes from the definition of "Federal intergovernmental mandate" duties that arise from participation in a voluntary Federal program. UMRA also excludes from the definition of "Federal private sector mandate" duties that arise from participation in a voluntary Federal program. Since the decision on whether to request that a product be included on the Schedule is voluntary, the Agency has determined that this proposed rule is not subject to the requirements of sections 202 or 205 of UMRA. This action is also not subject to the requirements of section 203 of UMRA because it contains no regulatory requirements that might significantly or uniquely affect small governments.

E. Executive Order 13132: Federalism

This action does not have federalism implications. It will not have substantial direct effects on the States, on the relationship between the national government and the States, or on the distribution of power and responsibilities among the various levels of government, as specified in Executive Order 13132. This proposal does not alter the general procedures already defined in the NCP of how state, local, and federal agencies cooperate in responding to oil spills and how to consult with the OSC and RRT when considering the use of products on the Schedule. Thus, Executive Order 13132 does not apply to this action. In the spirit of Executive Order 13132, and consistent with EPA policy to promote communications between EPA and State and local governments, EPA specifically solicits comment on this proposed action from State and local officials.

F. Executive Order 13175: Consultation and Coordination With Indian Tribal Governments

Subject to Executive Order 13175 (65 FR 67249, November 9, 2000), EPA may not issue a regulation that has tribal implications, that imposes substantial direct compliance costs, and that is not required by statute, unless the Federal government provides the funds necessary to pay the direct compliance costs incurred by tribal governments, or EPA consults with tribal officials early in the process of developing the proposed regulation and develops a tribal summary impact statement.

EPA has concluded that this action may have tribal implications. However, it will neither impose substantial direct compliance costs on tribal governments, nor preempt Tribal law, similarly to the effect on states. EPA will be consulting with tribal officials as it develops this regulation to permit them to have meaningful and timely input into its development. Consultation will include conference calls, webinars, and meetings with interested tribal representatives to ensure that their concerns are addressed before the rule is finalized. In the spirit of Executive Order 13175 and consistent with EPA policy to promote communications between EPA and tribal governments, EPA specifically solicits comment on this proposed rule from tribal officials.

G. Executive Order 13045: Protection of Children From Environmental Health Risks and Safety Risks

EPA interprets EO 13045 (62 FR 19885, April 23, 1997) as applying only to those regulatory actions that concern health or safety risks, such that the analysis required under section 5–501 of the EO has the potential to influence the regulation. This action is not subject to EO 13045 because it does not establish environmental standards, such as limits on levels of pollutants in the water, that are intended to directly mitigate health or safety risks.

H. Executive Order 13211: Actions That Significantly Affect Energy Supply, Distribution or Use

This proposed rule is not a "significant energy action" as defined in Executive Order 13211, "Actions Concerning Regulations that Significantly Affect Energy Supply, Distribution, or Use" (66 FR 28355, May 22, 2001) because it is not likely to have a significant adverse effect on the supply, distribution, or use of energy. The proposal focuses on maintaining the availability of certain response tools that can be considered when responding to oil discharges, minimizing any potential adverse impacts from their use, and resulting in greater overall environmental protection. Thus, the proposed rule would not cause reductions in the supply or production of oil, fuel, coal, or electricity; nor would it result in increased energy prices, increased cost of energy distribution, or an increased dependence on foreign supplies of energy.

I. National Technology Transfer and Advancement Act

Section 12(d) of the National Technology Transfer and Advancement Act of 1995 ("NTTAA"), Public Law 104–113, 12(d) (15 U.S.C. 272 note) directs EPA to use voluntary consensus standards in its regulatory activities unless to do so would be inconsistent with applicable law or otherwise impractical. Voluntary consensus standards are technical standards (*e.g.,* materials specifications, test methods, sampling procedures, and business practices) that are developed or adopted by voluntary consensus standards bodies. NTTAA directs EPA to provide Congress, through OMB, explanations when the Agency decides not to use available and applicable voluntary consensus standards.

This rulemaking involves technical standards. The Agency conducted a search to identify potentially applicable

voluntary consensus standards for efficacy testing. However, we identified no such standards. Therefore, EPA developed the Baffled Flask Efficacy Test and the Bioremediation Efficacy Test required in Appendix C of this proposed rule. Voluntary consensus standards developed by ASTM are recommended for several product property data points, such as pH, flash point and pour point. The product toxicity testing relies on existing protocols that are universally accepted. EPA welcomes comments on this aspect of the proposed rulemaking and, specifically, invites the public to identify potentially-applicable voluntary consensus standards for product efficacy and to explain why such standards should be used in this regulation.

J. Executive Order 12898: Environmental Justice

Executive Order 12898, "Federal Actions to Address Environmental Justice in Minority Populations and Low-Income Populations" (59 FR 7629 (February 11, 1994)) establishes federal executive policy on environmental justice. Its main provision directs federal agencies, to the greatest extent practicable and permitted by law, to make environmental justice part of their mission by identifying and addressing, as appropriate, disproportionately high and adverse human health or environmental effects of their programs, policies, and activities on minority populations and low-income populations in the United States.

EPA is committed to addressing environmental justice concerns and has assumed a leadership role in environmental justice initiatives to enhance environmental quality for all citizens of the United States. The Agency's goals are to ensure that no segment of the population, regardless of race, color, national origin, income, or net worth bears disproportionately high and adverse human health and environmental impacts as a result of EPA's policies, programs, and activities. In response to Executive Order 12898, EPA's Office of Solid Waste and Emergency Response (OSWER) formed an Environmental Justice Task Force to analyze the array of environmental justice issues specific to waste programs and to develop an overall strategy to identify and address these issues (OSWER Directive No. 9200.3–17). To address this goal, EPA conducted a qualitative analysis of the environmental justice issues under this proposed rule.

Under the NCP, RRTs and ACs are required to address, as part of their planning activities, the desirability of using appropriate chemical or biological agents, or other spill mitigating devices. In addition, the OSC, under authority granted by the NCP, must respond to an oil spill in a diligent and effective manner to protect human health and the environment. If chemical or biological agents are needed, the OSC must coordinate with the RRT and ACs before their use is authorized. In all cases, the RRT, ACs and OSC will address a broad array of oil spill response and mitigation issues, including the potential for environmental justice concerns. Historically, EPA has not found any evidence that the use of chemical or biological agents on the Schedule on oil spills in the U.S. has had any disproportionate effect on any environmental justice communities. However, EPA will continue to monitor the implementation of the rule to ensure the planned or actual use of chemical or biological agents has no disproportionate effect on any EJ communities.

EPA has determined that this proposed rule will not have disproportionately high and adverse human health or environmental effects on minority or low-income populations because it increases the level of environmental protection for all affected populations without having any disproportionately high and adverse human health or environmental effects on any population, including any minority or low-income population. Specifically, the proposed rule provides additional safeguards before any product can be listed on the Schedule, as well as allows OSCs and RRTs to request additional information to ensure that the use of any chemical or biological agent, or any other spill mitigating substance, in responding to oil discharges is protective of human health and the environment. This proposed rule is consistent with EPA's Environmental Justice Strategy and the OSWER Environmental Justice Action Agenda.

List of Subjects

40 CFR Part 110

Environmental protection, Oil pollution, and Reporting and recordkeeping requirements.

40 CFR Part 300

Air pollution control, Area contingency planning, Bioremediation, Chemicals, Dispersants, Environmental protection, Hazardous materials, Hazardous substances, Intergovernmental relations, Natural resources, Oil spills, Oil spill mitigating devices, Regional response teams, Sorbents, and Surface washing agents.

Dated: January 9, 2015.

Gina McCarthy,

Administrator.

For the reasons set out in the preamble, the Environmental Protection Agency proposes to amend 40 CFR parts 110 and 300 to read as follows:

PART 110—DISCHARGE OF OIL

* 1. The authority citation for part 110 continues to read as follows:

Authority: 33 U.S.C. 1321(b)(3) and (b)(4) and 1361(a); E.O. 11735, 38 FR 21243, 3 CFR parts 1971–1975 Comp., p. 793.

* 2. Revise §110.4 and its section heading to read as follows:

§110.4 Chemical or biological agents.

Addition of any chemical or biological agent, as defined in §300.5, to oil to be discharged that would circumvent the provisions of this part is prohibited.

PART 300—NATIONAL OIL AND HAZARDOUS SUBSTANCES POLLUTION CONTINGENCY PLAN

* 3. The authority citation for part 300 continues to read as follows:

Authority: 33 U.S.C. 1321(c)(2); 42 U.S.C. 9601–9657; E.O. 13626, 77 FR 56749, 3 CFR, 2013 Comp., p. 306; E.O. 12777, 56 FR 54757, 3 CFR, 1991 Comp., p. 351; E.O. 12580, 52 FR 2923, 3 CFR, 1987 Comp., p. 193.

Subpart A—Introduction

* 4. Amend §300.5 by:

* a. Adding in alphabetical order the definitions of "Bioaccumulation", "Bioconcentration", "Biodegradation", "Biological agents", and "Bioremediation";

* b. Revising the definitions of "Bioremediation agents", "Burning agents", "Chemical agents", and "Dispersants";

* c. Adding in alphabetical order the definition of "Herding agents";

* d. Removing the definitions for "Miscellaneous oil spill control agent";

* e. Adding in alphabetical order the definition of "Products";

* f. Revising the definition of "Sinking agents";

* g. Adding in alphabetical order the definition of "Solidifiers"; and

* h. Revising the definition of "Sorbents"

* i. Removing the definition for "Surface collecting agents".

* j. Revising the definition title "Surface washing agent" and its definition;

The revisions and additions read as follows:

§300.5 Definitions.

* * * * *

Bioaccumulation is the process of accumulation of chemicals in the tissue of organisms through any route, including respiration, ingestion, or direct contact with the ambient or contaminated medium.

Bioconcentration is the accumulation of chemicals in the tissues of organisms from water alone.

Biodegradation is a process by which microorganisms metabolically decompose contaminants into biomass and simpler compounds such as carbon dioxide, water, and innocuous end products.

Biological agents are microorganisms (typically bacteria, fungi, or algae) or biological catalysts, such as enzymes, able to enhance the biodegradation of a contaminated environment.

Bioremediation is the process of enhancing the ability of microorganisms to convert contaminants into biomass and innocuous end products by the addition of materials into a contaminated environment to accelerate the natural biodegradation process.

Bioremediation agents are biological agents and/or nutrient additives deliberately introduced into a contaminated environment to increase the rate of biodegradation and mitigate any deleterious effects caused by the contaminant constituents. Bioremediation agents include microorganisms, enzymes, and nutrient additives such as fertilizers containing bioavailable forms of nitrogen, phosphorus and potassium.

Burning agents are additives that improve the combustibility of the materials to which they are applied through physical or chemical means.

* * * * *

Chemical agents are elements, compounds, or mixtures designed to facilitate the removal of oil from a contaminated environment and mitigate any deleterious effects. Chemical agent categories include burning agents, dispersants, herding agents, sinking agents, solidifiers, surface washing agents, and bioremediation agents that consist of nutrient additives.

* * * * *

Dispersants are typically mixtures of solvents, surfactants, and additives that promote the formation of small droplets of oil in the water column by reducing the oil-water interfacial tension.

* * * * *

Herding agents are substances that are used to control the spreading of the oil across the water surface.

* * * * *

Products are chemical or biological agents manufactured using a unique composition or formulation.

* * * * *

Sinking agents are substances deliberately introduced into an oil discharge for the purpose of submerging the oil to the bottom of a water body.

* * * * *

Solidifiers are substances that through a chemical reaction cause oil to become a cohesive mass, preventing oil from dissolving or dispersing into the water column, and which are collected and recovered from the environment.

Sorbents are inert, insoluble substances that readily absorb and/or adsorb oil or hazardous substances, and that are not combined with or act as a chemical or biological agent. Sorbents are generally collected and recovered from the environment. Sorbents may be used in their natural bulk form, or as manufactured products in particulate form, sheets, rolls, pillows, or booms. Sorbents consist of:

(1) Natural organic substances (*e.g.,* feathers, cork, peat moss, and cellulose fibers such as bagasse, corncobs, and straw);

(2) Inorganic/mineral compounds (*e.g.,* volcanic ash, perlite, vermiculite, zeolite, clay); and

(3) Synthetic compounds (*e.g.,* polypropylene, polyethylene, polyurethane, polyester).

* * * * *

Surface washing agents are substances that separate oil from solid surfaces, such as beaches, rocks, metals, or concrete, through a detergency mechanism that lifts and floats oil for collection and recovery from the environment with minimal dissolution, dispersion, or transfer of oil into the water column.

* * * * *

Subpart J—Use of Dispersants, and Other Chemical and Biological Agents

* 5. Revise the heading of Subpart J as set out above.

* 6. Amend §300.900 by revising paragraphs (a) and (c), and by adding paragraph (d) to read as follows:

§300.900 General.

(a) Section 311(d)(2)(G) of the Clean Water Act (CWA) requires EPA to prepare a schedule identifying dispersants, other chemicals, other spill mitigating devices and substances if any, that may be used in carrying out the NCP; and the waters and quantities in which they may be used. This subpart establishes a schedule identifying chemical and biological agents, and procedures that, when taken together, identify the waters and quantities in which such dispersants, other chemicals, or other spill mitigating devices and substances may be used.

* * * * *

(c) This subpart applies to the use of chemical and biological agents as defined in Subpart A of this part, or other substances that may be used to remove, control, or otherwise mitigate oil discharges.

(d) [Reserved]

§300.905 [Removed]

* 7. Remove §300.905.

* 8. Revise §300.910 and the section heading to read as follows:

§300.910 Authorization for agent use.

Use of chemical or biological agents in response to oil discharges to waters of the U.S. or adjoining shorelines must be authorized by the OSC in accordance with the provisions of this section:

(a) *Use of Agents Identified on the Schedule on Oil Discharges Addressed by a Preauthorization Plan.* RRTs and Area Committees shall address in a preauthorization plan, as part of their planning activities, whether the use of chemical and biological agents listed on the Schedule on certain oil discharges is appropriate. RRTs and Area Committees shall, as appropriate, include applicable approved preauthorization plans in RCPs and ACPs. When a preauthorization plan is approved in advance for the use of certain agents under specified discharge situations, then the OSC may authorize the use of agents on the Schedule for their intended purpose without obtaining the incident specific concurrences described in paragraph (b) of this section.

(1) *Preauthorization Plan Development.* For discharge situations identified where such agents may be used, the preauthorization plan must specify limits for the quantities and the duration of use, and use parameters for water depth, distance to shoreline, and proximity to populated areas. In meeting the provisions of this paragraph, preauthorization plans should document how regional factors are addressed including likely sources and types of oil that might be discharged, various discharge scenarios, the existence and location of environmentally sensitive resources or restricted areas that might be impacted by discharged oil, and logistical factors including inventory, storage locations and manufacturing capability of available agents, availability of equipment needed for agent use, availability of adequately trained

operators, and means to monitor agent use in the environment.

(2) *Preauthorization Plan Approval.* The EPA representative to the RRT, the Department of Commerce and the Department of the Interior natural resource trustees and, as appropriate the RRT representative from the state(s) with jurisdiction over waters and adjoining shorelines within the preauthorization plan area shall review and either approve, approve with modification, or disapprove the preauthorization plans developed by the RRT and/or the Area Committees. Withdrawal of concurrence means the preauthorization plan becomes invalid and the authorization of use for chemical or biological agents must be performed according to paragraph (b) of this section. The RRTs and Area Committees shall address the withdrawal and the RRT shall notify the NRT of the final status of the preauthorization plan within 30 days from withdrawal.

(3) *Preauthorization Plans Reviews.* The RRT and/or the Area Committees must review, and revise as needed, preauthorization plans at least every 5 years; after a major discharge or after a Spill of National Significance (SONS); to address revisions of the Schedule; to reflect new listings of threatened and/or endangered species; and to address any other change that may impact the conditions under which the use of chemical and biological agents is preauthorized. The designated EPA RRT representative, the Department of Commerce and the Department of the Interior natural resource trustees, and the RRT representative from the state(s) with jurisdiction over the waters of the area to which a preauthorization plan applies shall review and either approve, approve with modification, or disapprove any revisions to the preauthorization plans.

(b) *Use of Agents Identified on the Schedule on Oil Discharges Not Addressed by a Preauthorization Plan.* For discharge situations that are not addressed by the preauthorization plan developed pursuant to paragraph (a) of this section, the OSC may authorize the use of appropriate chemical or biological agents identified on the Schedule for their intended purpose on an oil discharge with the concurrence of the designated EPA RRT representative and, as appropriate, the concurrence of the RRT representatives from the state(s) with jurisdiction over the waters and adjoining shorelines threatened by the release or discharge, and in consultation with the Department of Commerce and the Department of the Interior natural resource trustees. In meeting the provisions of this paragraph, the OSC must consider and document the parameters for the use of agents including the quantities to be used, the duration of use, the depth of water, the distance to shoreline and proximity to populated areas, and should address factors such as environmentally sensitive resources or restricted areas that might be impacted, agent inventory and storage locations, agent manufacturing capability, availability of equipment needed for agent use, availability of adequately trained operators and appropriate means to monitor agent use in the environment.

(c) *Burning Agents.* For authorized in-situ burns, the OSC may authorize the use of burning agents.

(d) *Exception.* The OSC may authorize the use of any chemical or biological agent, whether it is identified or not on the Schedule, without obtaining the immediate concurrence of the designated EPA RRT representative and, as appropriate, the RRT representatives from the state(s) with jurisdiction over the waters and adjoining shorelines threatened by the release or discharge, when, in the judgment of the OSC, the use of the agent is necessary to prevent or substantially reduce a threat to human life. If an OSC authorizes the use of an agent pursuant to this paragraph, he or she shall immediately notify, and document the circumstances requiring and the reasons for use of the agent to the EPA RRT representative and, as appropriate, the RRT representatives from the affected state(s) and, the Department of Commerce/Department of the Interior natural resources trustees. Use of any agent beyond 48 hours under this exception shall be in accordance with paragraphs (a) or (b) of this section.

(e) *Prohibited Agents.* Notwithstanding paragraph (d) of this section, the OSC may not authorize the use of the following:

(1) Sinking agents, or any other chemical agent, biological agent, or any substance that acts as a sinking agent when mixed with oil; and

(2) Chemical or biological agents that have either nonylphenol (NP) or nonylphenol ethoxylates (NPEs) as components.

(f) *Storage and Use of Agents.* The OSC may authorize for use only products that are certified by the responsible party to have been stored under the conditions provided by the submitter under §300.915(a)(6) and whose date of use does not exceed the expiration date listed on the container's label at the time of the incident. The responsible party must provide the OSC product documentation, developed in consultation with the submitter of the product to the Schedule, prior to OSC authorization of product use affirming it has maintained its integrity, including no changes in its composition, efficacy, and toxicity. The owner or operator of a facility or vessel must ensure samples of the expired product lot are tested following the applicable testing protocols in Appendix C, and that they are representative of all storage conditions at any end user location. If testing demonstrates the expired product has maintained its integrity, the product may be used for an additional 5 years from the date of the testing described above. The responsible party, or its representative, must re-label the tested product lots and maintain test results and document all of the information under §300.915(a)(17) and (a)(18) until used. The owner or operator of a facility or vessel must ensure the testing of re-labeled products every 5 years.

(g) *Supplemental Testing, Monitoring and Information.* The RRT may require supplementary toxicity and efficacy testing, or available data or information that addresses site, area, or ecosystem specific concerns relative to the use of a product for both planning and authorization of use. During a discharge incident, the RRT may request that the OSC require a responsible party to conduct additional monitoring associated with the use of a product. Such additional monitoring data may include supplemental toxicity and efficacy testing or submission of available data or information that addresses the discharge area or ecosystem specific concerns relative to the use of a product or that aids the OSC and/or the RRT in operational decisions.

(h) *Recovery of Agents from the Environment.* Depending on factors such as the safety of response personnel and harm to the environment, and as directed by the OSC, the responsible party shall ensure that any removal action adequately contains, collects, stores and disposes of agents that are intended to be recovered from the environment.

(i) *Reporting of Agent Use.* Unless already included in the OSC report required under §300.165 of this part, within 30 days of completion of agent operations, the authorizing OSC shall provide the RRT the following information on chemical and biological agents used in response to an oil discharge: product name, quantity and concentration used, duration of use, locations, and any data collected and analysis of efficacy or environmental effects.

* 9. Add §300.913 to read as follows:

§300.913 Monitoring the use of dispersants.

As directed by the OSC, the responsible party must monitor any subsurface use of dispersant in response to an oil discharge, surface use of dispersant in response to oil discharges of more than 100,000 U.S. gallons occurring within 24 hours, and surface use of dispersant for more than 96 hours in response to an oil discharge, and submit a Quality Assurance Project Plan for approval to the OSC covering the collection of all environmental data. When these dispersant use conditions are met, and for the duration of dispersant operations, the responsible party shall:

(a) Document the characteristics of the source oil; best estimate of the oil discharge flow rate, periodically reevaluated as conditions dictate, including a description of the method, associated uncertainties, and materials; dispersant(s) product used, rationale for dispersant product choice(s) including the results of any efficacy and toxicity tests specific to area or site conditions, recommended dispersant-to-oil ratio (DOR); and the application method and procedures, including a description of the equipment to be used, hourly application rates, capacities, and total amount of dispersant needed. For subsurface discharges also document the best estimate of the discharge flow rate of any associated volatile petroleum hydrocarbons, periodically reevaluated as conditions dictate, including a description of the method, associated uncertainties, and materials.

(b) In areas not affected by the discharge of oil, collect a representative set of background water column samples following standard operating and quality assurance procedures, at the closest safe distance from the discharge as determined by the OSC and in any direction of likely transport considering surface and subsurface currents and oil properties for the variables listed below. In the dispersed oil plume, collect daily water column samples following standard operating and quality assurance procedures, at such depths and locations where dispersed oil is likely to be present and analyze for:

(1) In-situ oil droplet size distribution, including mass or volume mean diameter for droplet sizes ranging from 2.5 to 2,000 mm, with the majority of data collected between the 2.5 and 100 mm size;

(2) In-situ fluorometry and fluorescence signatures targeted to the type of oil discharged and referenced against the source oil;

(3) Dissolved oxygen (DO);

(4) Total petroleum hydrocarbons, individual resolvable constituents including volatile organic compounds, aliphatic hydrocarbons, monocyclic, polycyclic, and other aromatic hydrocarbons including alkylated homologs, and hopane and sterane biomarker compounds;

(5) Carbon dioxide (CO_2) (subsurface only);

(6) Methane, if present (subsurface only);

(7) Heavy metals, including nickel and vanadium;

(8) Turbidity;

(9) Water temperature;

(10) pH; and

(11) Conductivity.

(c) In consultation with the OSC, and using best available technologies, characterize the dispersant effectiveness and oil distribution, considering the condition of oil, dispersant, and dispersed oil components from the discharge location;

(d) In consultation with the OSC, characterize the ecological receptors (*e.g.* aquatic species, wildlife, and/or other biological resources) and their habitats that may be present in the discharge area and their exposure pathways. Include those species that may be in sensitive life stages, transient or migratory species, breeding or breeding-related activities (*e.g.,* embryo and larvae development), and threatened and/or endangered species that may be exposed to the oil that is not dispersed, the dispersed oil, and the dispersant alone. Estimate an acute toxicity level of concern for the dispersed oil using available dose/response information relevant to potentially exposed species.

(e) Immediately report to the OSC any:

(1) Deviation of more than 10 percent from the mean hourly dispersant use rate for subsurface application, based on the dispersant volume authorized for 24 hours use, and the reason for the deviation; and

(2) Ecological receptors, including any threatened or endangered species that may be exposed based on dispersed plume trajectory modeling and level of concern information.

(f) Report daily to the OSC water sampling and data analyses collected in §300.913(b) and include:

(1) Specific hourly dispersant application rate and the total amount of dispersant used for the previous reporting period established by the OSC with concurrence from the EPA representative to the RRT;

(2) All collected data and analyses of those data within a timeframe necessary to make operational decisions (*e.g.,* within 24 hours of collection), including documented observations, photographs, video, and any other information related to dispersant use, unless an alternate timeframe is authorized by the OSC;

(3) For analyses that take more than 24 hours due to analytical methods, provide such data and results within 5 days, unless an alternate timeframe is authorized by the OSC; and

(4) Estimates of the daily transport of dispersed and non-dispersed oil and associated volatile petroleum hydrocarbons, and dispersants, using the best available trajectory modeling.

* 10. Revise §300.915 and the section heading to read as follows:

§300.915 Data and information requirements for Product Schedule listing.

If you are submitting an application for listing a product to the Schedule, you must provide EPA the information required under §300.955. Your submission must contain:

(a) *General Information for any Product Category.* (1) Your name, physical address, email, and telephone number;

(2) Your identity as the manufacturer of the product, a vendor, importer, or distributor of the product, and/or a designated agent acting on behalf of the manufacturer. Provide documentation of such identity;

(3) All name(s), brand(s), and/or trademark(s) under which the product is to be sold;

(4) Names, physical addresses, emails and telephone numbers of the primary distributors, vendors, importers, and/or designated agent acting on behalf of the manufacturer;

(5) A Safety Data Sheet (SDS) for the product;

(6) The maximum, minimum and optimum temperature, humidity and other relevant conditions for product storage and a brief description of the consequences to performance if the product is not stored within these limits;

(7) The anticipated shelf life of the product at the storage conditions noted in paragraph (a)(6) of this section and documentation for this determination;

(8) A sample product label for all name(s), brand(s), and/or trademark(s) under which the product is to be sold that includes manufacture and expiration dates, and conditions for storage. You may use an existing label provided it already contains the required dates and storage information;

(9) The chemical or biological agent category under which you want the product to be considered for listing on the Schedule, including detailed information on the specific process(es)

through which the product affects the oil, and the specific environment(s) (waters and/or adjoining shorelines) on which it is intended to be used. If your product meets the definition of more than one chemical or biological agent category and you want it considered for listing on the Schedule in more than one category, you must identify all applicable categories and provide the test data to meet the listing criteria appropriate to each category;

(10) Recommended product use procedures, including product concentrations, use ratios, types of application equipment, conditions for use, and any application restrictions. These procedures must address, as appropriate, variables such as weather, water salinity, water temperature, types and weathering states of oils or other pollutants, and product and oil containment, collection, recovery and disposal, and include supporting documentation and standard methods used to determine them;

(11) Environmental fate information, including any known measured data and supporting documentation, on the persistence, bioconcentration factor, bioaccumulation factor, and biodegradability of the product and all of its components in the environment;

(12) The physical/chemical properties of the product, as appropriate, and a citation for the standard methods used to determine them, including:

(i) Physical state and appearance;

(ii) Vapor pressure;

(iii) Flash point;

(iv) Pour point;

(v) Viscosity;

(vi) Specific gravity;

(vii) Particle size for solid components; and

(viii) pH.

(13) The identity and concentration of all components in the product, including each specific component name; corresponding Chemical Abstract Service (CAS) Registry Number; the maximum, minimum, and average weight percent of each component in the product; and the intended function of each component (*e.g.,* solvent, surfactant);

(14) For products that contain microorganisms, enzymes and/or nutrients, provide the following along with a citation or a description of the methodology used to determine:

(i) The name of all microorganisms by current genus and species, including any reclassifications, and any physical, chemical, or biological technique used to manipulate the genetic composition and the weight percent of each genus in the product;

(ii) The name of all enzymes and their International Union of Biochemistry (I.U.B.) number(s); Enzyme Classification (EC) code numbers; the source of each enzyme; units; and specific oil-degrading activity;

(iii) The name(s), maximum, minimum, and average weight percent of the nutrients contained in the product; and

(iv) Certification, including data, methodology, and supporting documentation, indicating that the product does not contain, at levels that exceed the National Ambient Water Quality Criteria lowest density value, bacterial, fungal, or viral pathogens or opportunistic pathogens including, but not limited to: enteric bacteria such as *Salmonella,* fecal coliforms, *Shigella,* or Coagulase positive *Staphylococci,* and Beta Hemolytic *Streptococci* and enterococci.

(15) Certification, including data, methodology, and supporting documentation, indicating that the product does not contain, at levels above National Water Quality Standards lowest acute value for aquatic life:

(i) Arsenic, cadmium, chromium, copper, lead, mercury, nickel, vanadium, zinc, and any other heavy metal reasonably expected to be in the product;

(ii) Cyanide;

(iii) Chlorinated hydrocarbons;

(iv) Pesticides;

(v) Polychlorinated Biphenyls (PCBs); and

(vi) Polynuclear aromatic hydrocarbons (PAHs).

(16) Certification, including data, methodology, and supporting documentation, indicating that the product does not contain any of the prohibited agents identified in §300.910(e);

(17) Information about the laboratory that conducted the required tests, including:

(i) Name of the laboratory, address, contact name, email, and phone number; and

(ii) The national and/or international accreditations held by the laboratory.

(18) All test data and calculations, including:

(i) Raw data and replicates, including positive controls;

(ii) Notes and observations collected during tests;

(iii) Calculated mean values and standard deviations;

(iv) Reports, including a summary of stock solution preparation;

(v) Source and preparation of test organisms;

(vi) Test conditions; and

(vii) Chain of custody forms.

(19) An estimate of the annual product production volume, the average and maximum amount that could be produced per day, and the time frame needed to reach that maximum production rate (days);

(20) Recognition received from EPA's Design for the Environment (DfE) if applicable; and

(21) International product testing or use data or certifications, if available, informing the performance capabilities or environmental benefits of the product.

(b) *Dispersant Testing and Listing Requirements*—(1) *Dispersant Efficacy test and listing criteria.* Test the dispersant product for efficacy using the Baffled Flask Test (BFT) method in Appendix C to part 300. To be listed on the Schedule, the dispersant must demonstrate for each oil and temperature a Dispersant Effectiveness (DE) at the 95% lower confidence level (LCL_{95}) greater than or equal to:

(i) 55% for Intermediate Fuel Oil 120 (IFO–120) at 5 °C;

(ii) 65% for IFO–120 at 25 °C;

(iii) 70% for Alaska North Slope (ANS) crude oil at 5 °C; and

(iv) 75% for ANS at 25 °C.

(2) *Dispersant Toxicity tests and listing criteria.* Use the methods specified in Appendix C to part 300 to test the dispersant alone, the dispersant mixed with ANS, and the dispersant mixed with IFO–120 for acute toxicity, using *Americamysis bahia* and *Menidia beryllina.* Use the methods specified in Appendix C to part 300 to test the dispersant alone for developmental toxicity using a sea urchin assay and for sub-chronic effects using *Americamysis bahia* and *Menidia beryllina.* To be listed on the Schedule, the lethal concentration for 50% of the test species (LC_{50}) at the lower 95% confidence interval for all acute toxicity tests must be greater than 10 ppm; the inhibition concentration for 50% of the test species (IC_{50}) at the lower 95% confidence interval must be greater than 10 ppm; and the sub-chronic No Observed Effect Concentration (NOEC) must be equal to or greater than 1 ppm.

(3) *Limitations.* Product listing would be for use only in saltwater environments.

(c) *Surface Washing Agent Testing and Listing Requirements*—

(1) *Surface Washing Agent Efficacy test and listing criteria.* To be listed on the Schedule, using a recognized standard methodology, the surface washing agent must meet an efficacy of greater than or equal to 30% in either fresh or saltwater or both depending on the intended product use.

(2) *Surface Washing Agent Toxicity test and listing criteria.* Using the toxicity test methodology in Appendix C to part 300, test the surface washing agent for acute toxicity against fresh water species *Ceriodaphnia dubia* and *Pimephales promelas,* or saltwater species *Americamysis bahia* and *Menidia beryllina,* or both, depending on the intended product use. To be listed on the Schedule, the surface washing agent must demonstrate an LC_{50} at the lower 95% confidence interval of greater than 10 ppm in either fresh or saltwater for all tested species.

(3) *Limitations.* Based on testing, product listing would be for use only in the fresh and/or saltwater environments for which it was tested and for which it met the efficacy and toxicity listing criteria.

(d) *Bioremediation Agent Testing and Listing Requirements*—(1) *Bioremediation Agent Efficacy test and listing criteria.* To be listed on the Schedule, a bioremediation agent must successfully degrade both alkanes and aromatics as determined by gas chromatography/mass spectrometry (GC/MS) in salt or fresh water or both, depending on the intended product use, following the test method specified in Appendix C to part 300. The percentage reduction of total alkanes (aliphatic fraction) from the GC/MS analysis must be greater than or equal to 95% at day 28, based on the ninety-fifth percentile Upper Confidence Limit (UCL_{95}) for both salt and freshwater. The percentage reduction of total aromatics (aromatic fraction) must be greater than or equal to 70% at day 28 for saltwater and greater than or equal to 40% for freshwater based on the UCL_{95}.

(2) *Bioremediation Agent Toxicity test and listing criteria.* The bioremediation agent must be tested for acute toxicity in saltwater, freshwater or both, depending on the intended product use, following the method specified in Appendix C to part 300. To be listed on the Schedule, the bioremediation agent must demonstrate an LC_{50} at the lower 95% confidence interval of greater than 10 ppm in either fresh or saltwater for all tested species.

(3) *Limitations.* Based on testing, product listing would be for use only in the fresh and/or saltwater environments for which it was tested and for which it met the efficacy and toxicity listing criteria.

(4) *Exceptions.* If the product consists solely of: ammonium nitrate, ammonium phosphate, ammonium sulfate, calcium ammonium nitrate, sodium nitrate, potassium nitrate, synthetically-derived urea, sodium triphosphate (or tripolyphosphate), sodium phosphate, potassium phosphate (mono- or dibasic), triple super phosphate, potassium sulphate, or any combination thereof, no technical product data are required, are generically listed as non-proprietary nutrients on the Schedule, and no further action is necessary.

(e) *Solidifier Testing and Listing Requirements.* (1) Solidifiers must be tested for acute toxicity in saltwater, freshwater or both, depending on the intended product use, following the method specified in Appendix C to part 300. To be listed on the Schedule, the solidifier must demonstrate an LC_{50} at the lower 95% confidence interval of greater than 10 ppm in either fresh or saltwater for all tested species.

(2) *Limitations.* Based on testing, product listing would be for use only in the fresh and/or saltwater environments for which it was tested and for which it met the toxicity listing criteria.

(f) *Herding Agent Testing and Listing Requirements.* (1) Herding agents must be tested for acute toxicity in saltwater, freshwater, or both, depending on the intended product use, following the method specified in Appendix C to part 300. The herding agent must demonstrate an LC_{50} at the lower 95% confidence interval of greater than 10 ppm in either fresh or saltwater for all tested species.

(2) *Limitations.* Based on testing, product listing would be for use only in fresh and/or saltwater environments for which it was tested and for which it met the toxicity listing criteria.

(g) *Sorbent Requirements.* Known sorbent materials and products will be identified on a publicly available Sorbent Product List for the use of such products when responding to an oil discharge as follows:

(1) For sorbent products that consist solely of the following materials, or any combination thereof, no technical data are required and no further action is necessary for use as a sorbent:

(i) Feathers, cork, peat moss, and cellulose fibers such as bagasse, corncobs, and straw;

(ii) Volcanic ash, perlite, vermiculite, zeolite, and clay; and

(iii) Polypropylene, polyethylene, polyurethane, and polyester.

(2) If the product consists of one or more natural organic substances, inorganic/mineral compounds, and/or synthetic compounds not specifically identified in paragraph (g)(1) of this section but you believe the product meets the definition of a sorbent then, as applicable under §300.955(a) and (b), you must submit the following information for consideration for listing it as a sorbent on the Sorbent Product List:

(i) The information required under paragraphs (a)(1) through (8), and paragraph (a)(13) of this section;

(ii) The certifications required under paragraphs (a)(14)(iv), (a)(15), and (a)(16) of this section; and

(iii) Information, including data, to support the claim your product meets the sorbent definition under §300.5.

§300.920 [Removed]

* 11. Remove §300.920.

* 12. Add §300.950 to subpart J to read as follows:

§300.950 Submission of Confidential Business Information (CBI).

(a) Except as provided in paragraph (b) of this section, all product information submitted to EPA as required under §300.915 will be disclosed to the public.

(b) You may only claim the concentration and the maximum, minimum, and average weight percent of each chemical component or microorganism in your product, as identified in §300.915(a)(13) or (14), to be CBI. EPA will handle such claims in accordance with 40 CFR part 2, subpart B.

(1) You must make your CBI claim at the time you submit your information to EPA to be listed on the Schedule.

(2) You must redact the CBI from all submitted information but include the CBI separately with your submission package. Clearly identify and mark the information as "Confidential Business Information" and place it in a separate inner envelope in your submission package labeled with "CONFIDENTIAL BUSINESS INFORMATION—TO BE OPENED BY THE PRODUCT SCHEDULE MANAGER ONLY."

* 13. Add §300.955 to subpart J to read as follows:

§300.955 Addition of a product to the Schedule.

(a) *Submission.* Submit your complete package to: U.S. Environmental Protection Agency, 1200 Pennsylvania Ave. NW., Mail Code: 5104A, Room 1448, William J. Clinton North, Washington, DC 20460, Attention: Product Schedule Manager.

(b) *Package contents.* Your package shall include, in this order:

(1) A cover letter on company letterhead signed and dated by you certifying that:

(i) All testing was conducted on representative product samples;

(ii) Testing was conducted at a nationally or internationally accredited laboratory in accordance with the

methods specified in Appendix C to part 300, and other applicable methods as appropriate; and

(iii) All test results and product technical data and information are true and accurate.

(2) A numbered Table of Contents showing the information and data submitted under §300.915(a) through (g);

(3) All required data and information arranged in the same order as specified in §300.915(a) through (g); and

(4) A separate envelope containing Confidential Business Information as specified in §300.950(b), if applicable.

(c) *EPA Review.* EPA shall, within 90 days of receiving a submission package:

(1) Review the package for completeness and compliance with all data and information requirements in §§300.915, 300.950, and this section, verify information, and request clarification or additional information as necessary;

(2) Make a product listing determination based on a technical evaluation of all data and information submitted, relevant information on impacts or potential impacts of the product or any of its components on human health or the environment, and the intended use of the product. EPA reserves the right to make a determination on whether the product will be listed, and under which category; and

(3) Notify you, in writing, of its decision to list the product on the Schedule and in which category or categories, or of its decision and supporting rationale to reject the submission. If your submission is rejected:

(i) You may revise the submission package to address test results, data, or information deficiencies and resubmit it.

(ii) EPA's 90-day review will not start until a complete package is resubmitted.

(d) *Request for review of decision.* If your product is rejected for listing on the Schedule, you may request that the EPA Administrator review the determination. Your request must be in writing within 30 days of receipt of notification of EPA's decision not to list the product on the Schedule. Your request must contain a clear and concise statement with supporting facts and technical analysis demonstrating why you believe EPA's decision was incorrect.

(1) The EPA Administrator or designee may request additional information from you and may offer an opportunity for you to meet with EPA.

(2) The EPA Administrator or his designee will notify you in writing of the decision within 60 days of receipt of your request, or within 60 days of receipt of requested additional information.

(e) *Changes to a product listing.* You must notify EPA in writing within 30 days of any changes to information submitted under §300.915(a)(1) through (8) and §300.915(a)(19) through (21) for a product on the Schedule. In the notification, you must detail the specific changes, the reasons for such changes and supporting data and information. EPA may request additional information and clarification regarding these changes. If you change the chemical components and/or concentrations, you must retest the reformulated product according to the requirements for the product category and submit a complete new package for a review and consideration for listing on the Schedule by EPA.

(f) *Transitioning Listed Products from the Current Schedule to the New Schedule.* All products on the current Schedule as of [EFFECTIVE DATE OF FINAL RULE] will remain conditionally listed until [DATE 24 MONTHS FROM THE EFFECTIVE DATE OF FINAL RULE] at which time all products that have not submitted and been listed in the new Schedule based on the amended test and listing criteria will be removed. Your product will be transitioned from the current Schedule to the new Schedule prior to [DATE 24 MONTHS FROM THE EFFECTIVE DATE OF FINAL RULE] after you submit a new, complete package according to the amended test and listing criteria and EPA makes a favorable finding to list the product on the new Schedule.

* 14. Add §300.965 to subpart J to read as follows:

§300.965 Mandatory product disclaimer.

The listing of a product on the Schedule does not constitute approval or recommendation of the product. To avoid possible misinterpretation or misrepresentation, any label, advertisement, or technical literature for the product must display in its entirety the disclaimer shown below. The disclaimer must be conspicuous and must be fully reproduced on all product literatures, labels, and electronic media including Web site pages.

DISCLAIMER [PRODUCT NAME] is listed on the National Contingency Plan (NCP) Product Schedule. This listing does NOT mean that EPA approves, recommends, licenses, or certifies the use of [PRODUCT NAME] on an oil discharge. This listing means only that data have been submitted to EPA as required by Subpart J of the NCP. Only a Federal On-Scene Coordinator (OSC) may authorize use of this product according to the NCP.

* 15. Add §300.970 to subpart J to read as follows:

§300.970 Removal of a product from the Schedule.

(a) The EPA Administrator may remove your product from the Schedule for reasons including, but not limited to:

(1) Misleading, inaccurate, or incorrect statements within the product submission to EPA or to any person or private or public entity regarding the composition or use of the product to remove or control oil discharges, including on labels, advertisements, or technical literature; or

(2) Alterations to the chemical components, concentrations, or use conditions of the product without proper notification to EPA as required by §300.955(e); or

(3) Failure to print the disclaimer provided in §300.965 on all labels, advertisements, or technical literature, or

(4) New or previously unknown relevant information concerning the impacts or potential impacts of the product to human health or the environment.

(b) EPA will notify you in writing, at your address of record, of its reasons for deciding to remove the product from the Schedule. If EPA receives no appeal from you in 30 days, the product will be removed from the Schedule without further notice to you.

(c) You may appeal the decision to remove your product from the Schedule within 30 days of receipt of EPA's notification. Your appeal must contain a clear and concise statement with supporting facts and technical analysis demonstrating why you believe EPA's decision was incorrect. The EPA Administrator will notify you in writing of his decision within 60 days of your appeal, or within 60 days of receipt of any requested additional information.

* 16. Revise Appendix C to Part 300 and appendix heading to read as follows:

Appendix C to Part 300—Requirements for Product Testing Protocols and Summary Test Data: Dispersant Baffled Flask Efficacy and Toxicity Tests; Standard Acute Toxicity Test for Bioremediation Agents, Surface Washing Agents, Herding Agents, and Solidifiers; and Bioremediation Agent Efficacy Test.

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1.0 *Applicability and Scope.* This Appendix establishes laboratory protocols required under Subpart J (Use of Dispersants and Other Chemical and Biological Agents) of 40 CFR part 300 (National Oil and Hazardous Substances Pollution Contingency Plan) to make listing determinations for the Product Schedule. The protocols apply, based on product type, to dispersants, bioremediation agents, surface washing agents, herding agents, and solidifiers as defined in Subpart A (Introduction) of 40 CFR part 300.

2.0 *Baffled Flask Dispersant Efficacy Test (BFT)*

2.1 *Summary.* This laboratory protocol establishes procedures to evaluate the degree to which a product effectively disperses oil spilled on the surface of seawater, using a modified 150-mL screw-cap trypsinizing flask (an Erlenmeyer flask with baffles) with a glass and Teflon® stopcock near the bottom to allow removal of subsurface water samples without disturbing the surface oil layer. The efficacy of a dispersant is measured using two types of oils (Intermediate Fuel Oil 120 and Alaska North Slope) at two temperatures (5 °C and 25 °C). Six replicates are required at each condition with two method blank replicates at each temperature. A layer of oil is placed on the surface of artificial seawater, and the dispersant is added to the slick at a dispersant:oil ratio (DOR) of 1:25 (4%) by volume. A standard orbital shaker table provides turbulent mixing at a speed of 250 revolutions per minute (rpm) for 10 minutes, immediately after which it is maintained stationary for 10 minutes to allow non-dispersed oil to rise to the water's surface. An undisturbed water sample is removed from the bottom of the flask through the stopcock, extracted with dichloromethane (DCM), and analyzed for oil content by UV-visible absorption spectrophotometry at wavelengths ranging between 340 and 400 nm.

2.2 *Apparatus.* All equipment must be maintained and calibrated per standard laboratory procedures.

2.2.1 *Modified Trypsinizing Flask.* A modified 150 mL glass screw-capped Erlenmeyer flask with baffles (*e.g.,* Wheaton No. 355394 or equivalent) fitted with a 2 mm bore Teflon® stopcock and glass tubing, the center of which is no more than 1.3 cm from the bottom, as shown in Figure 1.

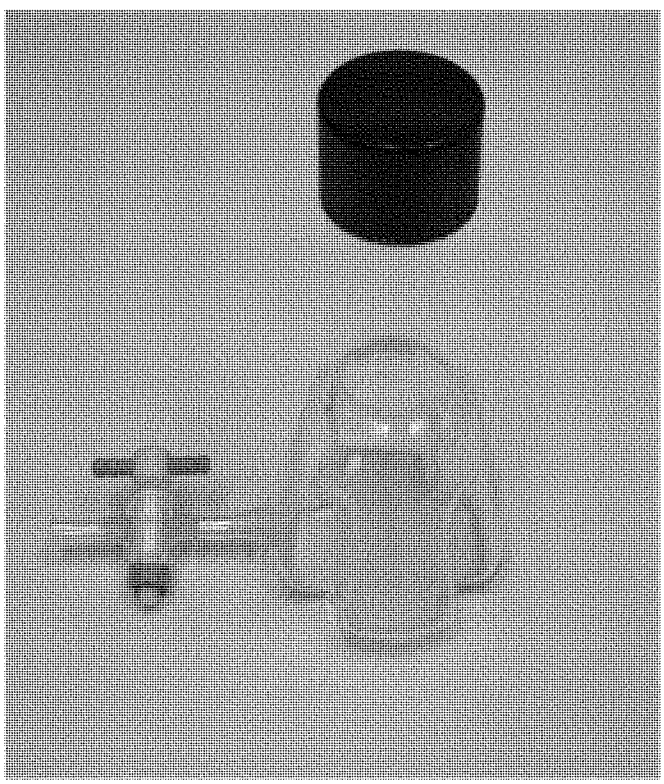

Figure 1. A Baffled Trypsinizing Flask

2.2.2 *Orbital Shaker Table.* An orbital shaker table with a variable speed control unit capable of maintaining 250 rpm. The orbital diameter must be approximately 1.0 inch (2.5 cm) +/ ¥ 0.1 inch (0.25 cm).

2.2.3 *Spectrophotometer.* A UV-visible spectrophotometer capable of measuring absorbance between 340 and 400 nm (*e.g.*, Shimadzu UV–1800, Agilent 8453, or equivalent). Use standard transmission-matched quartz 10-mm path length rectangular cells with PTFE cover for absorbance measurements.

2.2.4 *Glassware.* Including: 25-ml graduated mixing cylinders (a graduated cylinder with a ground glass stopper); 50- and 100-ml graduated cylinders; 125-mL separatory funnels with Teflon stopcocks; 10 ml volumetric flasks; 30 ml crimp style glass serum bottles; 1-, 2-, 5-mL pipettes; other miscellaneous laboratory items.

2.2.5 *Micropipettor.* Use a micropipettor capable of dispensing 4 mL of dispersant and 100 mL of oil (*e.g.*, Brinkmann Eppendorf repeater pipettor with 100 mL and 5 mL syringe tip attachments or equivalent).

2.2.6 *Syringes.* 25-, 100-, 250-, 1000-, 2500-, 5000- ml gas-tight syringes.

2.2.7 *Constant temperature rooms or incubators to hold the shaker at* 5 °C and 25 °C.

2.2.8 *Analytical Balance.*

2.2.9 *Chemical fume hood.*

2.3 *Reagents*

2.3.1 *Artificial seawater.* Use the artificial seawater GP2 formulation shown in Table 1 of this Appendix.

2.3.2 *Test oils.* Use the two EPA standard reference oils, Alaska North Slope oil (ANS) and Intermediate Fuel Oil 120 (IFO 120). To obtain these oils at no charge (except for a minimal shipping fee), see the instructions at *http://www.epa.gov/emergencies/content/ncp/index.htm.* Selected properties are summarized in Table 2 of this Appendix.

2.3.3 *Dichloromethane (DCM) (also known as methylene chloride), pesticide quality.*

2.3.4 *Positive Control Dispersant.* Dispersant sample with a known, reproducible efficacy. To obtain this control sample at no cost (except for shipping), see the instructions at *http://www.epa.gov/emergencies/content/ncp/index.htm.*

2.4 *Container Handling and Storage*

2.4.1 *Glassware.* If the glassware has been used with oil before, rinse with DCM to remove as much of the oil adhering to the sides of the flask as possible; waste DCM may be used. Soak in warm water with detergent and individually wash with bristled brushes. First rinse with tap water, then follow with two de-ionized water rinses. Dry either on a rack or in a 110 °C drying oven). After drying, rinse with fresh DCM (use sparingly).

2.4.2 *Serum bottles and other non-volumetric glassware.* Bake for at least 4 hours in a muffle furnace at 450 °C.

2.5 *Calibration Curve for the UV-visible spectrophotometer*

2.5.1 *Stock Standard Solution Preparation.* Stock standard solution concentrations are based on the mass measurements after each addition and density determinations of the oil/dispersant/DCM solution using a density bottle or a 1-mL gas tight syringe. An example calculation is given in Table 3 of this Appendix according to the following equation:

$$theoretical\ concentration, \frac{mg}{mL} = \frac{mass\ of\ oil,\ g * 1000\ mg/g}{total\ mass,\ g / \rho_{solution,\ g/mL}} \qquad \text{(Equation 1)}$$

Use the reference oils and the specific dispersant being tested for a particular set of experimental test runs. Prepare the stock solution of dispersant-oil mixture in DCM, starting with 2 ml of the oil, then adding 80 ml of the dispersant followed by 18 ml of DCM. Two sets of standards are needed, one for each oil and dispersant combination.

2.5.2 Six-*point Calibration Curve.* For each reference oil, add specific volumes of its stock standard solution (given in Table 4 of this Appendix) to 30 ml of artificial seawater in a 125 ml separatory funnel. Extract the oil/water mixture with triplicate 5 ml volumes of DCM. Follow each DCM addition by 15 seconds of vigorous shaking, carefully releasing the initial pressure inside the separatory funnel by partially removing the glass stopper inside a fume hood after the first few shakes. Then, allow a 2-minute stationary period for phase separation for each extraction. Drain the extracts into a 25-mL graduated mixing cylinder. Release any entrained bubbles of DCM from the water layer by sideways shaking of the funnel. Use precaution not to drain water into the DCM extract as it can affect the absorbance readings. Adjust the final volume of the collected extracts to 20 mL in the mixing cylinder using DCM. Determine specific masses for oil concentrations in the standards as volumes of oil/dispersant solution multiplied by the concentration of the stock solution. An example calculation is given in Table 4 of this Appendix. Two calibration curves are needed, one for each oil and dispersant combination.

2.6 *Sample Preparation and Testing.* See section 2.7 of this Appendix for a detailed description of the spectrophotometer's linear calibration procedure.

2.6.1 Six replicates of each oil and test dispersant are required at each temperature plus two additional tests of method blanks (artificial seawater without oil and dispersant), one at each temperature. A completed test consists of 26 baffled flask tests (a total of six replicates for each of two reference oil/test dispersant combinations at two temperatures (5 °C and 25 °C), plus two method blanks).

2.6.2 A positive control run of 6 replicates of a dispersant with known dispersion efficacy (see 2.3.4 above for how to obtain) is prepared with both oils at both temperatures to verify the protocol is being correctly performed. The lab must certify that a positive control was successfully conducted within a year of any testing submitted for a dispersant listing.

2.6.3 Attach a 3-inch length of Teflon tubing to the stopcock of each of the 150-mL baffled flasks. Add 120 mL of artificial seawater to each flask. Put screw cap on flasks and place them at the appropriate temperature (either 5 °C or 25 °C) for equilibration.

2.6.4 Calibrate and adjust the shaker table to 250 ± 10 rpm.

2.6.5 Prepare and time separately each baffled flask. Sequentially add 100 mL of oil and 4 mL of dispersant to the flask layering them onto the center of the seawater to give a dispersant-to-oil ratio (DOR) of 1:25. Avoid any oil or dispersant splashing on the flask walls, as it may reduce efficacy or cause errors in the calculated results. Discard the sample and repeat the setup if: (1) Any oil or dispersant splashing occurs during the additions, or (2) the dispersant contacts the water first rather than the oil.

2.6.6 For the oil, fill the tip of the pipettor, using a wipe to remove any oil from the sides of the tip. Holding the pipettor vertically, dispense several times back into the reservoir to ensure that the oil flows smoothly. Insert the syringe tip vertically into the baffled flask, and let the bottom of the pipettor rest on the neck of the flask. Slowly and carefully dispense the oil one time onto the center of the water's surface. IFO 120 takes longer to drip, and ANS will splash if dispensed too fast. The remainder of the oil can either be returned to the oil bottle or set aside for use in the next test flask. Note to 2.6.6: If a Brinkmann Eppendorf repeater pipettor is used for dispensing the oil, attach a 5-mL syringe tip, and set the dial to 1.

2.6.7 For the dispersant, use the same procedure as for the oil to dispense onto the center of the oil slick surface. As the dispersant first contacts the oil, it will usually push the oil to the sides of the flask. Replace the screw cap onto the flask.

Note to 2.6.7: If a Brinkmann Eppendorf repeater pipettor is used for dispensing the dispersant, attach a 100-mL syringe tip, and set the dial to 2.

2.6.8 Carefully place flask securely onto the shaker and agitate for 10 ± 0.25 minutes at 250 ± 10 rpm.

2.6.9 Remove the flask from the shaker table and allow a stationary, quiescent period of 10 ± 0.25 minutes to allow undispersed and/or recoalesced oil droplets to refloat to the surface.

2.6.10 Carefully open the screw cap, then the stopcock at the bottom, and discard the first several mL of seawater into a waste beaker to remove non-mixed water-oil initially trapped in the stopcock tubing. Collect a volume slightly greater than 30-mL into a 50-mL graduated cylinder. Adjust the collected volume to the 30-mL mark by

removing excess with a disposable glass Pasteur pipette. A web-like emulsion may form at the solvent/water interface during the water sample extraction. Avoid pulling any emulsion phase into the DCM extract as it may cloud the DCM-extract leading to error.

2.6.11 Transfer the water-oil sample from the graduated cylinder into a 125-mL glass separatory funnel fitted with a Teflon stopcock.

2.6.12 Add 5 mL DCM to the separatory funnel. Start shaking, releasing pressure into the fume hood by loosening the glass stopper. Shake vigorously at least 20 times for 15 seconds.

2.6.13 Allow the funnel to remain in a stationary position for 2 minutes to allow phase separation of the water and DCM.

2.6.14 Drain the DCM layer from the separatory funnel into a 25 mL mixing cylinder. Avoid pulling any emulsion phase into the DCM extract as it may cloud the DCM extract.

2.6.15 Repeat the DCM-extraction process two or three additional times until the DCM is clear. Collect each extract in the graduated cylinder. After the final extraction, lightly shake the separatory funnel sideways once or twice to dislodge entrained bubbles of DCM and drain.

2.6.16 Adjust the final volume to a known quantity, 20 or 25 mL, in the mixing cylinder. IFO 120 samples may require dilution when dispersed chemically because of their high absorbance properties. Using a syringe, dispense 2.5 mL or 5.0 mL of an IFO 120 sample into a 10-mL volumetric flask, and fill with DCM to make either a 1:4 or 1:2 dilution, respectively. ANS samples adjusted to 25 mL typically don't require dilution.

2.6.17 If analysis cannot be conducted immediately, store the extracted DCM samples at 4 °C until time of analysis. Glass-stoppered mixing cylinders may be used for short-term storage or prior to bringing the extracts up to volume. After bringing to volume, transfer the DCM extracts to 25–30 ml crimp-style serum vials with aluminum/Teflon seals.

2.6.18 Complete all analysis within 10 consecutive days from when the sample was collected.

2.7 *UV-Visible Spectrophotometer Linear Stability Calibration*

2.7.1 A six-point calibration of the UV-visible spectrophotometer is required at least once per day for each oil. The stability calibration criterion is determined with the six oil standards identified in Table 4 of this Appendix.

2.7.2 Turn on spectrophotometer and allow it to warm up for at least 30 minutes before beginning analysis. Blank the instrument for the wavelengths between 340 and 400 nm with DCM.

2.7.3 If refrigerated, allow all extracts, standards and samples to warm to room temperature.

2.7.4 Determine the absorbance of the six standards between the wavelengths of 340 and 400 nm. This can be done by either one of the following methods:

2.7.4.1 *Trapezoidal Rule.* Program the spectrophotometer to take readings every 5 l or 10 l and calculate the area under the curve using the Trapezoidal rule:

$$\int_{340\lambda}^{400\lambda} f(x)dx \approx \frac{H}{2}\sum_{k=1}^{N}\left(f(x_{k+1})+f(x_k)\right) \qquad \text{(Equation 2)}$$

where N+1 = number of absorbance measurements to delineate N equally spaced sections of the curve, and H = the distance (l) between each reading. For H = 5, N+1 = 13 measurements, for H = 10, N+1 = 7. The following formula illustrates readings taken every 10 l.

$$Area = \frac{(Abs_{340}+Abs_{350})*10}{2} + \frac{(Abs_{350}+Abs_{360})*10}{2} + \cdots \frac{(Abs_{390}+Abs_{400})*10}{2} \qquad \text{(Equation 3)}$$

When using readings taken every 5 l, each absorbance sum is multiplied by 5.

2.7.4.2 Automatic *Integration.* Program the spectrophotometer to automatically integrate the area under the curve between 340 nm and 400 nm.

2.7.4.3 If the wavelengths must be manually set on the spectrophotometer, the older method of only measuring at 340 l, 370 l, and 400 l may be used. Then calculate using the trapezoidal rule for N + 1 = 3, H = 30. While the resulting area count with the older method is less accurate, the final results are similar since the inaccuracy is systematic.

2.7.5 After determining the area count for each standard, determine the response factor (RF) for the oil at each concentration using the following equation:

$$RF = \frac{Theoretical\ Concentration, \frac{g}{mL}\ (Eq.1)}{area\ (Eq.3)} \qquad \text{(Equation 4)}$$

2.7.6 Spectrophotometer stability for the initial calibration is acceptable when the RFs of the six standard extracts are less than 10% different from the overall mean value for the six standards, as calculated in Equation 5 of this Appendix and depicted in the example in Table 4 of this Appendix.

$$\%\ difference = \frac{|RF-\overline{RF}|}{\overline{RF}} * 100 \qquad \text{(Equation 5)}$$

2.7.7 If this criterion is satisfied, begin analysis of sample extracts. Absorbances greater than or equal to 3.5 are not included because absorbance saturation occurs at and above this value. If any of the standard oil extracts fails to satisfy the initial-stability criterion, the source of the problem (*e.g.,* preparation protocol for the oil standards, spectrophotometer stability, etc.) must be corrected before analysis of the sample extracts begins.

2.7.8 Determine the slope of the calibration points by using linear regression forced zero intercept:

$$Y(area\ under\ absorbance\ curve) = m(slope) * x(concentration\ of\ oil) \qquad \text{(Equation 6)}$$

2.8 Spectrophotometric *Analysis and Calculations*

2.8.1 Once a successful calibration curve for each of the two reference oils has been created and verified, measure experimental replicates for each of the reference oils at each temperature followed by a standard check sample.

2.8.2 The same procedure is followed for the positive controls.

2.8.3 Determine the area for the absorbance values obtained for the experimental samples by using Equation 2 of this Appendix and illustrated by Equation 3 of this Appendix.

2.8.4 Calculate the Total Oil dispersed and the percentage of oil dispersed (%OD) based on the ratio of oil dispersed in the test system to the total oil added to the system, as follows:

$$Total\ Oil\ Dispersed, mg = \frac{Area\ (Eq.2)}{Calibration\ Curve\ Slope} * V_{DCM} * \frac{V_{tw}}{V_{ew}} \qquad \text{(Equation 7)}$$

where:

V_{DCM} = final volume of the DCM extract (mL)

V_{tw} = total seawater in Baffled Flask (120 mL)

V_{ew} = volume seawater extracted (30 mL)

$$\%OD = \frac{Total\ Oil\ Dispersed}{\rho_{oil} * V_{oil}} * 100 \qquad \text{(Equation 8)}$$

where:

r_{Oil} = density of the specific test oil, mg/mL and

V_{Oil} = Volume (mL of oil added to test flask (100 mL = 0.1 mL))

2.8.5 The %ODs for the six replicates within a particular treatment are then subjected to an outlier test, the Grubb's Test or Maximum Normal Residual test (6). A convenient Internet-based calculator of a Grubbs outlier may be found at: *http://www.graphpad.com/quickcalcs/Grubbs1.cfm.* If an outlier is detected ($p < 0.05$), analyze an additional replicate to obtain the required six replicates.

2.8.6 Report the Dispersion Efficacy value for each oil and each temperature, which is the lower 95% confidence level of the 6 independent replicates (DE_{LCL95}) for each oil/temperature combination. Error bars are not needed as reporting the lower confidence level computationally takes the variability of the replicates into account as shown in Equation 9 of this Appendix.

$$DE_{LCL95} = \overline{\%OD} - \left(\frac{t_{(n-1,1-\alpha)} * s}{\sqrt{n}}\right) \qquad \text{(Equation 9)}$$

where $\overline{\%OD}$ = mean percentage oil dispersed for the n = 6 replicates, S = standard deviation, and $t_{(n-1,1-\alpha)}$ = 100 * (1-a)th percentile from the t-distribution with n-1 degrees of freedom. For 6 replicates, $t_{n-1,1-\alpha}$ = 2.015, where a = 0.05. An example of the calculations is given in Table 5 of this Appendix.

2.9 Performance *Criterion*

The dispersant product tested will remain in consideration for listing on the NCP Product Schedule if the dispersant efficacy (DE_{LCL95}), as calculated in section 2.8.6 of this Appendix, is:

Oil	Temp (°C)	DE_{LCL95} (%)
ANS	5	≥ 70
ANS	25	≥ 75
IFO120	5	≥ 55
IFO120	25	≥ 65

2.10 *Quality Control (QC) Procedures for Oil Concentration Measurements*

2.10.1 *Absorbance readings.* Perform at least 5% of all UV-visible spectrophotometric measurements in duplicate as a QC check on the analytical measurement method. The absorbance values for the duplicates must agree within ± 5% of their mean value.

2.10.2 *Method blanks.* Analytical method blanks involve an analysis of artificial seawater blanks (artificial seawater without oil or dispersant in a baffled flask) through testing and analytical procedures. Analyze method blanks with a frequency of at least two per completed test. Oil concentrations in method blanks must be less than detectable limits.

2.10.3 *Accuracy.* Determine accuracy by using a mid-point standard calibration check after each set of replicate samples analyzed. The acceptance criterion is based on a percent recovery of 90–110% using the following equation:

$$\%recovery = 100 * \frac{measured\ concentration\ of\ check\ standard}{theoretical\ concentration\ of\ check\ standard} \qquad \text{(Equation 10)}$$

2.10.4 Calibration *QC checks.* Before analyzing samples, the spectrophotometer must meet an instrument stability calibration criterion using the oil standards. The instrument stability for initial calibration is acceptable when the RFs (Equation 5 of this Appendix) for each of the six standard concentration levels are less than 10% different from the overall mean value.

TABLE 1—CONSTITUENT CONCENTRATIONS FOR GP2 ARTIFICIAL SEAWATER

[Based on Spotte et al., 1984]

Constituent	Concentration (g/L)
NaCl	21.03
Na_2SO_4	3.52
KCl	0.61
KBr*	0.088
$Na_2B_4O_7 \cdot 10H_2O$*	0.034
$MgCl_2 \cdot 6H_20$	9.50
$CaCl_2 \cdot 2H_2O$	1.32
$SrCl_2 \cdot 6H_2O$*	0.02
$NaHCO_2$ *	0.17

*Use Stock Solution, 1 mL/L GP2 for 100X stock solution for Bromide, Borate, and Strontium.

10 mL/L GP2 for bicarbonate—10X stock solution as it is not soluble in a 100X solution.

Adjust to pH 8.0 prior to autoclaving.

TABLE 2—TEST OIL CHARACTERISTICS

Oil	Density, mg/mL @ 15 °C	API gravity (deg)	Viscosity @ 15 °C, (cSt)	Category by API gravity	Category by kinematic viscosity
ANS	884	28.2	40	Medium	Light
IFO120	948	17.5	1520	Heavy	Heavy

TABLE 3—STOCK SOLUTION PREPARATION

Item	Amount
Mass of Bottle, g	29.9666
Mass of Bottle + oil, g	31.5734
Mass of bottle + disp + oil + DCM, g	55.0425
Mass of oil, g	1.6068

TABLE 3—STOCK SOLUTION PREPARATION—Continued

Item	Amount
Mass of disp + oil + DCM, g	25.0759
Mass of 1 mL syringe, g	14.5563
Mass of 1 mL syringe + solution, g	15.8779

TABLE 3—STOCK SOLUTION PREPARATION—Continued

Item	Amount
Density of solution, g/mL	1.3216
Volume of solution, mL	18.9740
Conc. Of stock solution, mg/mL	84.6850

Table 4. Dispersant Calibration Example for Both Oils

ANS	Stock Solution Concentration mg/mL				84.6848				
Standard – Stock vol. (uL)	Theoretical Conc.	Abs 340 nm	…..	Abs 400 nm	Calculated Trapezoid Area	RF	Avg. RF	Dev. From Avg. RF	Slope
20	0.085	0.231	…..	0.09	8.55	0.0099	0.0101	2.27	99.11
40	0.169	0.452	…..	0.175	16.68	0.0102		0.15	
80	0.339	0.864	…..	0.334	31.91	0.0106		4.72	
120	0.484	1.307	…..	0.503	48.16	0.0100		0.88	
160	0.677	1.818	…..	0.711	67.38	0.0101		0.82	
200	0.847	2.251	…..	0.888	84.30	0.0100		0.91	
IFO120	Stock Solution Concentration mg/mL				95.7542				
Standard – Stock vol. (uL)	Theoretical Conc.	Abs 340 nm	…..	Abs 400 nm	Calculated Trapezoid Area	RF	Avg. RF	Dev. From Avg. RF	Slope
10	0.048	0.278	…..	0.109	10.33	0.0046	0.0048	3.64	205.67
20	0.096	0.536	…..	0.209	19.87	0.0048		0.22	
40	0.192	1.066	…..	0.414	39.39	0.0049		1.10	
60	0.287	1.608	…..	0.628	59.72	0.0048		0.04	
80	0.383	2.154	…..	0.836	79.73	0.0048		0.10	
100	0.479	2.649	…..	1.021	97.24	0.0049		2.39	

Table 5. Sample Calculation with ANS

Rep	Abs 340nm	..	Abs 400nm	Area from spec	Dilution Factor	Extract Volume (ml)	Area	Conc, mg/mL.	Mass in 30 mL, mg	Total Oil Dispersed, mg	Efficiency %	Avg.	Std. Dev.	Coeff. Of Var	LCL 95
1	2.096	..	0.814	77.51	1	25	77.59	0.72	18.12	72.49	82.00	81.01	3.17	3.91	78.40
2	1.985	..	0.774	73.72	1	25	73.70	0.69	17.21	68.86	77.89				
3	2.012	..	0.781	74.50	1	25	74.54	0.70	17.41	69.64	78.78				
4	2.18	..	0.841	80.25	1	25	80.19	0.75	18.73	74.93	84.76				
5	1.999	..	0.785	73.87	1	25	73.93	0.69	17.27	69.07	78.13				
6	2.173	..	0.841	79.86	1	25	79.96	0.75	18.68	74.71	84.51				
dup	2.162	..	0.841	80.143	1	25	80.16	0.75	18.72	74.89	84.72	0.04			
	2.18	..	0.841	80.245	1	25	80.19	0.75	18.73	74.93	84.76				

2.11 *References for Section 2.0*

(1) U.S. Environmental Protection Agency (1994), "Swirling Flask Dispersant Effectiveness Test," *Title 40 Code of Federal Regulations,* Pt. 300, Appendix C, pp 47458–47461.

(2) Sorial, G.A., A.D. Venosa, K.M, Koran, E. Holder, and D.W. King. 2004. "Oil spill dispersant effectiveness protocol: I. Impact of operational variables." ASCE J. Env. Eng. 130(10):1073–1084.

(3) Sorial, G.A., A.D. Venosa, K.M, Koran, E. Holder, and D.W. King. 2004. "Oil spill dispersant effectiveness protocol: II. Performance of revised protocol." ASCE J. Env. Eng. 130(10):1085–1093.

(4) Venosa, A.D., D.W. King, and G.A. Sorial. 2002. "The baffled flask test for dispersant effectiveness: A round robin evaluation of reproducibility and repeatability." Spill Sci. & Technol. Bulletin 7(5–6):299–308.

(5) Spotte, S., G. Adams, and P.M. Bubucis. 1984. "GP2 medium is an synthetic seawater for culture or maintenance of marine organisms," Zoo Biol, 3:229–240.

(6) Grubbs, F. 1969. "Sample Criteria for Testing Outlying Observations," Annals of Mathematical Statistics, pp. 27–58.

3.0 Dispersant Toxicity Testing

3.1 *Summary.* This laboratory protocol includes testing for: (1) Dispersant standard static acute toxicity tests for the mysid shrimp, *Americamysis bahia* (48-hr duration) and the inland silverside, *Menidia beryllina* (96-hr duration); (2) dispersant/oil mixture static acute toxicity tests for *Americamysis bahia* and *Menidia beryllina* (48-hr and 96-hr duration, respectively); (3) dispersant developmental assay for the purple sea urchin, *Strongylocentrotus purpuratus,* (72-hr duration); and (4) dispersant 7-day static subchronic tests with *Americamysis bahia* and *Menidia beryllina* (Table 6 of this Appendix).

TABLE 6—TOXICITY TESTING REQUIREMENTS FOR DISPERSANTS

Test substance	Test procedure			
	96-hr static acute: *Menidia beryllina*	48-hr static acute: *Americamysis Bahia*	72-hr sea urchin *Developmental Assay*	7-day sub-chronic: *M. beryllina* & *A. bahia*
Dispersant only	*yes*	*yes*	*yes*	*yes.*
Dispersant/Reference Oil Mixture	*yes*	*yes*	*no*	*no.*

3.2 *Preparation of Stock Solutions*

3.2.1 *Dispersant.* Prepare a 1000 mL/L primary stock solution prior to test initiation by adding 1.1 mL of dispersant to 1100 mL of dilution water consisting of salinity adjusted uncontaminated natural or artificial seawater, in a glass vessel. Using a laboratory top stirrer equipped with a stainless steel blade, center the stirrer blade in the mixing vessel one inch off the bottom. Initially mix the resulting stock solution for approximately five seconds at speeds of < 10,000 rpm to avoid foaming. Thereafter, set the speed to provide a 70% vortex. Using a glass pipette, remove appropriate aliquots of stock solution from between the mixing vessel wall and edge of the vortex and place directly into the dilution water within an exposure vessel. Suspend mixing of the stock solution after the removal of each aliquot. Base the preparation of exposure solutions on the nominal concentration of the stock solution and follow procedures outlined in sections 3.5 and 3.6 of this Appendix.

3.2.2 *Dispersant-Reference Oil(s) Mixtures.* Use IFO 120 and ANS oils. To obtain these oils at no charge (except for a minimal shipping fee) see *http://www.epa.gov/emergencies/content/ncp/index.htm.* Assessment of dispersant-reference oil mixture (DOM) toxicity is determined for each reference oil using the aqueous phase of a chemically enhanced-water accommodated fraction (CE–WAF). Repeat the following procedure for each reference oil tested. Fit a glass aspirator bottle (approximately 23L) equipped with a hose bib at the base with a length of silicon tubing containing a hose clamp. Fill the bottle with 19L of seawater leaving a 20% headspace above the liquid, place on a magnetic stir plate then add and center a stir bar. Add the respective crude oil at 25g/L using a silicon tube attached to a glass funnel that reaches just below the water surface. Using this method reduces the production of air bubbles on the oil surface slick. Adjust the stir plate to obtain an oil vortex of 25% of the total volume of the seawater, then add the dispersant to be tested at a ratio of 1:10 dispersant:oil (2.5 g/L). Securely seal the bottle to reduce the loss of volatiles using a silicon stopper and wraps of Parafilm and stir for 18 hours, then allow the solution to settle for 6 hours. Maintain the temperature at 25 °C during stirring and settling. Purge the hose at the base of the bottle of any material followed by removal of the CE–WAF (aqueous phase) into a clean glass container without disturbing the surface oil slick. The CE–WAF should be remixed and 1–2 L removed for chemical analysis of total petroleum hydrocarbons (TPH) following the procedures outlined in section 3.4 of this Appendix. The remaining volume will be used for the preparation of exposure solutions following procedures outlined in section 3.3 of this Appendix. To reduce time and cost, mix sufficient amounts of dispersant product-reference oil mixture CE–WAF to allow preparation of exposure solutions for conducting simultaneous acute tests with both *Americamysis bahia* and *Menidia beryllina.*

3.3 *Preparation of Exposure Concentrations*

3.3.1 *Concentration Selection.* Preliminary rangefinder tests may be necessary using a series of logarithmic concentrations (*e.g.* 0.1, 1, 10, 100 ml dispersant product/L or mg TPH/L) to determine the appropriate exposure concentration range necessary to determine LC_{50} values and 95% confidence intervals. For definitive tests, conduct a minimum of five test concentrations using a geometric ratio between 1.5 and 2.0 (*e.g.* 2, 4, 8, 16, and 32). Note that when testing only the dispersant product, the highest test concentration must not exceed the dispersant's self-dispersibility limit.

3.3.2 *Exposure Concentrations.* Exposure solutions are prepared by adding the appropriate amount of stock solution directly to dilution water in each test chamber. Mix each exposure solution using five rotations in one direction followed by five rotations in the opposite direction using a solid glass stir rod.

3.3.3 *Reference Toxicants.* Separate toxicity tests must be performed with a reference toxicant for each species tested. Conduct additional reference toxicity tests any time a change in the population or source of a test species occurs. Use sodium dodecyl sulfate (SDS), also known as dodecyl sodium sulfate (DSS), and sodium lauryl sulfate (SLS) as the reference toxicant for exposures conducted with *Menidia beryllina* and *Americamysis bahia.* Use copper chloride as the reference toxicant for exposures conducted with the sea urchin developmental test. Use reagent grade quality SDS and copper chloride for tests. Information on procedures for conducting reference toxicant tests with these species can be found in the specific EPA methods documents cited in sections 3.5.1, 3.6.1 and 3.7.1 of this Appendix.

3.4 *Chemical Analysis of Stock Solutions.* Add the 1L sample of CE–WAF (Section 3.2.2 of this Appendix) solutions directly to amber glass bottles with Teflon®-lined cap. Collect a replicate sample in the event of accidental loss or if reanalysis of the stock solution becomes necessary. Adjust sample to a pH=2 using 50% hydrochloric acid, immediately refrigerate and analyze within 48 hours of collection. Analyze samples for C9–C32 TPH by gas chromatography-flame ionization detection (GC–FID) following EPA SW–846, Method 8015B–DRO (4). Report TPH concentration of stock solutions as milligrams TPH/L and use in the calculation of exposure concentrations for all toxicity tests conducted with CE–WAF.

3.5 *Static Acute Tests With M. beryllina and A. bahia*

3.5.1 *General.* Use EPA's *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA–821–R–02–012) (1) for testing each species separately with dispersant product or a mixture of dispersant product and reference oil (DOM).

3.5.2 *Test Solutions.* Modify procedures in EPA–821–R–02–012 specifically dealing with the handling and toxicity testing of effluents or receiving water samples as follows: Prepare stock solutions following section 3.2 of this Appendix and exposure concentrations following section 3.3 of this Appendix.

3.5.3 *Number of Treatments, Replicates and Organisms.* Conduct a minimum of three replicates of at least five exposure treatments plus a minimum of three replicate dilution water controls. Expose ten organisms per replicate treatment.

3.5.4 *Exposure Period.* Test duration is 48-hr for *Americamysis bahia* and 96-hr for *Menidia beryllina.* Mortality must be recorded at each 24 hour period of each test.

3.5.5 *Test Acceptability.* For each test performed, survival of control animals must be >90% and test results must allow determination of statistically valid LC_{50} and 95% confidence interval values except in cases where the LC_{50} is >1000 ml/L or is determined to be greater than the limits of water solubility of dispersibility.

3.5.6 *Static Acute Test Summary.* A summary of required test conditions is provided in Table 7 of this Appendix.

3.6 *Sea Urchin Developmental Test With Dispersant Product*

3.6.1 *General.* Use Section 15, ''Purple Urchin, *Strongylocentrotus purpuratus* and Sand Dollar, *Dendraster excentricus* Larval Development Test Method'' of EPA's *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms* (EPA/600/R–95–136) (2).

3.6.2 *Test Organism.* Tests of dispersant products are to follow methods for the purple urchin only. Tests with the sand dollar are not required.

3.6.3 *Test Solutions.* Modify procedures in EPA/600/R–95–136, Section 15 specifically dealing with the handling and toxicity testing of effluents or receiving water samples as follows: Prepare stock solutions following section 3.2.1 of this Appendix and exposure concentrations following section 3.3 of this Appendix.

3.6.4 *Number of Treatments and Replicates.* Conduct a minimum of four replicates of five exposure treatments plus a minimum of four replicate dilution water controls.

3.6.5 *Exposure Duration and Test Endpoint.* Examine the effects of the dispersant product on normal development of sea urchin embryos over a period of 72 hours. An IC_{50} (the exposure concentration at which normal development is inhibited in 50% of the embryos) with 95% confidence intervals are to be determined in place of an IC_{25}. The concentration of dispersant causing inhibition of development in 50% of exposed embryos (IC_{50}) with the lower and upper 95% confidence intervals (LCI_{95} and $ULCI_{95}$) must be calculated at the end of the exposure period. Mortality determinations are not required.

3.6.6 *Test Acceptability.* Requirements of the assay are: (i) ≥ 80% normal larval development in the control treatment, (ii) the minimum significant difference (MSD) that can be statically detected relative to the control is ≤25%, (iii) test results which support the determination of a statistically valid IC_{50} and 95% confidence interval unless the LC_{50} is >1000 ml/L or is greater than the limits of water solubility of dispersibility.

3.6.7 *Urchin Developmental Test Summary.* A summary of required test conditions is provided in Table 7 of this Appendix.

3.7 *Seven-Day Subchronic Tests With M. beryllina and A. bahia*

3.7.1 *General.* Use Section 13, Method 1006.0, ''Inland Silverside (*Menidia beryllina*) Larval Survival and Growth Method,'' and Section 14, Method 1007.0, ''Mysid (*Mysidopsis* [renamed *Americamysis*] *bahia*) Survival, Growth, and Fecundity Method'' of EPA's *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* (EPA–821–R–02–014) (3) for testing of dispersant product.

3.7.2 *Test Solutions.* Modify procedures in EPA–821–R–02–014, sections 13 and 14 specifically dealing with the handling and toxicity testing of effluents or receiving water samples as follows: Prepare stock solutions following section 3.2.1 of this Appendix and exposure concentrations following section 3.3 of this Appendix. Exposure solutions should be renewed every 24 hr for the duration of the test.

3.7.3 *Number of Treatments, Replicates and Organisms.* (i) *Menidia beryllina:* Conduct a minimum of four replicates of at least five exposure treatments plus a minimum of four replicate dilution water controls. Expose ten *M. beryllina* per replicate treatment. (ii) *Americamysis bahia:* Conduct a minimum of eight replicates of at least five exposure treatments plus a minimum of eight replicate dilution water controls. Expose five *A. bahia* per replicate treatment.

3.7.4 *Exposure Duration and Test Endpoint.* The test duration is seven days for both species. Test endpoints for *Menidia beryllina* are survival and growth (dry weight) and for *Americamysis bahia* is survival, growth (dry weight) and fecundity. Calculate an LC_{50} and 95% confidence interval for survival and IC_{25} and IC_{50} with 95% confidence intervals for growth (and fecundity for *A. bahia* only). Report the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) for each endpoint.

3.7.5 *Test Acceptability.* Requirements of the assay are: (i) ≥80% survival in the control treatment for each species, (ii) dry weights must meet the specific requirements as stipulated in Method 1006.0 for *Menidia beryllina* and Method 1007.0 for *Americamysis bahia,* (iii) egg production must occur in 50% of female *Americamysis bahia* in the replicate control treatments.

3.7.6 *Subchronic Test Summary.* A summary of required test conditions for each species is provided in Table 7 of this Appendix.

3.8. *Laboratory Report.* The laboratory must include, for each toxicity test report, all applicable information, data and analyses as follows:

3.8.1 *Test Objective:* Protocol title and source, endpoint(s);

3.8.2 *Product Information:* Product name, manufacturer contact information, lot number, production date, date received/chain of custody;

3.8.3 *Contract Facility:* Contact information;

3.8.4 *Dilution Water:* Source, pretreatment, physical and chemical characteristics (pH, salinity);

3.8.5 *Test Conditions:* Date and time of test (start and end), test chambers type and volume, volume of solution per chamber, number of organisms per chamber, number of replicate chambers per treatment, feeding frequency, amount and type of food, test concentrations, test temperature (mean and range), test salinity (mean and range);

3.8.6 *Test Organisms:* Common and scientific name, source contact information, age and date purchased, acclimation conditions (*e.g.,* temperature, salinity, both mean and range), age at test start;

3.8.7 *Reference toxicant:* Date received, lot number, date of most recent test, results and current Cumulative Sum Chart, dilution water used, physical and chemical methods used;

3.8.8 *Quality Assurance:* Verification of laboratory accreditation, including subcontractor facilities;

3.8.9 *Test Results:* Raw data in tabular and graphical form, daily records of affected organisms in each concentration replicate and controls, table of required endpoints (*i.e.,* LC_{50} with 95% confidence interval (CI), IC_{25} and IC_{50} with 95% CI, LOEC and NOEC), statistical methods used to calculate endpoints, summary tables of test conditions and QA data;

3.8.10 *Analytical Results:* Method summary including Limit of Detection (LOD)/Limit of Quantitation (LOQ), deviations and reasons if any, sample summary, results including chromatograms and data qualifiers, QA summary including calibration curves, method blank and surrogate recovery, analytical results summary; and

3.8.11 *Conclusions:* Relationship between test endpoints and threshold limit.

TABLE 7—SUMMARY OF TEST CONDITIONS—DISPERSANT TOXICITY

	Acute *M. beryllina*	Acute *A. bahia*	Subchronic *M. beryllina*	Subchronic *A. bahia*	Development *S. purpuratus*
Test type	Static non-renewal	Static non-renewal	Static renewal (daily)	Static renewal (daily)	Static non-renewal.
Test duration	96 hours	48 hours	7 days	7 days	72 ± 2 hours.
Salinity	20 ± 2‰	20 ± 2‰	20 ± 2‰	20 ± 2‰	34 ± 2‰.
Temperature	25 ± 1 °C. Test temperatures must not deviate (maximum minus minimum temperature) by for than 3 °C during the test.				15 ± 1 °C.
Light quality	Ambient laboratory illumination				
Light intensity	10–20 μE/m²/s				
Photoperiod	16 h light, 8 h darkness, with phase in/out period recommended				
Test chamber size [1]	250 mL	250 mL	600 mL–1 L	400 mL	30 mL.
Test solution volume [1].	200 mL	200 mL	500–750 mL	150 mL	10 mL.
Age of test organism [2].	9–14 days	1–5 days	7–11 days	7 days	1 hr old fertilized eggs.
No. organisms per test chamber.	10	10	10	5	25 embryos per mL.
No. of replicate chambers per concentration.	3	3	4	8	4.
Feeding regime	Refer to specific feeding procedures provided in each test method				None.
Aeration	None, unless DO falls below 4.0 mg/L, then aerate all chambers. Rate:<100 bubbles/minute				
Test concentrations	5 exposure concentrations and a control (minimum required)				
Test acceptability (required).	≥90% survival in controls.	≥90% survival in controls.	For controls: ≥80% survival; average dry weight ≥0.5mg where test starts with 7 day old larvae, or ≥ 0.43 mg for larvae preserved for ≤7days.	For controls: ≥80% survival; average dry weight ≥0.20 mg; ≥ 50% of control females produce eggs.	≥80% normal shell development in controls.

[1] Recommended minimum value.
[2] *Less than or equal to 24-hr range in age.*

3.9 *References for Section 3.0*

(1) U.S. EPA. 2002. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms.* Fifth Edition. U.S. Environmental Protection Agency, Washington, DC (EPA–821–R–02–012).

(2) U.S. EPA. 1995. *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms.* First Edition. U.S. Environmental Protection Agency, Washington, DC (EPA/600/R–95–136)

(3) U.S. EPA. 2002. *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* U.S. Environmental Protection Agency, Washington, DC (EPA–821–R–02–014).

(4) U.S. EPA. 2008. *Test Methods for Evaluating Solid Waste, Physical/Chemical Methods* U.S. Environmental Protection Agency, Washington, DC (SW–846) *http://www.epa.gov/osw/hazard/testmethods/sw846/online/index.htm.*

4.0 *Standard Acute Toxicity Testing of Surface Washing Agents, Bioremediation Agents, Herding Agents, and Solidifiers.*

4.1 *Summary.* This laboratory protocol includes testing for: (1) Saltwater standard static acute toxicity tests for test products with the mysid shrimp, *Americamysis bahia* (48-hr duration) and the inland silverside, *Menidia beryllina* (96-hr duration); and (2) freshwater standard static acute toxicity tests for test products with the daphnid, *Ceriodaphnia dubia* (48-hr duration) and the fathead minnow, *Pimephales promelas* (96-hr duration) (see Table 8 of this Appendix).

TABLE 8—TOXICITY TESTING REQUIREMENTS FOR SURFACE WASHING AGENTS, HERDING AGENTS, BIOREMEDIATION AGENTS AND SOLIDFIERS

Application environment	Test procedure			
	96-hr static acute: *Menidia beryllina*	*48-hr static acute: Americamysis bahia*	96-hr static acute: *Pimephales promelas*	48-hr static acute: *Ceriodaphnia dubia*
Saltwater only	*yes*	*yes*	*no*	*no.*
Freshwater only	*no*	*no*	*yes*	*yes.*
Freshwater and saltwater use	*yes*	*yes*	*yes*	*yes.*

4.2 *Dilution Water.* Use Section 7 of EPA's *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA–821–R–02–012) [1] for preparation of the appropriate dilution water for each species tested. Use of clean natural or synthetic seawater for tests conducted with saltwater species is acceptable.

4.3 *Preparation of Stock Solutions*

4.3.1 Liquid *Surface Washing Agents and/or Herding Agents.* Prepare a 1000 mL/L stock solution prior to test initiation by adding 1.1 mL of test product to 1100 mL of dilution water in a glass vessel. Place on a magnetic stir plate then add and center a stir bar and adjust the stir plate to obtain a vortex of 25% of the total volume of the liquid. Mix the resulting stock solution for approximately five minutes at room temperature. Using a glass pipette, remove appropriate aliquots of stock solution from between the mixing vessel wall and edge of the vortex and place directly into the dilution water within an exposure vessel. Base the preparation of exposure solutions on the nominal concentration of the stock solution and follow procedures outlined in sections 4.6 and/or 4.7 of this Appendix, as appropriate.

4.3.2 *Bioremediation Agents.* For products consisting of two or more liquid and/or solid components, prepare the product following the manufacturers recommended procedure and ensure the test product mixture is completely blended. Prepare a 1000 mL/L stock solution prior to test initiation by adding 1.1 mL of the test product mixture to 1100 mL of dilution water in a glass vessel. Place on a magnetic stir plate then add and center a stir bar and adjust the stir plate to obtain a vortex of 25% of the total volume of the liquid. Mix the resulting stock solution for approximately five minutes at room temperature. Using a glass pipette, remove appropriate aliquots of stock solution from between the mixing vessel wall and edge of the vortex and place directly into the dilution water within an exposure vessel. Base the preparation of exposure solutions on the nominal concentration of the stock solution and follow procedures outlined in sections 4.5 and/or 4.6 of this Appendix, as appropriate.

4.3.3 *Solid Phase Products.* Assessment of the toxicity of solidifiers and other solid phase products are determined using the aqueous phase of water-accommodated fractions (WAFs) of the test product. Fit a glass aspirator bottle (approximately 23L) equipped with a hose bib at the base with a length of silicon tubing containing a hose clamp. Fill the bottle with 19L of dilution water leaving a 20% headspace above the liquid, place on a magnetic stir plate then add and center a stir bar. Add the test product at 25 g/L and securely seal the bottle using a silicon stopper and wraps of parafilm. Adjust the stir plate to obtain a vortex of 25% of the total fluid volume, stir for 18 hours then settle for 6 hours. Maintain the temperature at 25 °C during stirring and settling. Purge the hose at the base of the bottle of any material followed by removal of the WAF (aqueous phase) into a clean glass container without disturbing the product on the surface. The WAF should be remixed and used for the preparation of exposure solutions following procedures outlined in section 4.4 of this Appendix.

4.4 *Preparation of Exposure Concentrations*

4.4.1 *Concentration Selection.* Preliminary rangefinder tests may be necessary using a series of logarithmic concentrations (*e.g.* 0.1, 1, 10, 100 ml test product/L) to determine the appropriate exposure concentration range necessary to determine LC_{50} values and 95% confidence intervals. For definitive tests, conduct a minimum of five test concentrations using a geometric ratio between 1.5 and 2.0 (*e.g.* 2, 4, 8, 16, and 32). Note that when testing the product, the highest test concentration should not exceed the test product's self-dispersibility limit.

4.4.2 *Exposure Concentrations.* Exposure solutions are prepared by adding the appropriate amount of stock solution directly to dilution water in each test chamber. Mix each exposure solution using five rotations in one direction followed by five rotations in the opposite direction using a solid glass stir rod.

4.4.3 *Reference Toxicants.* Separate toxicity tests must be performed with a reference toxicant for each species tested. Conduct additional reference toxicity tests any time a change in the culture population or source of a test species occurs. Use reagent grade quality sodium dodecyl sulfate (SDS), also known as dodecyl sodium sulfate (DSS), and sodium lauryl sulfate (SLS) as the reference toxicant. Information on procedures for conducting reference toxicant tests with these species can be found in section 4 of EPA's *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA–821–R–02–012) (3).

4.5 *Saltwater Static Acute Tests With Menidia beryllina and Americamysis bahia*

4.5.1 *General.* Use EPA's *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA–821–R–02–012) (1) for testing each species separately with the test product.

4.5.2 *Test Solutions.* Modify procedures in EPA–821–R–02–012 specifically dealing with the handling and toxicity testing of effluents or receiving water samples as follows: Prepare stock solutions following the appropriate sections (4.3.1, 4.3.2, or 4.3.3) of this Appendix and exposure concentrations following section 4.4 of this Appendix.

4.5.3 *Number of Treatments, Replicates and Organisms.* Conduct a minimum of three replicates of at least five exposure treatments plus a minimum of three replicate dilution water controls. Expose ten organisms per replicate treatment.

4.5.4 *Exposure Period.* Test duration is 48-hr for *A. bahia* and 96-hr for *M. beryllina.* Mortality must be recorded at each 24 hour period of each test.

4.5.5 *Test Acceptability.* For each test performed, survival of control animals must be > 90% and test results must allow determination of statistically valid LC_{50} and 95% confidence interval values except in cases where the LC_{50} is >1000 ml/L or is determined to be greater than the limits of water solubility or dispersibility.

4.5.6 *Static Acute Test Summary.* A summary of required test conditions is provided in Table 9 of this Appendix.

4.6 *Freshwater Static Acute Tests With Pimephales promelas and Ceriodaphnia dubia*

4.6.1 *General.* Use EPA's *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA–821–R–02–012) (1) for testing each species separately with the test product.

4.6.2 *Test Solutions.* Modify procedures in EPA–821–R–02–012 specifically dealing with the handling and toxicity testing of effluents or receiving water samples as follows: Prepare stock solutions following the appropriate sections (4.3.1, 4.3.2, or 4.3.3) of this Appendix and exposure concentrations following section 4.4 of this Appendix.

4.6.3 *Number of Treatments, Replicates and Organisms. P. promelas:* Conduct a minimum of three replicates of at least five exposure treatments plus a minimum of three replicate dilution water controls. Expose ten organisms per replicate treatment. *C. dubia:* Conduct a minimum of four replicates of at least five exposure treatments plus a minimum of four replicate dilution water controls. Expose five organisms per replicate treatment.

4.6.4 *Exposure Period.* Test duration is 48-hr for *C. dubia* and 96-hr for *P. promelas.* Mortality must be recorded at each 24 hour period of each test.

4.6.5 *Test Acceptability.* For each test performed, survival of control animals must be > 90% and test results must allow determination of statistically valid LC_{50} and 95% confidence interval values except in cases where the LC_{50} is >1000 ml/L or is determined to be greater than the limits of water solubility of dispersibility.

4.6.6 *Static Acute Test Summary.* A summary of required test conditions is provided in Table 9 of this Appendix.

4.7 *Laboratory Report*

The laboratory must include, for each toxicity test report, all applicable information, data and analyses as follows:

4.7.1 *Test Objective:* Protocol title and source, endpoint(s);

4.7.2 *Product Information:* Product name, manufacturer contact information, lot number, production date, date received/chain of custody;

4.7.3 *Contract Facility:* Contact information;

4.7.4 *Dilution Water:* Source, pretreatment, physical and chemical characteristics (pH, salinity);

4.7.5 *Test Conditions:* Date and time of test (start and end), test chambers type and volume, volume of solution per chamber, number of organisms per chamber, number of replicate chambers per treatment, feeding frequency, amount and type of food, test concentrations, test temperature (mean and range), test salinity (mean and range);

4.7.6 *Test Organisms:* Common and scientific name, source contact information, age and date purchased, acclimation conditions (*e.g.*, temperature, salinity, both mean and range), age at test start;

4.7.7 *Reference toxicant:* Date received, lot number, date of most recent test, results

and current Cumulative Sum Chart, dilution water used, physical and chemical methods used;

4.7.8 *Quality Assurance:* Verification of laboratory accreditation, including subcontractor facilities;

4.7.9 *Test Results:* Raw data in tabular and graphical form, daily records of affected organisms in each concentration replicate and controls, table of required endpoints (*i.e.,* LC_{50}, 95% CI, inhibited concentration for 50% of the species (IC_{50}), lower observed effect concentration (LOEC) and no observed effect concentration (NOEC)), statistical methods used to calculate endpoints, summary tables of test conditions and QA data; and

4.7.10 *Conclusions:* Relationship between test endpoints and threshold limit.

TABLE 9—SUMMARY OF TEST CONDITIONS—SURFACE WASHING AGENTS, HERDING AGENTS, BIOREMEDIATION AGENTS AND SOLIDIFIERS TOXICITY

	Saltwater Acute *M. beryllina*	Saltwater Acute *A. bahia*	Freshwater Acute *P. promelas*	Freshwater Acute *C. dubia*
Test type	Static non-renewal	Static non-renewal	Static non-renewal	Static non-renewal.
Test duration	96 hours	48 hours	96 hours	48 hours.
Salinity	20 ± 2‰	20 ± 2‰	NA	NA.
Temperature	25 ± 1 °C. Test temperatures must not deviate (maximum minus minimum temperature) by more than 3 °C during the test.			
Light quality	Ambient laboratory illumination			
Light intensity	10–20 μE/m²/s			
Photoperiod	16 h light, 8 h darkness, with phase in/out period recommended			
Test chamber size [1]	250 mL	250 mL	250 mL	30 mL.
Test solution volume [1]	200 mL	200 mL	200 mL	15 mL.
Age of test organism [2]	9–14 days	1–5 days	1–14 days	<24 hours.
No. organisms per test chamber	10	10	10	5.
No. of replicate chambers per concentration (minimum).	3	3	3	4.
Feeding regime	Refer to specific feeding procedures provided in each test method			
Aeration	None, unless DO falls below 4.0 mg/L, then aerate all chambers. Rate: <100 bubbles/minute			
Test concentrations	5 exposure concentrations and a control (minimum required)			
Test acceptability (required)	≥90% survival in controls			

[1] Recommended minimum value.
[2] Less than or equal to 24-hr range in age.

4.8 *References for Section 4*

(1) U.S. EPA. 2002. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms.* Fifth Edition. U.S. Environmental Protection Agency, Washington, DC (EPA–821–R–02–012).

5.0 *Bioremediation Agent Efficacy Test Protocol*

5.1 *Summary.* This protocol quantifies changes in weathered Alaska North Slope (ANS) crude oil composition of alkanes and aromatics resulting from the use of a bioremediation agent in either artificial seawater or freshwater. The manufacturer may test either one or both freshwater or saltwater, depending on the product's intended use. Biodegradation of the alkanes and aromatics is monitored for 28 days at 20–23 °C. Product flasks at Day 28 are compared to Day 0 flasks to determine reductions in alkanes and aromatics. A positive control of a known oil-degrading bacterial culture supplied by EPA is tested. A negative, sterile control is also set up containing exposure water, weathered crude oil, product, and a sterilant, sodium azide. The purpose of the negative, killed control is to make sure the disappearance of the oil constituents at day 28 is due to biodegradation and not some physical loss such as volatilization. The day 28 GC/MS results from the killed control must not be less than 90% of the day 0 results. The sample preparation procedure extracts the oil phase into the solvent dichloromethane (DCM) (also known as methylene chloride) with a subsequent solvent exchange into hexane. The hexane extracts are analyzed by a high resolution gas chromatograph/mass spectrometer (GC/MS) operated in the selected ion monitoring mode (SIM) at a scan rate of >5 scans per second. Note to 5.1: Artificially distilled at 521 °F (272 °C) to remove the low molecular weight hydrocarbons to approximate natural weathering processes that occur after a spill.

5.2 Apparatus. All equipment must be maintained and calibrated per standard laboratory procedures.

5.2.1 Assorted flasks and other glassware;

5.2.2 Graduated cylinders (100 mL);

5.2.3 Deionized water; 250 mL borosilicate glass Erlenmeyer flasks;

5.2.4 Pasteur pipettes;

5.2.5 Multichannel pipettor (5–50 mL and 50–200 mL);

5.2.6 Autoclave; environmental room or incubator;

5.2.7 Balance accurate to 0.1 mg;

5.2.8 Orbital shaker table with clamps sized to hold flasks securely;

5.2.9 GC/MS instrument equipped with a DB–5 capillary column (30 m, 0.25 mm ID, and 0.25 mm film thickness) or equivalent, and a split/splitless injection port operating in the splitless mode, such as a Agilent 6890 GC/5973 MS (or equivalent) equipped with an auto-sampler for testing multiple samples; and

5.2.10 Fixed Rotor Centrifuge.

5.3 *Reagents and Culture Medium*

5.3.1 *Stock Seawater Preparation.* Prepare the artificial seawater GP2 (Spotte *et al.,* 1984) following the procedures in section 2.3 of this Appendix, to obtain the final concentration of the salts listed in Table 1 of this Appendix, except for the sodium bicarbonate ($NaHCO_3$) which is prepared separately. Autoclave the artificial seawater. Filter sterilize the concentrated solution of sodium bicarbonate through a 0.45 mm membrane filter and add to the autoclaved and cooled artificial seawater GP2 to obtain the final concentration listed in Table 1 of this Appendix.

5.3.2 *Seawater for the positive control flasks.* Prepare sodium triphosphate (a.k.a.,

sodium tripolyphosphate) ($Na_5P_3O_{10}$), potassium nitrate (KNO_3), and ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) as a concentrated solution. Filter sterilize through a 0.45 mm membrane filter and add to autoclaved artificial seawater to obtain the final nutrient concentrations listed in Table 10 of this Appendix. Calibrate the pH meter at room temperature (approximately 20–23 °C) using commercial buffers of pH 4.0, 7.0, and 10.0, as appropriate, prior to use. Adjust the pH of the artificial seawater with concentrated hydrochloric acid (HCl) or 10 normality sodium hydroxide (10 N NaOH), as appropriate.

TABLE 10—ARTIFICIAL SEAWATER NUTRIENT CONCENTRATIONS

Constituent	Final concentration, g/L
*$FeCl_3 \cdot 6H_2O$	0.050
KNO_3	2.890
*$Na_5P_3O_{10}$	0.297

*Added aseptically after the GP2 has been autoclaved to limit phosphorus and iron precipitation.

5.3.3 Seawater *for bioremediation agents that do not include nutrients.* If a bioremediation agent contains living microorganisms but not nutrients (or limiting concentrations of nutrients), then nutrients may be added by the manufacturer. However, the total concentration of the nutrients added to the bioremediation agent must not exceed the final concentrations listed in Table 11 of this Appendix.

TABLE 11—ARTIFICIAL SEAWATER NUTRIENT CONCENTRATIONS FOR BIOREMEDIATION AGENTS HAVING NO NUTRIENTS INCLUDED

Constituent	Final concentration, g/L
as Iron (Fe)	0.010
as Nitrogen (N)	0.400
as Phosphorus (P)	0.075

If nutrients are supplied by the product manufacturer, the specific composition and concentration used in the efficacy testing must be submitted.

5.3.4 *Freshwater Preparation.* The artificial freshwater, which is a modification of Bushnell-Haas medium (Haines *et al.*, 2005), is prepared following the concentrations listed in Table 12 of this Appendix and then autoclaved. The pH is adjusted to 7.4 before autoclaving. Constituents removed from the original formulation are KNO_3, K_2HPO4 and KH_2PO_4.

TABLE 12—CONSTITUENT CONCENTRATIONS FOR ARTIFICIAL FRESHWATER

[Bushnell-Haas]

Constituent	Final concentration (mg/L)
$MgSO_4 \cdot 7H_2O$	200
$CaCl_2 \cdot 2H_2O$	20
$FeCl_3 \cdot 6H_2O$	50
$MnSO_4 \cdot H_2O$	0.0302
H_3BO_3	0.0572
$ZnSO_4 \cdot 7H_2O$	0.0428
$(NH_4)_6Mo_7O_2$	0.0347

5.3.5 *Freshwater for the positive control.* To prepare the freshwater for the positive controls, prepare the nutrients potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4) and potassium nitrate (KNO_3) as a concentrated solution. Filter sterilize and add to autoclaved artificial freshwater to obtain the final concentrations given in Table 13 of this Appendix. Calibrate the pH meter at room temperature (approximately 20–23 °C) using commercial buffers of pH 4.0, 7.0, and 10.0, as appropriate, prior to use. Adjust the pH of the artificial freshwater to 7.4 with 1 N HCl or 1 N NaOH, as appropriate.

TABLE 13—FRESHWATER NUTRIENT CONCENTRATIONS

Constituent	Final concentration (g/L) [1]
KNO_3	2.89
KH_2PO_4	1.00
K_2HPO_4	1.00

[1] Adjust pH to 7.4 prior to autoclaving

5.3.6 *Freshwater for bioremediation agents that contain living microorganisms but not nutrients or limiting concentrations of nutrients.* If a bioremediation agent does not include nutrients, then nutrients may be added. However, the total concentration of the nutrients added to the bioremediation agent must not exceed the final concentrations provided in Table 14 of this Appendix.

TABLE 14—ARTIFICIAL FRESHWATER NUTRIENT CONCENTRATIONS FOR BIOREMEDIATION AGENTS HAVING NO NUTRIENTS INCLUDED

Constituent	Final concentration, g/L [1]
as Iron (Fe)	not added since iron is already in the freshwater solution.
as Nitrogen (N)	0.400.
as Phosphorus (P)	0.400.

[1] Adjust to pH 7.4 prior to autoclaving.

If nutrients are supplied by the product vendor, the specific composition and concentration used in the efficacy testing must be submitted.

5.3.7 *Oil Preparation.* The test oil, weathered ANS521 crude oil, can be obtained from EPA at no charge (except for a minimal shipping fee). See *http://www.epa.gov/emergencies/content/ncp/index.htm* for more information.

5.3.8 *Sodium azide sterilant.* Prepare a stock solution of NaN_3 for addition to the negative killed control. The final concentration in the killed controls will be 0.5 g/L.

5.4 *Experimental Setup and Procedure*

5.4.1 Autoclave clean borosilicate glass Erlenmeyer flasks (250 mL) for 20 minutes at 121 °C at 15 psig.

5.4.2 Label flasks with the appropriate code (negative control, positive control, or product; day to be sampled (0 or 28); letter indicating replicate number) to reflect the following treatment design in Table 15 of this Appendix:

TABLE 15—BIOREMEDIATION EFFICACY TEST—SUMMARY OF EXPERIMENTAL SETUP

Treatment	Number of replicates at sampling times		Analysis
	Day 0	Day 28	
Negative (killed) Control (oil + exposure water + product + EPA culture + NaN_3 sterilant)	0	3	GC/MS
*Positive control (oil + exposure water + nutrients + EPA culture)	6	6	GC/MS
Test Type 1: Product containing living microorganisms (oil + exposure water + living product + supplemented nutrients (if necessary)).	6	6	GC/MS
Test Type 2: Product containing proprietary nutrients but no live microorganisms (oil + exposure water + product + EPA culture).	6	6	GC/MS
Test Type 3: Product (such as an enzyme) containing no live microorganisms and no nutrients (oil + exposure water + product).	6	6	GC/MS

*The laboratory must report positive control test results conducted within the year of any test results for bioremediation products, for one or both types of water as applicable.

5.4.3 Aseptically dispense 100 mL of pre-sterilized artificial exposure water (seawater or freshwater) into each flask. For the positive control flasks, use exposure water containing nutrients. Alternatively, the artificial exposure water can be dispensed into clean flasks and then autoclaved.

5.4.4 Tare the labeled flasks containing exposure water and other additions, as necessary, on the balance with a minimum accuracy of 0.01 g. Add drop-wise 0.50 g oil (this results in a final oil concentration of 5 g/L) using a sterile Pasteur pipette to the center of the flask taking care to avoid splashing the oil onto the sides of the flasks. Record the precise weight. ANS521 may be previously warmed in a hot water bath to facilitate its flow. Take precautions when handling and charging the flasks to minimize the likelihood of contamination by exogenous microbes, including using a new sterile pipette for each series of flasks.

5.4.5 Preparation of the EPA culture for both the positive control flasks and the flasks containing non-living bio-stimulation products. Use two vials containing approximately 5 mL of the known EPA culture frozen in glycerol. Thaw both vials at room temperature, transfer the contents of both thawed vials to a single sterile centrifuge tube, rinse tubes with two volumes each of sterile exposure water, centrifuge at between 6,000 and 7,000 ×s gravity (6,000–7,000 × *g*) for 15 minutes using a fixed rotor to fully pellet the cells.

5.4.6 Positive control flasks contain exposure water, oil, nutrients, and the EPA culture.

5.4.7 Negative killed control flasks for all products shall contain exposure water, oil, product, the EPA culture for products not containing a living culture, and the sodium azide sterilant at a final concentration of 0.5 g/L. Add the sodium azide sterilant prior to adding any product or EPA culture. For the negative killed control flasks and product flasks, prepare and add the product to the flasks in a concentration specified by the manufacturer or vendor.

5.4.8 For non-living products that contain nutrient only, use the EPA culture as the inoculum.

5.4.9 For other non-living products (*e.g.*, enzymes), do not add nutrients or the EPA culture as the inoculum as they are not needed.

5.4.10 For products containing microbial cultures, prepare 6 flasks the same way as in Steps a-d, but without the EPA culture. A product that contains its own nutrients must not be amended with nutrients, unless the product contains insufficient nutrients. Since this is a closed flask test, nutrients could be limiting if they are at the same concentration as used in the field. This could cause the product to fail the test. Thus, the manufacturer has the option to supplement its product with a higher concentration of nutrients than that contained in the product. Any nutrient supplements to a product must be reported and must not exceed the concentration limits in Table 10 (for seawater) and 13 (for freshwater) of this Appendix, as applicable.

5.4.11 Cap all flasks either with cotton stoppers or loosely applied aluminum foil to allow gas exchange with the atmosphere. Set aside the T=0 flasks for immediate extraction and analysis. Place the rest of the flasks onto the orbital shaker table. Do not tip the flasks excessively to avoid stranding oil above the mixing area of the flask. Set the orbital shaker to 200 rpm and shake the flasks for 28 days at 20–23° C.

5.4.12 Submit all information on added cultures and nutrients for testing in the data report.

5.5 *Sampling and Chemical Analysis*

5.5.1 *Summary.* At each sampling event (Days 0 and 28), product and control flasks are sacrificed for analysis of residual oil concentrations (SOP 4 of this Appendix). Record all physical observations for each flask (such as degree of emulsification, whether the oil has congealed into tar balls, wall growth, color, etc.) at each sampling. The analytical procedure is summarized in Table 16 of this Appendix. Dichloromethane (DCM) is the solvent used for the initial extraction. Solvent-exchange the extract into hexane prior to injection into the gas chromatograph. The solvent exchange is done to prevent asphaltenes from contaminating the column.

TABLE 16—BIOREMEDIATION EFFICACY—SUMMARY OF ANALYTICAL PROCEDURES

Matrix	Measurement	Sampling/measurement method	Analysis method	Sample container/quantity of sample	Preservation/storage	Holding times
DCM	N/A	Solvent Exchange to Hexane.	N/A	Capped Vial with Teflon septa, 30 mL.	4 °C	6 months.
Hexane	Hydrocarbon Concentration	SOP 4	GC/MS	Capped Vial with Teflon septa, 10 mL.	4 °C	6 months.

5.5.2 *Hydrocarbon Extraction.* To measure extraction efficiency, 500 mL of the surrogate recovery standard (compounds and concentrations described in SOP 1 in this Appendix) is added to each flask. Add 50 mL DCM to each flask. Use magnetic stir bars to stir the flasks for 20 minutes to transfer the oil into the DCM phase. Allow the flasks to stand one hour for phase separation. If an emulsion remains after one hour, centrifuge the emulsion in Teflon® centrifuge tubes for at least ten minutes in a low speed centrifuge at 3,000 × gravity (3,000 × *g*) to break the emulsion and recover the DCM phase. Pass the DCM extract through a funnel plugged with glass wool and containing approximately 20 g anhydrous, granular sodium sulfate (Na_2SO_4) to remove water. Collect the extract in 30 mL serum vials, capped with Teflon lined septa and aluminum crimp seals, and store at 4 °C for up to 6 months.

5.5.3 *Solvent Exchange.* Perform a solvent exchange (DCM to hexane) prior to GC/MS analysis to prevent injection of asphaltenes into the GC/MS column. Using a Class A volumetric pipette, transfer10 mL of DCM extract to 16 x 125 mm test tubes with Teflon lined screw caps. Place the test tubes in a 37° C water bath under a stream of dry nitrogen gas. Reduce the sample to 1 mL and return to volume with hexane. Repeat the blow down and re-suspension twice to exchange the hydrocarbons from DCM into the hexane.

5.5.4 *Hydrocarbon Analysis.* Quantify the concentrations of 25 alkanes (including hopane) and 31 aromatics (SOP 4, Table SOP 4.1 of this Appendix) using an Agilent 6890 GC/5973 MS or equivalent equipped with a 30-m × 0.25-mm ID × 0.25-mm film thickness DB–5 or equivalent fused silica column. To prepare the samples, transfer 1.0 mL of the hexane extract into a 2 mL autosampler vial with Teflon lined cap. Add 20 mL of internal standard solution to each vial with a syringe or positive displacement pipettor. SOP 2 of this Appendix outlines the procedure for preparing the internal standard solution. Load vials onto the autosampler tray and analyze in selected ion monitoring mode (SIM). Sum the individual alkane concentrations for the total alkane concentration and the individual aromatic concentrations for total aromatic concentrations in each flask.

5.6 *Quality Assurance/Quality Control (QA/QC)*

5.6.1 *Objectives.* The critical variables to be analyzed for each set of experimental conditions are the individual petroleum hydrocarbons, *i.e.,* the alkanes ranging in carbon number from nC–14 to nC–35, plus pristane and phytane, and the 2- to 4-ring polycyclic aromatic hydrocarbons (PAHs) and their alkylated homologs as listed in SOP 4 of this Appendix. The quality assurance objectives for precision, accuracy, and detection limits are ± 20%, 75–125% recovery, and 22.5 mg/L, respectively. For more details, refer to the SOPs of this Appendix.

5.6.2 *Precision Objectives.* Precision is presented as relative percent difference (RPD) for duplicate measurements and as relative standard deviation (RSD, or coefficient of variance) for triplicate measurements, applicable to replication of treatments as separate samples.

5.6.3 *Accuracy Objectives.* These are based on the check standards and standard oil samples run concurrently with the sample analyses for GC/MS analysis of critical

compounds. Critical compounds in the check standards and in the oil standards must fall within 75–125% of expected values for the analysis to be valid. Six surrogate compounds (SOP 1 of this Appendix) added to each sample before extraction can also serve as a surrogate for determining accuracy. The measured surrogate concentrations must fall within 75–125% of expected values.

5.6.4 *Calibration Range.* Conduct all measurements within the linear calibration range of the instrument. The calibrated concentration range for GC/MS analysis is 1 mg/L to 30 mg/L. If the measured concentration of any critical compound is above the calibration range, dilute the sample and re-analyze to quantify that particular compound within the linear calibration range.

5.6.5 *Quality Control.* Table 17 of this Appendix summarizes the QC checks for each measurement. See the corresponding SOP in this Appendix for detailed descriptions of QC checks, frequency, acceptance criteria, and corrective actions.

TABLE 17—QA/QC CHECKS

Sample matrix	Measurement	QA/QC check	Frequency	Acceptance criteria	Corrective action
DCM	GC/MS hydrocarbon analysis.	Blanks	Once per calibrated run	Peak area of interfering peaks <10% of lowest standard peak area.	Flush with solvent, clean injection port, and/or bake column.
DCM	GC/MS hydrocarbon analysis.	DFTPP Check Standard	Once per calibrated run	Must pass all DFTPP criteria.	If any criteria fail, retune and rerun DFTPP check standard.
DCM	GC/MS hydrocarbon analysis.	Initial Calibration Samples.	Once per calibrated run	Response Factor RSD ≤25%.	If RSD for any one compound >25%, recalibrate.
DCM	GC/MS hydrocarbon analysis.	Calibration Check Standards.	Every 10–15 samples	± 25% of expected values.	If >5 compounds are out of range, recalibrate and rerun samples.
Hexane	GC/MS hydrocarbon analysis.	Surrogates	Every Sample	± 30% of expected values.	Re-inject.
Hexane	GC/MS hydrocarbon analysis.	Biomarker Concentration.	Every Sample	± 25% of average values.	Re-inject.

5.7 *Pass/Fail Criteria*

5.7.1 Calculate the mean and standard deviation of the hopane-normalized total aromatics (sum of all resolved aromatics) and hopane-normalized total alkane concentrations (sum of all resolved alkanes) from the 6 independent replicates at days 0 and 28. To normalize, divide the sum of the alkane analytes and the sum of the aromatic analytes in each replicate by the hopane concentration in the corresponding replicate.

5.7.2 From those data, calculate the 95% Upper Confidence Level (UCL_{95}) at days 0 and 28 using the following formula (Equation 11 of this Appendix):

$$UCL_{95} = \bar{x}_{t(0and28)} + \left(\frac{t_{95,5df} \times \sigma}{\sqrt{n}} \right) \qquad \text{(Equation 11)}$$

Where:

x_{t28} = total hopane-normalized alkane or total hopane-normalized aromatic mean of 6 replicates at days 0 and 28,

$t_{95,\ 5\ df}$ = the 95% one-tailed t-value with 5 degrees of freedom (2.015),

s = the standard deviation of the 6 replicates at day 0 and 28, and

n = no. of replicates = 6.

5.7.3 Using Equation 12 of this Appendix, calculate the % reduction of each oil fraction from day 0 to day 28, using the day 0 and 28 UCL_{95} hopane-normalized values for each fraction:

$$\%\ reduction = 100 \times \left[1 - \left(\frac{t_{28(UCL95)}}{t_{0(UCL95)}} \right) \right] \qquad \text{(Equation 12)}$$

Where:

$t_{28(UCL95)}$ = UCL_{95} of the hopane-normalized total alkane or total aromatic mean of 6 replicates on day 28, and

$t_{0(UCL95)}$ = UCL_{95} of the hopane-normalized total alkane or total aromatic mean of 6 replicates on day 0.

5.7.4 A product is successful in saltwater if the % reduction of total alkanes (aliphatic fraction) from the GC/MS analysis is greater than or equal to 95% and the % reduction of total aromatics (aromatic fraction) is greater than or equal to 70% at day 28 based on the UCL_{95} (Equation 12 of this Appendix). For freshwater, a product is successful if the % reduction of total alkanes and total aromatics is greater than or equal to 95% and 40%, respectively. The benchmark reduction ranges in aliphatic and aromatic fractions for the positive control are the same as for the products specified above. The average concentration of the biomarker hopane at day 28 must not differ from the average concentration at day 0 by more than 12% in the positive control. If the conditions for the positive control are not met, the entire procedure must be repeated.

5.8 *Data Verification and Reporting.* GC/MS data files are generated by MS ChemStation software (the Agilent standard software for GC/MS) or equivalent for each injection. Data files contain summed ion chromatograms and selected ion chromatograms. Calibration curves are generated within MS ChemStation software, and all data files are calculated against the calibration curve by MS ChemStation. Data verification would be done by crosschecking between analysts for 10% of the raw data and its reduction process.

5.9 *Laboratory Report.* The summary of findings from a product test must include the data listings for each analyte that was analyzed (*i.e.,* all individual alkanes and aromatics in the list of required analytes). Express all concentrations as mg analyte/L exposure water.

5.10 *Standard Operating Procedures (SOPs) 1–4*

5.10.1 *SOP 1. Preparation of Surrogate Recovery Standards*

5.10.1.1 *Preparation:*

5.10.1.1.1 Solvents: Dichloromethane (DCM), Optima (Fisher) grade or equivalent.

5.10.1.1.2 Reagents: D36-Heptadecane (C17), D50-Tetracosane (C24), D66-Dotriacontane (C32), D10–1-Methylnaphthalene, D10-Phenanthrene, D10-Pyrene, 5-beta-cholestane (coprostane).

Note: Deuterated reagents are available from Cambridge Isotope Laboratories, Andover, MA.

5.10.1.1.3 Equipment: Micro-spatula, Small beakers, Glass funnel, Analytical balance (0.0001g), Vials with Teflon-lined caps, Teflon wash bottle with Optima grade DCM, Volumetric flask (250 mL), class A, Pasteur pipettes.

5.10.1.2 *Procedure:*

5.10.1.2.1 Using a calibrated analytical balance, weigh 100 mg (0.100 g) of each reagent into separate 10–25 mL beakers.

5.10.1.2.2 Dissolve the reagents in their beakers by adding 10 mL DCM. Use a Pasteur pipette to transfer the solutions to a single 250 mL volumetric flask.

5.10.1.2.3 Wash the beakers 3 or 4 times with DCM. Use a Pasteur pipette to transfer each of the washings to the 250 mL volumetric flask.

5.10.1.2.4 Dilute the solution to the 250 mL volume mark on the volumetric flask with DCM.

5.10.1.2.5 Use a glass stopper to seal the flask and homogenize the solution by inverting the flask 5 or more times. The final concentration of this solution is 400 mg/L for each of the reagents.

5.10.1.2.6 Transfer the solution into 40 mL storage vials and cap with Teflon-lined caps and label each with the date of preparation, operator, sample names, and concentrations.

5.10.1.2.7 Weigh each vial and record its weight on the label. This weight is used to monitor possible evaporation during storage.

5.10.1.2.8 Store these vials at 0 °C or lower.

5.10.1.2.9 Before using, allow the solution to come to room temperature, and then shake it well.

5.10.1.2.10 Weigh the vial before using it, and compare the weight with the last weight recorded on the vial.

5.10.1.2.11 If the weights are consistent, the integrity of the solution can be assumed. If not, investigate and resolve the cause. Prepare a new solution if the integrity has been compromised.

5.10.1.3 *Quality Control:* Inject 20 mL of the surrogate stock solution into 1 mL DCM. Add 20 mL of the internal standard solution (SOP 2 of this Appendix). Analyze this solution by GC/MS using a calibrated method (SOPs 3 and 4 of this Appendix). The expected concentration of each of the corresponding surrogate compounds is 8 ± 2 mg/L. If the measured value does not fall within this range, prepare and measure another independent surrogate solution. If the measured concentration of the second surrogate solution is within the allowable tolerance range, the calibration and instrument conditions are acceptable; properly discard the first surrogate solution. If the concentration of the second surrogate solution is also out of range, then clean and recalibrate the instrument until the problem is resolved.

5.10.2 *SOP 2. Preparation of Internal Standard Solution*

5.10.2.1 *Preparation*

5.10.2.1.1 Solvents: Dichloromethane (DCM), Optima (Fisher) grade or equivalent.

5.10.2.1.2 Reagents: D34 n-Hexadecane (C16), D42 n-Eicosane (C20), D62 n-Triacontane (C30), D8-Naphthalene, D10-Anthracene, D12-Chrysene, 5-alpha-Androstane.

Note: Deuterated reagents are available from Cambridge Isotope Laboratories, Andover, MA.

5.10.2.1.3 Equipment: Micro-spatula, Small beakers, Glass funnel, Analytical balance (0.0001g), calibrated and checked for accuracy, Amber vials with Teflon-lined caps, labeled, Teflon wash bottle with DCM, Volumetric flask (200 mL), class A, Pasteur pipettes.

5.10.2.2 *Procedure:*

5.10.2.2.1 Using a calibrated analytical balance, weigh 100 mg (0.100 g) of each of the reagents into separate small beakers.

5.10.2.2.2 Dissolve the reagents in their beakers by adding 10 mL DCM; using a Pasteur pipette, transfer the solutions to a single 200 mL volumetric flask.

5.10.2.2.3 Wash the beakers 3 or 4 times with DCM; use a Pasteur pipette to transfer each of the washings to the 200 mL volume mark on the volumetric flask.

5.10.2.2.4 Dilute the solution with DCM to the 200 mL volume.

5.10.2.2.5 Seal the flask with a glass stopper and homogenize the solution by inverting the flask a minimum of 5 times. The final concentration of this solution is 500 mg/L of each reagent.

5.10.2.2.6 Transfer the solution into 40 mL storage vials and cap with Teflon-lined caps. Label each vial with the date of preparation, operator, sample names, and concentrations.

5.10.2.2.7 Weigh each vial, and record its weight on the label. This weight is used to monitor possible evaporation during storage.

5.10.2.2.8 Store this solution at 0 °C or lower.

5.10.2.2.9 Before using, allow the solution to come to room temperature, and then shake it well.

5.10.2.2.10 Weigh the vial before using it, and compare the weight with the last weight recorded on the vial.

5.10.2.2.11 If the weights are consistent, the integrity of the solution can be assumed. If not, investigate and resolve the cause. Prepare a new solution if the integrity has been compromised.

5.10.2.3 *Quality Control:* Inject 20 mL of the internal standard solution into 1 mL DCM. Analyze this solution by GC/MS. The only peaks corresponding to the internal standards must appear. If other peaks appear, particularly close to the internal standard peaks, discard the internal standard solution and prepare a new solution.

5.10.3 *SOP 3. Preparation of Working Standards, Check Standards, and Oil Standards for GC/MS Consistency.*

5.10.3.1 *Preparation:*

5.10.3.1.1 Solvent: Dichloromethane (DCM), Optima (Fisher) grade or equivalent.

5.10.3.1.2 Stock solutions:

5.10.3.1.2.1 Oil analysis standard: 44 compounds, 100 mg/L in hexane/DCM (9:1), four, 1-mL vials required. Available from Absolute Standards, Inc., Hamden, CT, Part # 90311.

5.10.3.1.2.2 Nine compound PAH standard: 1,000 mg/L in DCM, one vial. Available from Absolute Standards, Inc., Hamden, CT, Part # 90822.

5.10.3.1.2.3 1,2-Benzodiphenylene sulfide, (synonym for naphthobenzothiophene). Prepare a 2 mg/mL stock solution. Available from Sigma-Aldrich Co., Part # 255122, purity 99%.

5.10.3.1.2.4 Hopane solution (17 b (H), 21b (H), 0.1 mg/mL in isooctane. Available from Sigma-Aldrich Co. Part # 07562.

5.10.3.1.2.5 Surrogate solution: 400 mg/L of each reagent in DCM (see SOP 1 of this Appendix).

5.10.3.1.2.6 Internal standard solution, 500 mg/L in DCM (see SOP 2 of this Appendix).

5.10.3.1.3 Alaska North Slope Crude Oil 521 (ANS521).

5.10.3.1.4 Equipment:

5.10.3.1.4.1 Glass storage vials with Teflon-lined caps (2 mL and 40 mL capacity);

5.10.3.1.4.2 Volumetric flasks, Class A, 5 mL, 10 mL, and 100 mL.

5.10.3.1.4.3 Glass syringes capable of dispensing 25–500 mL with an accuracy and precision of ± 1%, or equivalent.

5.10.3.1.4.4 Wheaton repetitive dispenser, Model 411 STEP–PETTE or equivalent.

5.10.3.1.4.5 Teflon wash bottle filled with Optima grade DCM or equivalent grade DCM.

5.10.3.1.4.6 Pasteur pipettes.

The volumes of stock solutions required to make the working standards are listed in Table SOP 3.1 of this Appendix.

TABLE SOP 3.1—AMOUNT OF STOCK SOLUTIONS REQUIRED TO MAKE THE WORKING STANDARDS

Stock standards	A	B	C	D	E		F
Working standards concentration, mg/L	Oil Analysis Mix (44 compounds, 100 mg/L) µL	Aromatics Mix (9 compounds, 1000 mg/L) µL	1,2-Benzodiphenylene sulfide (NBT) (2 mg/mL) µL	Surrogate solution (100 mg/L) µL	Hopane solution (100 mg/L) µL	Volumetric Flask Volume mL	ISTD (500 mg/L) µL
STD 30 (no hopane)	1500	150	75	375	0	5	100
STD 20 (5 mg/L hopane)	1000	100	50	250	250	5	100
STD 10 (2.5 mg/L hopane)	500	50	25	125	125	5	100
STD 5* (1 mg/L hopane)	500	50	25	125	100	10	200
STD 5-Utility (1 mg/L hopane)	500	50	25	125	100	10 (used for preparation of STD 2.5 & STD 1).	0
STD 2.5 (0.5 mg/L hopane)	Use 5 mL of STD 5-Utility and dilute to 10 mL						200
STD 1 (0.2 mg/L hopane)	Use 2 mL of STD 5-Utility and dilute to 10 mL						200

*Make extra STD 5 for use as check standard.

5.10.3.2 *Procedure for Working Standards and Check Standards:*

5.10.3.2.1 Label three 5 mL volumetric flasks as STD30, STD20, STD10, and two 10 mL volumetric flasks as STD5, and STD5-utility.

5.10.3.2.2 Add 1–2 mL of DCM to each volumetric flask.

5.10.3.2.3 Using glass syringes, add the appropriate volume of stock solution A (as listed in Table SOP 3.1 of this Appendix) to the flasks labeled STD30, STD20, STD10, STD5, and STD5-utility.

5.10.3.2.4 Wash the walls of the inner neck of the flasks with several drops of DCM to rinse off the residue of the stock solution into the flasks.

5.10.3.2.5 Repeat Step 3 and Step 4 to dispense stock solutions B–E (do not add stock solution F, internal standard solution, at this step).

5.10.3.2.6 Dilute to volume with DCM for all the above flasks, seal with glass stoppers, and invert several times to homogenize the solutions.

5.10.3.2.7 Label two additional 10 mL volumetric flasks as STD2.5 and STD1. Wet with 1–2 mL DCM.

5.10.3.2.8 Dispense 5 mL of STD5-utility solution into flask STD2.5, and 2 mL of STD5-utility solution into flask STD1.

5.10.3.2.9 Dilute to volume with DCM, seal with glass stoppers, and invert several times to homogenize the solutions.

5.10.3.2.10 Using a 100 mL glass syringe, dispense 100 mL of internal standard solution into flasks STD30, STD20, and STD10. Dispense 200 mL into flasks STD5, STD2.5, and STD1 to give a final concentration of 10 mg/L internal standard.

5.10.3.2.11 Seal with glass stoppers, and invert the flasks several times to homogenize the solutions.

5.10.3.2.12 Transfer the solutions into 2 mL storage vials, and cap with Teflon-lined caps.

5.10.3.2.13 Label each vial with date of preparation, analyst, sample names, and concentrations.

5.10.3.2.14 Weigh each storage vial and record its weight on the label. This weight is used to monitor possible evaporation during storage.

5.10.3.2.15 Store this solution at 0 °C or below.

5.10.3.2.16 Before using, allow the solution to come to room temperature, and shake it well.

5.10.3.2.17 Weigh the vial before opening, and compare the weight with the last weight recorded on the vial. If the weights are consistent, the integrity of the solution can be assumed. If not, investigate and resolve the cause. Do not use the solution if the integrity has been compromised.

5.10.3.3 *Procedure for Oil Standard.* In a 100 mL volumetric flask, weigh 0.500 g of the standard ANS521 crude oil, add 2 mL of surrogate solution (see SOP 1 of this Appendix), and bring to volume with DCM. Add 2 mL of internal standard solution (see SOP 2 of this Appendix). Follow steps 5.10.3.2.11 through 5.10.3.2.17 of this SOP, substituting 40 mL storage vials for the 2 mL vials.

5.10.3.4 *Quality Control/Quality Assurance:*

5.10.3.4.1 Run the six standard solutions using the GC/MS method (SOP 4) on a tuned GC/MS. Use the EnviroQuant software or equivalent to calculate the average Relative Response Factor (RRF) and the relative standard deviation (RSD) of the RRFs for each analyte over the six concentrations. The RRF is defined as:

$$RRF = \frac{\text{area analyte}}{\text{area internal standard}} \times \frac{\text{concentration of internal standard}}{\text{concentration of analyte}} \qquad \text{(Equation 13)}$$

5.10.3.4.2 The RSD of the RRFs for all analytes must be 25% or less according to EPA Method 525.2.

5.10.4 *SOP 4. GC/MS Method for the Analysis of Crude Oil Samples.*

5.10.4.1 *Instrument Specifications:*

5.10.4.1.1 Use an Agilent 6890 GC coupled with an Agilent 5973 mass selective detector (MSD) and an Agilent 6890 series auto sampler or equivalent, equipped with a DB–5 capillary column (30 m, 0.25 mm I.D., and 0.25 mm film thickness) or equivalent, and a split/splitless injection port operating in the splitless mode. Data acquisition occurs either in the SIM (selected ion monitoring) mode for quantitative analysis or in SCAN mode for qualitative analysis. In SIM mode, the dwell time of each ion is set to be 10 milliseconds and the ions are split up into groups by retention time. One way to divide the ions is by retention time grouping as shown in Table SOP 4–1 of this Appendix. The number of ions in each ion group must be constant, yielding the same scan rate for each group.

TABLE SOP 4–1—IONS ASSOCIATED WITH RETENTION TIME GROUPS

Group	Ions
1	57, 66, 128, 136, 142, 152, 156, 166, 170, 184
2	57, 66, 166, 170, 178, 180, 184, 188, 192, 194, 198, 208
3	57, 66, 178, 184, 188, 192, 194, 198, 202, 206, 208, 212, 220, 226

TABLE SOP 4–1—IONS ASSOCIATED WITH RETENTION TIME GROUPS—Continued

Group	Ions
4	57, 66, 192, 198, 202, 206, 208, 212, 216, 220, 226, 230, 234, 245
5	57, 66, 191, 217, 228, 240, 242, 248, 256, 262, 264, 270, 276, 284

5.10.4.1.2 Table SOP 4.2 of this Appendix summarizes the instrumental conditions for crude oil analysis. Use only ultra-high purity helium (99.999% pure) as the carrier gas. In series, connect a moisture trap, an oxygen trap, and an organic trap to the carrier gas line before it enters the column.

TABLE SOP 4.2—INSTRUMENTAL CONDITIONS FOR CRUDE OIL ANALYSIS

Instrument	Agilent 6890 Series II Gas Chromatograph (GC) with an Agilent 5973MSD and an Agilent 6890 auto sampler, or equivalent.
Column	DB–5 capillary column (30 m, 0.25 mm I.D., and 0.25-μm film thickness) or equivalent.
Carrier Gas	Helium, ultra-high purity grade (99.999%).
Inlet Temperature	300 °C.
Transfer Line (detector) Temperature.	310 °C.
Oven Temperature Program.	50 °C for 4 minutes, then 7 °C/min to 310 °C, hold for 18 minutes.
Flow Rate	Constant flow at 1mL/min. Linear velocity: 36.2 cm/sec.
Injection Volume	1 μL.
Split/Splitless Mode	Splitless.
Total Run Time	59.18 minutes.

5.10.4.2 *Procedure for preparing the instrument:*

5.10.4.2.1 Lower the injection port temperature and the oven temperature to 50° C or less to avoid oxidation of the column.

5.10.4.2.2 Replace the liner with a clean, silanized liner. Do not touch the liner with bare fingers. A small piece of muffled glass wool may be inserted to protect the column.

5.10.4.2.3 Return the injection port and oven to the appropriate temperatures.

5.10.4.2.4 Wait five minutes after the temperature equilibrates before using the instrument.

5.10.4.3 *Procedure for tuning the MSD:*

5.10.4.3.1 Perform an air/water check. The value reported for the relative abundance of water (m/z 18), nitrogen (m/z 28), oxygen (m/z 32), or carbon dioxide (m/z 44) shall be less than 5% of the base peak for the system to be considered leak free.

5.10.4.3.2 Tune the MSD using the Standard Autotune program and the decafluorotriphenylphosphine (DFTPP) Tune program to reduce instrument variability. The Autotune report file is referenced by the instrument when performing an air/water check and thus must be run at least once per month. Run standards and samples using DFTPP Tune parameters, and retune the instrument using DFTPP Tune at least once per week. The tune programs use three fragment ions of perfluorotributylamine (PFTBA) as a standard for tuning: m/z 69, 219, and 502. Tune reports must meet the following criteria:

5.10.4.3.2.1 Symmetrical peaks;

5.10.4.3.2.2 Mass assignments within ± 0.2 amu's from 69, 219, and 502;

5.10.4.3.2.3 Peak widths within 0.5 ± 0.1 amu's;

5.10.4.3.2.4 Relative abundance is 100% for ion 69, at least 35% for ion 219, and at least 1% for ion 502;

5.10.4.3.2.5 Relative abundances for isotope masses 70, 220, and 503 ± 0.2 amu's are 0.5–1.5%, 2–8%, and 5–15%, respectively; and

5.10.4.3.2.6 Air and water peaks at m/z = 18, 28, 32, and 44 amu's must be very small and consistent with historical values.

5.10.4.4 *Maintaining a log book.* Maintain an instrument logbook, and make entries for each use. Include the following information in the logbook: operator name, helium cylinder tank pressure and outlet pressure, vacuum gauge reading, any maintenance performed on the instrument (such as changing the injection port liner, gold seal, guard column, source cleaning), sequence name, data path, samples in order of injection, method information, GC column number, and the Standard Auto Tune report and DFTPP Tune report.

5.10.4.5 *Running a Solvent Blank:* Following a liner change or at the start of a new run, run an injection of a pure solvent to confirm that the system is free of excessive or interfering contamination. Analyze the solvent in SCAN mode using the same temperature program used for sample analysis. If contamination is present, analyze additional samples of fresh solvent until the interfering contamination is removed.

5.10.4.6 *Checking the DFTPP Tune:* Prior to running the first calibration standard, verify the instrument tune conditions by running a 10 ng/mL DFTPP check standard to check the mass measuring accuracy of the MS, the resolution sensitivity, the baseline threshold, and the ion abundance ranges. Run the standard using the DFTPP method provided with the instrument. Each of the criteria identified in Table SOP 4.2 of this Appendix must be met before using the instrument for analysis:

TABLE SOP 4.3—ION ABUNDANCE CRITERIA FOR DFTPP

Mass, M/z	Relative to mass	Relative abundance criteria	Purpose of checkpoint
51	442	10–80% of the base peak	Low mass sensitivity.
68	69	<2% of mass 69	Low mass resolution.
70	69	<2% of mass 69	Low mass resolution.
127	442	10–80% of the base peak	Low-mid mass sensitivity.
197	198	<2% of mass 198	Mid mass resolution.
198	442	Base peak or >50% of 442	Mid mass resolution and sensitivity.
199	198	5–9% of mass 198	Mid mass resolution and isotope ratio.
275	442	10–60% of the base peak	Mid-high mass sensitivity.
365	442	>1% of the base peak	Baseline threshold.
441	443	Present and < mass 443	High mass resolution.
442	442	Base peak or >50% of 198	High mass resolution and sensitivity.
443	442	15–24% of mass 442	High mass resolution and isotopic ratio.

5.10.4.7 Calibrating *with a Multiple-Point Calibration Curve.* A 5- or 6-point calibration curve is obtained by running 5 or 6 working standards (see SOP 3) on the tuned GC/MS instrument. Calculate the relative response factor (RRF) for each compound relative to its corresponding deuterated internal standard as indicated in Table SOP 4.3 of this Appendix. The relative standard deviation (RSD) of the RRFs for each compound must be less than 25%. Run an independently prepared check standard immediately after the calibration standards to validate the accuracy of the calibration curve.

5.10.4.8 *Running Samples.* Once the calibration curve has been validated, samples can be analyzed. Dispense 1,000 mL of sample extract into labeled auto-sampler vials. Add

20 mL of the internal standard solution (see SOP 2 of this Appendix) to the extract using a syringe or a positive displacement pipettor. Run a check standard every 10 samples to ensure the consistency of the instrument. The RRF for each compound in the check standard must be within 25% of the average RRF obtained in the initial calibration.

5.10.4.9 *Quantification:* Once a calibration table has been generated, quantify each data file using the "Calculate and Generate" function in the MS ChemStation software, or equivalent software. Review individual peak integration manually to ensure proper baseline integration. The quantification of a compound is based on the peak area of the primary ion (Q Ion) indicated in Table SOP 4.4 of this Appendix.

TABLE SOP 4.4—TARGET COMPOUND LIST

Compound name	Quantitation ion	Reference compound for response factor	Internal standard for quantitation
N D34 C16	66	N D34 C16	D34 n C16 Q Ion 66.
n-C14	57	n C14.	
n-C15	57	n C15.	
n-C16	57	n C16.	
N D34 C17	66	N D34 C17.	
n-C17	57	n C17.	
Pristane	57	Pristane.	
n-C18	57	n C18.	
Phytane	57	Phytane.	
n C19	57	n C19.	
N D42 C20	66	N D42 C20	D42 n C20 Q Ion 66.
n C20	57	n C20.	
n C21	57	n C21.	
n C22	57	n C22.	
n C23	57	n C23.	
N D50 C 24	66	N D50 C 24.	
n C24	57	n C24.	
n C25	57	n C25.	
n C26	57	n C26.	
n C27	57	n C27.	
n C28	57	n C28.	
n C29	57	n C29.	
N D62 C30	66	N D62 C30	D62 n C30Q Ion 66.
n C30	57	n C30.	
n C31	57	n C31.	
N D66 C32	57	N D66 C32.	
n C32	57	n C32.	
n C33	57	n C33.	
n C34	57	n C34.	
n C35	57	n C35.	
D8 Naphthalene	136	D8 Naphthalene	D8 Naphthalene Q Ion 136.
Naphthalene	128	Naphthalene.	
D10 1-Methylnaphthalene	152	D10 1-Methylnaphthalene.	
C1 Naphthalene*	142	C1 Naphthalene.	
C2 Naphthalene*	156	C2 Naphthalene.	
C3 Naphthalene*	170	C3 Naphthalene.	
C4 Naphthalene*	184	C3 Naphthalene.	
D10 Anthracene	188	D10 Anthracene	D10 Anthracene Q Ion 188.
D10 Phenanthrene	188	D10 Phenanthrene.	
Phenanthrene	178	Phenanthrene.	
C1 Phenanthrene*	192	C1 Phenanthrene.	
C2 Phenanthrene*	206	C2 Phenanthrene.	
C3 Phenanthrene*	220	C2 Phenanthrene.	
C4 Phenanthrene*	234	C2 Phenanthrene.	
Fluorene	166	Fluorene.	
C1 Fluorene*	180	Fluorene.	
C2 Fluorene*	194	Fluorene.	
C3 Fluorene*	208	Fluorene.	
Dibenzothiophene	184	Dibenzothiophene.	
C1 Dibenzothiophene*	198	Dibenzothiophene.	
C2 Dibenzothiophene*	212	Dibenzothiophene.	
C3 Dibenzothiophene*	226	Dibenzothiophene.	
Naphthobenzothiophene (NBT)	234	Naphthobenzothiophene.	
C1 NBT*	248	Naphthobenzothiophene.	
C2 NBT*	262	Naphthobenzothiophene.	
C3 NBT*	276	Naphthobenzothiophene.	
Fluoranthene	202	Fluoranthene.	
D10 Pyrene	212	D10 Pyrene.	
Pyrene	202	Pyrene.	
C1 Pyrene*	216	Pyrene.	
C2 Pyrene*	230	Pyrene.	
D12 Chrysene	240	D12 Chrysene	D12 Chrysene Q Ion 240.
Benzo(a)anthracene/Chrysene*	228	Chrysene.	
C1 Chrysene*	242	Chrysene.	

TABLE SOP 4.4—TARGET COMPOUND LIST—Continued

Compound name	Quantitation ion	Reference compound for response factor	Internal standard for quantitation
C2 Chrysene*	256	Chrysene.	
C3 Chrysene*	270	Chrysene.	
C4 Chrysene*	284	Chrysene.	
5a-androstane	245	5a-androstane	5a-androstane Q Ion 245
Coprostane	219	Coprostane.	
Hopane	191	Hopane.	

*Summed compounds; draw an integration line underneath all peaks with selected ion.

5.10.4.10 Equation 14 of this Appendix is used to calculate the concentration of analytes in units of mg/g oil added:

$$Concentration\ of\ analyte\ (\mu g / g\ oil) = \frac{100 \times A_{analyte} \times C_{istd}}{A_{istd} \times RRF} \qquad \text{(Equation 14)}$$

where:

$A_{analyte}$ = the peak area of the analyte,
C_{istd} = the concentration of the internal standard,
A_{istd} = the area of the internal standard,
RRF = the relative response factor, and
100 is the conversion factor to convert mg/L DCM to mg/g oil added.

5.10.4.11 If some analytes are not commercially available, the RRFs of other compounds (usually the parent compound) are used to quantify those analytes. For example, the RRF of C3-naphthalene may be used to calculate the concentrations of C3- and C4-naphthalenes. See Table SOP 4.4 of this Appendix for details. The quantification of these alkylated PAHs is relative because it is assumed that the molecular ions of the alkylated PAHs have the same RRFs as the parent compound ions. Nevertheless, these relative concentrations are useful for monitoring the fate of these compounds during the course of any analysis, as long as their concentrations are measured in a consistent way throughout the analysis.

5.10.4.12 Concentration calculations for all target compounds are performed using EnviroQuant software or equivalent. Data for each sample can be printed directly using a customized report template. Data can also be automatically entered into a spreadsheet within the EnviroQuant software.

5.10.5 *Quality Assurance/Quality Control.* The following criteria must be met before any samples are analyzed:

5.10.5.1 Air/water check to verify the system is leak free.

5.10.5.2 AutoTune and DFTPP Tune pass all criteria.

5.10.5.3 DFTPP check standard passes all criteria.

5.10.5.4 Solvent blank scan indicates the GC/MS system is free of interfering contamination.

5.10.5.5 Prepare and monitor a control chart of a standard oil analysis. Concentrations of the analytes in the control chart must be no more than 25% different from their historical averages.

5.10.5.6 Relative response factors for analytes in the check standards inserted between every 10 samples must be no more than 25 percent different from the average RRF of those same analytes in the calibration curve. Peak shapes must be symmetrical.

5.11 *References for Section 5*

(1) Haines, J.R., E.J. Kleiner, K.A. McClellan, K.M. Koran, E.L. Holder, D.W. King, and A.D. Venosa. 2005. "Laboratory evaluation of oil spill bioremediation products in salt and freshwater systems." J. Ind. Microbiol. Biotech 32: 171–185.

Appendix E to Part 300 [Removed]

* 17. Remove Appendix E to Part 300.

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Development of Aquatic Toxicity Benchmarks for Oil Products Using Species Sensitivity Distributions

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ABSTRACT

Determining the sensitivity of a diversity of species to spilled oil and chemically dispersed oil continues to be a significant challenge in spill response and impact assessment. We used standardized tests from the literature to develop species sensitivity distributions (SSDs) of acute aquatic toxicity values for several petroleum products and 2 Corexit oil dispersants. Fifth percentile hazard concentrations (HC5s) were computed from the SSDs and used to assess relative oil product toxicity and in evaluating the feasibility of establishing toxicity benchmarks for a community of species. The sensitivity of mysids (Americamysis bahia) and silversides (Menidia beryllina) were evaluated within the SSDs to determine if these common test species were appropriate surrogates for a broader range of species. In general, SSD development was limited by the availability of acute toxicity values that met standardization criteria for a diversity of species. Pooled SSDs were also developed for crude oil and Corexit dispersants because there was only small variability in the HC5s among the individual oil or dispersant products. The sensitivity of mysids and silversides varied across the oil and dispersant products, with the majority of toxicity values greater than the HC5. Application of SSDs appears to be a reasonable approach to developing oil product toxicity benchmarks, but additional toxicity data are needed for a larger range of species conducted under standardized test conditions. Integr Environ Assess Manag 2013;9:610–615. © 2013 SETAC

Keywords: Acute toxicity Aquatic Oil Species sensitivity distribution

Environmental Management

INTRODUCTION

Determining the effects of spilled and chemically dispersed petroleum on aquatic communities continues to be a significant challenge in oil spill response and impact assessment. The Deepwater Horizon spill highlighted the diversity of ecological receptors and complexity of aquatic habitats that may be impacted by a spill, including the deep ocean, offshore pelagic areas, and coastal wetlands and estuaries (Barron 2012; Carriger and Barron 2011). Issues include the adequacy of relying on a few standard test species such as mysids (Americamysis bahia) and silversides (Menidia beryllina) to represent the array of communities of aquatic organisms (Hemmer et al. 2011). Other issues include whether there are intrinsic differences in sensitivity between polar and temperate aquatic species and communities (de Hoop et al. 2011).

A species sensitivity distribution (SSD) is an assemblage of single-species toxicity data for a specific chemical or product and is used to predict a hazardous concentration (HCp) affecting a specified proportion (p) of species in the community represented by the SSD. Species sensitivity distribution have been used internationally to establish both screening-level benchmarks and regulatory guidelines such as US Ambient Water Quality Criteria (Barron and Wharton 2005). Typically the 5th percentile of the model-fitted distribution (HC5) is used as the hazardous concentration applied in typical risk assessments, although endangered species risk assessments and other applications may use different hazardous concentrations (e.g., HC1 to protect 99% of species) (Suter et al. 2002). Impacts predicted from using SSDs have been shown to be significantly associated with observed effects in the field (Posthuma and de Zwart 2012). Historically, the application of SSDs in aquatic toxicology has been limited by requirements of sufficient diversity of species to approximate the distribution of sensitivities within an aquatic community. Sensitivities are represented by the compound specific toxicity values, typically median lethal and median effect values for acute toxicity. Various authors have recommend a minimum of 5 to greater than 30 species in an SSD to produce reliable HC values, and 8 to 10 species a typical minimum with requisite diversity in the taxa of invertebrates and fish (Newman et al. 2000; Suter et al. 2002; Wheeler et al. 2002; Duboudin et al. 2004). Species sensitivity distribution and the resulting HC5 are also known to be dependent on the model choice and quality of data used in building the SSD, such as the level of toxicity data standardization (e.g., conformance to criteria for ranges of test conditions and methods) (Newman et al. 2000; Wheeler et al. 2002; Duboudin et al. 2004).

Species sensitivity distribution have not been generally applied in petroleum ecotoxicology despite the potential for developing community-based toxicity benchmarks. Typically only a few standard test species and those species from the area of oil production or transport have been evaluated. The objectives of this study were to assess whether sufficient data exist to develop SSDs for individual oils and dispersants using standardized acute toxicity values and compare HC5s among these products. Only aquatic invertebrates and fish were included to maximize data standardization and comparability across SSDs. An additional objective was to determine the relative sensitivity of mysids and Menidia based on their position within the SSDs. These 2 species are standard test

All Supplemental Data may be found in the online version of the article.
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organisms used frequently in petroleum and dispersant impact and regulatory assessments and are generally assumed to be representative of a broad range of species.

METHODS

Data sources and standardization criteria

A general literature and data search was carried out for acute toxicity values for aquatic invertebrates and fish tested with chemical oil dispersants, middle distillates, crude oils, and heavy residual fuel oils. Toxicity values were reported as total petroleum hydrocarbons (TPH) or equivalent, rather than specific analytes. Sources of information included Edwards et al. (2003), Fuller et al. (2004), George-Ares and Clark (2000), Hemmer et al. (2011), Liu (2003), Malins (1977), Moles (1998), Moles et al. (1979), NRC (1989, 2005), Rice et al. (1979), and Scarlett et al. (2005). For oils, standard water accommodated fraction (WAF) tests following the general methods of Anderson et al. (1974) were targeted because they were considered to be most representative of intrinsic species sensitivity and prolonged exposure durations. Preliminary search results indicated that a relatively few oil and dispersant products had the minimum diversity of species with standardized toxicity values needed to build SSDs (e.g., 8 to 15) (USEPA 1985; Wheeler et al. 2002). Oils used in this assessment had a minimum of 10 species and included a middle distillate (fuel oil number 2 [FO2]), heavy crude oil (Alaska North Slope crude [ANSC]; Prudhoe Bay crude [PBC]), light crude oil (Cook Inlet crude [CIC]; South Louisiana crude [SLC]), and heavy residual oil (Bunker C [BC]). Two dispersants, Corexit 9500 and 9527 (Nalco Company, Naperville, IL) were also evaluated because of a relatively large diversity of species with reported values and recent use in the Deepwater Horizon spill (Hemmer et al. 2011). Toxicity values were selected for use in SSDs if they met the standardization criteria listed in Table 1, and all data used in SSDs are provided in Table S1 in the Supplementary data. Each species was categorized as either an invertebrate or fish, and either cold or warm and/or temperate based on recommended test temperatures and species geographic distribution. Geometric mean values were computed for species with multiple reported values for a specific oil or dispersant before use in an SSD.

Table 1. Standardization criteria for toxicity data used in developing SSDs

Parameter	Criterion
Species	Aquatic invertebrates, fish; name verified in ITIS [a]
Life stage	Larval, juvenile, adult (no embryos)
Test duration	Invertebrate: 48–96 h; Fish: 96 h
Exposure regime	Continuous WAF exposure [b]
Oil weathering	None
Oil/water mixing	WAF or equivalent
Oil exposure analytes	Analytical confirmation as TPH [c]
Toxicity endpoint	Medial lethal concentration [d]

ITIS ¼ Integrated Taxonomic Information System; SSD ¼ species sensitivity distribution; TLm ¼ median tolerance limit; TPH ¼ total petroleum hydrocarbons or equivalent; WAF ¼ water accommodated fraction.
[a]Available from: http://www.itis.gov
[b]Static, static renewal, recirculated renewal; WAF or equivalent such as water soluble fraction; spiked/continuous dilution tests excluded
[c]Dispersants: nominal.
[d]Reported as LC50 or TLm (oil, mg TPH/L; dispersant, mL/L).

Generating SSDs and HCs

Species sensitivity distributions were developed for 2 Corexit dispersants and 5 oils; ANSC and PBC were combined for analysis because of similar chemical composition and oil source location. Additionally, combined oil or dispersant SSDs were generated using all species values for the 3 crude oils and 2 Corexits. Species sensitivity distributions were generated and HC5 values were computed from the 5th percentile of the fitted log-normal distribution using the CADDIS SSD Generator (Causal Analysis/Diagnosis Decision Information System; http://www.epa.gov/caddis/da_software_ssdmacro.html).

RESULTS

Comparison of SSDs and HC5s

A total of 227 toxicity values determined under standardized test conditions (Table 1) were used in the development of SSDs and HC5 values. Toxicity values encompassed 67 species across the 5 oils and 2 dispersants. All SSDs had similar proportions of invertebrates and fish, with an apparent random distribution of sensitivity (Figures 1 to 3). Model fits of the probability distributions were excellent with R^2 values of 0.9 or greater (Table 2). Balance between cold and warm and/or temperate species varied among the oil and dispersant SSDs. SSDs for ANSC/PBC, CIC, and FO2 were dominated by cold water species, whereas SLC, and Corexit 9500 were dominated by warm water and temperate species (Figures 1 to 3). Data were limited to 10 species for BC, with only 1 cold water species represented (Figure 2). HC5 values for both FO2 (0.29 mg/L) and BC (0.56 mg/L) were lower than those for crude oils (0.9 mg/L to 3.5 mg/L), with upper prediction limits that did not encompass the crude oil HC5 (Table 2). On a ppm basis, HC5 values (mL dispersant/L) for the 2 Corexit products were nearly identical and ranged from 1.2- to 7.3-fold higher than TPH-based crude oil HC5s (Table 2).

Relative sensitivity of mysids and Menidia

Toxicity values for both Menidia and mysids (either A. bahia or the sympatric A. almyra) determined under standardized test conditions were available for all oil and dispersant products except CIC. The relative sensitivity of each species was not consistent among the oil and dispersants, although ranges in toxicity values within a SSD were relatively narrow (Table 3 and Figures 1 to 3). Mysid sensitivity ranged from the 4th to 39th percentile, with an average of 25.8 across the oil products and 2 dispersants (Table 3). Menidia ranged from the 13th to 97th percentile, with an average of 54.2 across oil and dispersant products (Table 3). There was relatively consistent sensitivity between species, with Menidia ranging from 1.5- to 3.3-fold less sensitive than mysids based on ranking within the SSD and 1.6- to 5.8-fold based on toxicity values (Table 3).

DISCUSSION

Species sensitivity distributions have been used internationally to develop screening level benchmarks and to derive water

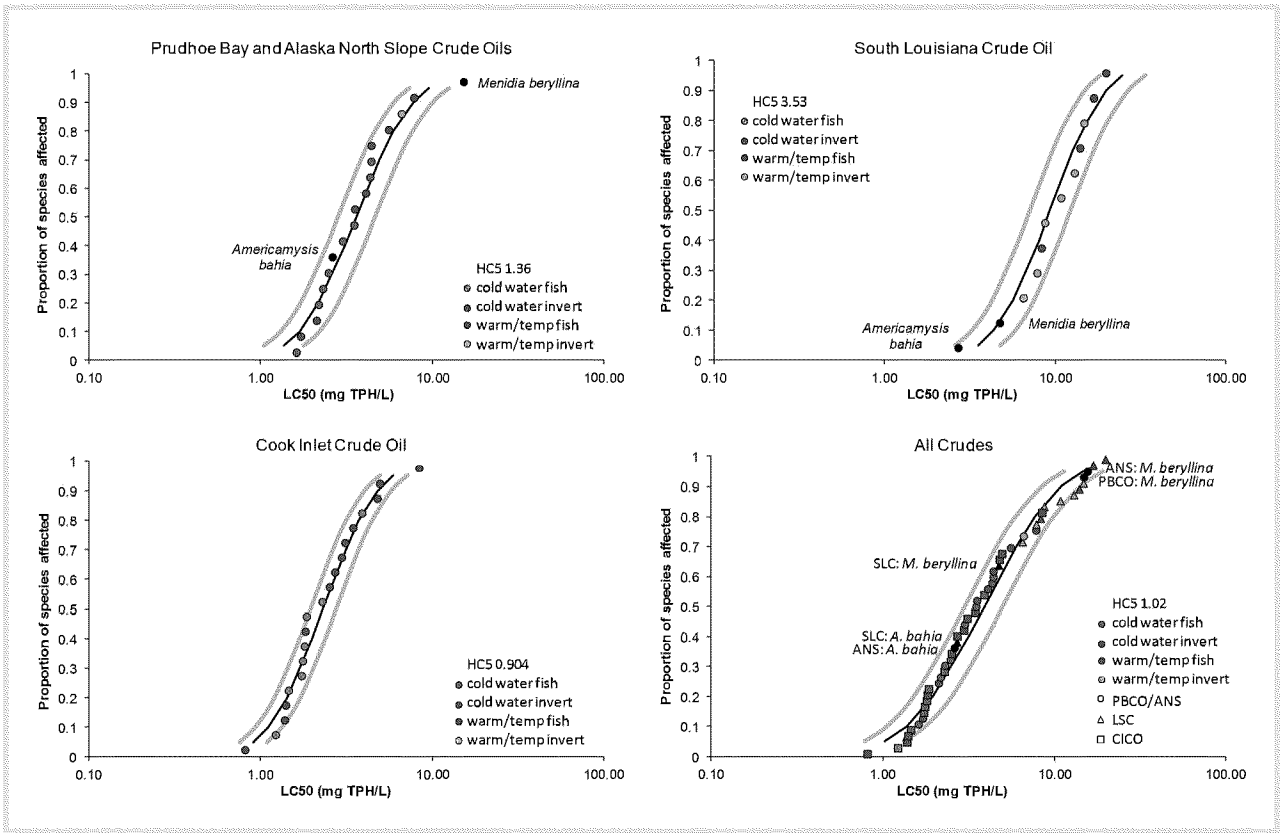

Figure 1. Species sensitivity distributions for 3 individual crude oils and the 3 oils combined (all crudes). Standardized values for mysids (A. bahia) and Menidia are identified. SSD for all crudes presented for illustrative purposes to show the distribution of individual mysid and Menidia values within the SSD.

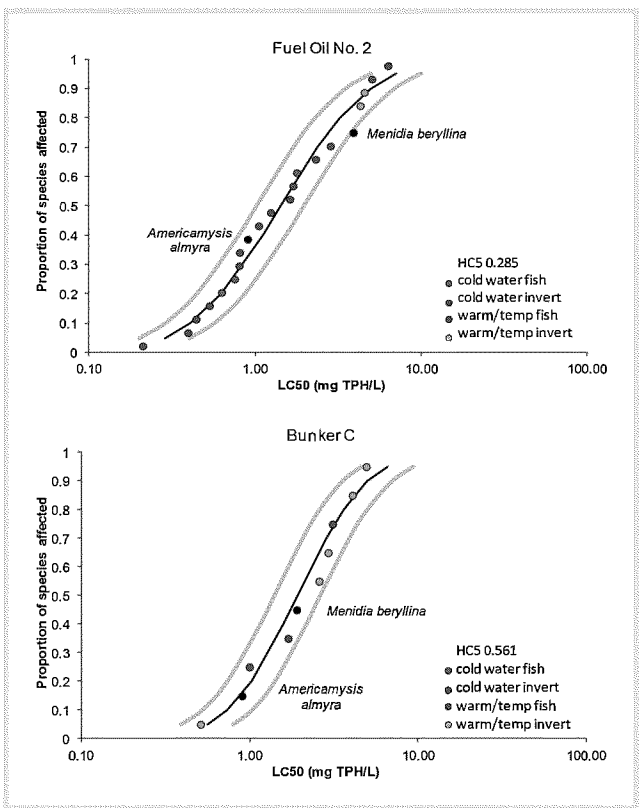

Figure 2. Species sensitivity distributions for the middle distillate Fuel Oil number 2, and the heavy residual oil Bunker C. Toxicity values for mysids (A. almyra) and Menidia are identified; data for A. bahia that met standardization criteria were not available for these oil products.

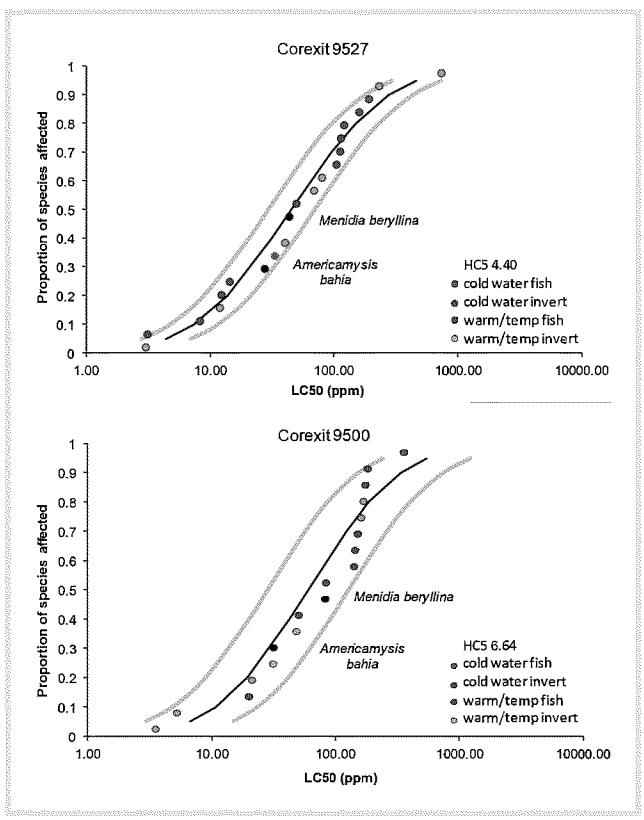

Figure 3. Species sensitivity distributions for 2 Corexit oil dispersants (ppm; mL dispersant/L). Toxicity values for mysids (A. bahia) and Menidia are identified.

Table 2. Summary of HC5, upper and lower PI, number of species in the SSD, slope of regression, y-intercept, and MSE

Oil product	HC5	Upper PI	Lower PI	Species in the SSD (n)	Slope	Intercept	R^2	MSE
All crudes	1.03	1.34	0.797	39	2.85	3.31	0.968	0.033
ANSC/PBC	1.38	1.77	1.04	18	3.89	2.84	0.948	0.055
South Louisiana crude	3.53	4.77	2.61	12	3.88	1.23	0.946	0.058
Cook Inlet crude	0.904	1.09	0.753	20	4.03	3.53	0.973	0.029
Fuel oil number 2	0.285	0.403	0.202	22	2.36	4.64	0.966	0.035
Bunker C	0.561	0.802	0.392	10	3.08	4.13	0.958	0.047
Corexit 9500 and 9527	5.22	9.42	2.89	28	1.64	2.18	0.948	0.053
Corexit 9500	6.64	15.0	2.94	18	1.72	1.94	0.907	0.098
Corexit 9527	4.40	6.99	2.77	22	1.63	2.31	0.971	0.030

ANSC ¼ Alaska North Slope crude; HC5 ¼ 5th percentile hazard concentrations; MSE ¼ mean square error; PBC ¼ Prudhoe Bay crude; PI ¼ prediction limits; R^2 ¼ coefficient of determination; SSD ¼ species sensitivity distribution.
HC5 and PI values in ppm for dispersants (mL/L) and oil products (mg TPH/L).

quality standards and other regulatory guidelines (Barron and Wharton 2005) but have seen limited use in oil and dispersant regulation and impact assessment. The few published oil SSDs have been developed using toxicity values for multiple oils, rather than single oil products, and variable standardization criteria were applied in building the database for the SSD. These studies have shown that the development of oil SSDs is feasible and can be useful in assessing endpoint and species sensitivity in aquatic organisms exposed to oil products. For example, Smit et al. (2009) used SSDs to compare the sensitivity of oil-related biomarkers to chronic no effect concentrations. Recently de Hoop et al. (2011) used SSDs to evaluate the sensitivity of polar invertebrates and fish compared with temperate species to WAF of crude oil and 2 naphthalene components of oil. They reported that polar and temperate species sensitivities were within a factor of 3 based on HC5 and HC50 values (de Hoop et al. 2011). In general, these studies and the current investigation highlight the relatively limited species diversity in available toxicity records for individual oil products.

A fundamental challenge in building and applying SSDs is defining the range of test data that is appropriate to modeling the environmental problem (Suter et al. 2002). The current study focused on acute toxicity of oil (as mg TPH/L) determined from WAFs tests similar to those described by Anderson et al. (1974) and Barron and Kaaihue (2003). Spiked oil tests with continuous dilution are an alternative testing regime not included in the current study (Singer et al. 1991). These tests expose organisms to declining oil concentrations (e.g., 4 h half life) and typically report toxicity values as amount

Table 3. Relative sensitivity of mysids (A. bahia or A. almyra) and Menidia to individual oil and dispersant products and combined product types, determined by their position within the species sensitivity distribution (as taxa proportion)

	Taxa proportion (%)		Acute toxicity (ppm)†	
Oil Product	Mysid [a]	Menidia	Mysid	Menidia
All crudes	32	73	2.7	7.5
ANSC/PBC	36	97	2.6	15.2
South Louisiana crude	4	13	2.7	4.7
Cook Inlet crude	NA	NA	NA	NA
FO2	39	75	0.9	3.9
Bunker C	15	45	0.9	1.9
Corexit 9500 and 9527	27	52	29.8	66.2
Corexit 9500	31	47	31.4	81.9
Corexit 9527	30	46	27.4	43.3

ANSC ¼ Alaska North Slope crude; FO2 ¼ fuel oil number 2; HC5 ¼ 5th percentile hazard concentrations; MSE ¼ mean square error; NA ¼ no standardized toxicity values for mysids, Menidia; PBC ¼ Prudhoe Bay crude; PI ¼ prediction limits; R^2 ¼ coefficient of determination; SSD ¼ species sensitivity distribution.
Reported as median lethal concentration (LC50) or median tolerance limit (TLm). Oil, mg TPH/L; dispersant, mL/L.
[a] Mysids: Americamysis bahia, except FO2 and Bunker C: A. almyra.

of oil applied to the test chamber. There is a growing database of spiked oil tests, but more research is needed to determine if relative species sensitivity comparisons would yield similar results as WAF-based tests that use exposures and analytical endpoints that more closely follow pure chemical testing methods.

Other key issues with developing oil SSDs and deriving toxicity benchmarks include data quality, model selection, number of species, and representativeness needed to predict effects in the field and protect important species (Suter et al. 2002). Species sensitivity distributions are models of the variance among species (Suter et al. 2002), and the log-normal model fit of the probability distribution within each SSD was excellent. Only aquatic invertebrates and fish were included to maximize data standardization and comparability across SSDs. Toxicity values from standardized tests with algae were not available for all the products evaluated in the current study, and perusal of data for other oils and dispersants suggested generally similar or lower sensitivity than invertebrates and fish. Thus HC5 values derived from SSDs without aquatic plants are expected to be protective of aquatic plant communities.

The range in standardized toxicity values comprising each oil SSD was generally within an order of magnitude and HC5 values were similar across the oil products. In contrast, each dispersant SSD showed a nearly 30-fold variation in species sensitivity, and HC5 values were higher than for the oils. All the SSDs had reasonable balance between invertebrates and fish (26% to 55% of species), with no consistent trend in taxa or species sensitivity. This is in contrast to some pure compound SSDs where specific taxa such as crustaceans and insects show substantially greater sensitivity than fish to chemicals such as insecticides. The acute toxicity of oil appears to occur via a narcosis mode of action that is not taxa specific, in contrast to more specific acting chemicals that can show substantial life stage and species differences in uptake, receptor modulation, and biotransformation pathways (Barron et al. 2004).

Species composition of the SSD can substantially influence the HC5 and should reflect the community to be protected (Suter et al. 2002; Posthuma and de Zwart 2012). The species composition of the crude oil SSDs in the current study generally reflected the region of oil production. SSDs for ANSC/PBC and CIC were dominated by Alaska species, whereas SLC was dominated by Gulf of Mexico species. de Hoop et al. (2011) reported minimal differences between HC5 values (0.37 mg TPH/L to 0.55 mg TPH/L) derived from polar or temperate SSDs, thus applicability of cold (e.g., FO2) or warm and/or temperate dominated SSD (e.g., Corexit 9500) maybe less of an issue for oil and dispersant products. Mysids and Menidia have been used as standard test species in oil toxicology for decades, and are the prescribed test species for dispersant testing under the US National Contingency Plan Subpart J regulations (Hemmer et al. 2011). Toxicity values for mysids fell within the lower 2 quartiles of the SSDs, whereas Menidia were consistently less sensitive, suggesting that these species are reasonable, but not highly sensitive surrogates.

Although SSD development was limited by the availability of toxicity values that met standardization criteria for a diversity of species, HC5 values were relatively similar for the 3 crude oils. This suggests that combining toxicity values and developing combined oil SSDs, at least within oil product types (e.g., light, medium, heavy crude oils; middle distillates; heavy residual oils) is feasible. The resulting HC5 values from these SSDs could then serve as the basis for establishing toxicity benchmarks. Acute benchmarks could be derived analogous to US Ambient Water Quality Criteria (USEPA 1985) approach for single compounds as 50% of the HC5 or lower confidence limit of the HC5. Chronic toxicity benchmarks for assessing sublethal toxicity during prolonged exposures could be developed by applying appropriate safety factors or acute to chronic ratios (USEPA 1985; Raimondo et al. 2007). Although the pooling of toxicity values for oil types suggested above also appears feasible for dispersants, it is unclear if the approach would work broadly. Exceptions may be those products with very similar composition such as the 2 Corexits. A large range of dispersant products exist as mixtures with differing composition, and toxicity values for standard test species vary widely (Hemmer et al. 2011).

The Deepwater Horizon spill renewed concerns about the complexity and diversity of ecological communities and environments that may be exposed to oil, as well as the need for rapid monitoring of water column toxicity during prolonged dispersant applications to continuing releases of oil (Barron 2012; Carriger and Barron 2011). The development of HC5-based benchmarks is an established practice internationally in setting screening levels for single compounds and regulatory guidelines (USEPA 1985; Barron and Wharton 2005) and appears a feasible approach for establishing aquatic toxicity benchmarks for oil products. Developed oil benchmarks could then be compared to water column concentrations of oil or chemically dispersed oil collected during spill response activities to provide a rapid assessment of impacts and risks. Previous studies with a diversity of oils and dispersants have shown similar acute toxicity values for physically and chemically dispersed oil, thus oil benchmarks should be applicable under both conditions (Hemmer et al. 2011). An alternative to an approach using TPH-based toxicity values could include the use of petrogenic PAH-based HC5s when data for a diversity of species becomes available. Impacts predicted from using SSDs for single chemicals have been shown to be significantly associated with observed effects in the field (Posthuma and de Zwart 2012), although HC5 values can either be protective or sometimes underprotective of sensitive taxa in model ecosystems and field studies (Mebane 2010; Jansch et al. 2006). Comparison of SSD-based benchmarks for oil products to field effects should be a research priority in the future.

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To: Conmy, Robyn[Conmy.Robyn@epa.gov]
From: Barron, Mace
Sent: Thur 4/21/2016 1:53:17 PM
Subject: RE: DWH source oil...

;;;;
Since Michael Hemmer retired, and our previous IH guy left, we have been just sitting on old dispersants and the BP oil. Out of the blue the BP contractor asked about their oil: I said we could either transfer it to another EPA facility (you) or send it back.

They asked for it back.

So we are cleaning house on the legacy DWH stuff. Crazy one of the dispersants Z-400 was in a plastic bottle and we found it leaking. All those are going into haz waste.

Hope all is well!

From: Conmy, Robyn
Sent: Thursday, April 21, 2016 8:40 AM
To: Barron, Mace
Subject: RE: DWH source oil...

Sweet! It's like you're having a garage sale. ☺

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From: Barron, Mace
Sent: Thursday, April 21, 2016 9:32 AM
To: Conmy, Robyn <Conmy.Robyn@epa.gov>
Subject: DWH source oil...

Will also send you a couple mL of oil collected at the well head by ROV. We got about 5 mL of the precious during the spill!

We had that chemically characterized as part of the attached work.

Unfortunately, it was stored in same vial (head space), so it may be compromised. You can check it out when you get it.

Mace

To: Conmy, Robyn[Conmy.Robyn@epa.gov]
From: Barron, Mace
Sent: Tue 5/3/2016 1:11:00 PM
Subject: RE: IOSC

;;;;
Will do!

From: Conmy, Robyn
Sent: Tuesday, May 03, 2016 5:35 AM
To: Barron, Mace
Subject: RE: IOSC

Yes, oils should be able to cover most if not all of the conference travel for IOSC. Why don't you go ahead a put in an abstract as a place holder. We can plan for your atetndence but can always withdraw if something wonky with the projected travel happens.

Cheers,

Robyn

<>/<>/<>/<>/<>/<>/<>/<>/<>/<>/<>/<>/<>/<>/<>/<>/<>/<>

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conmy.robyn@epa.gov

From: Barron, Mace
Sent: Monday, May 02, 2016 9:06 AM
To: Conmy, Robyn <Conmy.Robyn@epa.gov>
Subject: RE: IOSC

That is awesome on the two dispersants.

Did you hear back how much the cost estimates from Hydrosphere were? (just so we can estimate our budget for any future tests). They were supposed to give us two quotes. Please let me know if I can as Eadie.

I would love to go to IOSC, but only get one meeting a year (and likely would be SETAC). Any possibility of the oil program supporting part of the travel as you did for Tampa?

Am happy to review for STICs.

Mace

From: Conmy, Robyn
Sent: Friday, April 29, 2016 6:46 AM
To: Barron, Mace <Barron.Mace@epa.gov>
Subject: IOSC

Hey Mace,

Are you planning on attending the IOSC conference in May 2017? Short abstracts are due this May 20th. I am submitting one (can you serve as a STICS technical reviewer BTW?) and would encourage you to do the same on the oil tox program. We should have some great results by then! If you are interested, oils can cover most if not all of the travel (provided we have our 2017 budget by May next year).

In other news, we have procured Corexit and Finasol for the tox work!!!! They should be shipped to us next week. 2 gallons of each. I can't believe it. Still working on remaining 2 but this can get hydrosphere started.

R

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COMPARATIVE TOXICITY OF EIGHT OIL DISPERSANTS, LOUISIANA SWEET CRUDE OIL (LSC), AND CHEMICALLY DISPERSED LSC TO TWO AQUATIC TEST SPECIES

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(Submitted 5 January 2011; Returned for Revision 7 February 2011; Accepted 16 June 2011)

Abstract—The present study describes the acute toxicity of eight commercial oil dispersants, South Louisiana sweet crude oil (LSC), and chemically dispersed LSC. The approach used consistent test methodologies within a single laboratory in assessing the relative acute toxicity of the eight dispersants, including Corexit 9500A, the predominant dispersant applied during the DeepWater Horizon spill in the Gulf of Mexico. Static acute toxicity tests were performed using two Gulf of Mexico estuarine test species, the mysid shrimp (Americamysis bahia) and the inland silversides (Menidia beryllina). Dispersant-only test solutions were prepared with high-energy mixing, whereas water-accommodated fractions of LSC and chemically dispersed LSC were prepared with moderate energy followed by settling and testing of the aqueous phase. The median lethal concentration (LC50) values for the dispersant-only tests were calculated using nominal concentrations, whereas tests conducted with LSC alone and dispersed LSC were based on measured total petroleum hydrocarbon (TPH) concentrations. For all eight dispersants in both test species, the dispersants alone were less toxic (LC50s: 2.9 to >5,600 ml/L) than the dispersant–LSC mixtures (0.4–13 mg TPH/L). Louisiana sweet crude oil alone had generally similar toxicity to A. bahia (LC50: 2.7 mg TPH/L) and M. beryllina (LC50: 3.5 mg TPH/L) as the dispersant–LSC mixtures. The results of the present study indicate that Corexit 9500A had generally similar toxicity to other available dispersants when tested alone but was generally less toxic as a mixture with LSC. Environ. Toxicol. Chem. 2011;30:2244–2252. # 2011 SETAC

Keywords—Oil dispersants Corexit 9500A Menidia Mysids South Louisiana sweet crude

INTRODUCTION

An estimated 4.9 million barrels of South Louisiana sweet crude (LSC) oil was released into the northern Gulf of Mexico between April 20 and July 15, 2010 as a result of the explosion and collapse of the Deepwater Horizon (DWH) oil exploration platform (www.restorethegulf.gov). During the catastrophe, a number of recovery strategies to contain the oil were employed, including direct capture of oil from the wellhead (17%), surface skimming (3%), booming and burning (5%), and the application of chemical oil dispersants (8%) (www.restorethegulf.gov). The use of dispersants in oil spill response involves tradeoffs between the direct effects of oil to bays, estuaries, and beaches and effects of dispersants and dispersed oil to pelagic and deep sea environments [1–3]. To mitigate the environmental impact of floating oil on sensitive shoreline habitats along the northern Gulf coast, the decision was made to apply Corexit 9500A, an oil dispersant listed on the U.S. Environmental Protection Agency's (U.S. EPA) National Contingency Plan (NCP) Product Schedule (http://www.epa.gov/emergencies/content/ncp/product_schedule.htm). Approximately 4,059,854 L of Corexit 9500A was applied to floating oil offshore, and an additional 2,914,767 L was injected directly into the oil and gas plume at the wellhead 1,544 m below the surface (www.restorethegulf.gov).

The U.S. Clean Water Act and Oil Pollution Act of 1990 requires the maintenance of a federal NCP for response to oil spills that identifies specific commercial products used for control of oil discharges and the quantities and water bodies in which the products may be used. These products consist of dispersants, surface washing agents, surface collecting agents, bioremediation agents, and other miscellaneous oil spill control agents. Under the NCP, the U.S. Environmental Protection Agency has statutory responsibility for obtaining toxicity and efficacy information from the manufacturers before placing a dispersant on the National Product Schedule. Toxicity data requirements and test procedures are stipulated under 62 FR11576, Appendix C, of Part 300, which consists of static acute toxicity tests conducted with the dispersant product and a separate test with no. 2 fuel oil using two estuarine test species [4]. The National Product Schedule acts as a preapproval mechanism, allowing the Federal On-Scene Coordinator working with state and local governments to respond quickly to a spill situation using the best available technology.

Currently, 15 dispersants are listed on the National Product Schedule (http://www.epa.gov/emergencies/content/ncp/product_schedule.htm). Although the exact compositions of most commercially available oil dispersants are proprietary, they typically contain a high percentage of one or more uncharged or charged anionic surfactants of different solubility. Surfactants are amphiphatic molecules possessing both hydrophilic and hydrophobic groups that act to decrease tension between the water and oil interface, stimulating the development of small oil-surfactant micelles less than 100 mM [5,6]. The greater surface area to volume of the droplets enhances entrainment of the micelles in the water column while also increasing the oil's availability to physical and microbial degradation. Inclusion of solvents such as petroleum distillates in dispersant mixtures assists in dissolving surfactants through reduction in viscosity.

Here we report a two-phase toxicity study to determine the hazards of eight commercial dispersant products using two Gulf of Mexico aquatic species. Phase 1 included acute toxicity tests performed with each of the dispersants in the absence of oil. The second phase of testing consisted of acute toxicity tests

All Supplemental Data may be found in the online version of this article.

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conducted with LSC oil only and chemically dispersed LSC using each of the eight oil dispersants. The approach used consistent test methodologies within a single laboratory, which provided a means to assess and compare independently acute toxicity estimates of the dispersants, LSC, and dispersant–oil mixtures.

MATERIALS AND METHODS

Study design

In the present study, the acute toxicity of eight dispersants, LSC, and dispersant–LSC mixtures was examined using two Gulf of Mexico aquatic species: the mysid shrimp, Americamysis bahia, an aquatic invertebrate, and the inland silverside, Menidia beryllina, a small estuarine fish. These species are standard test organisms used in a variety of U.S. EPA toxicity test methods. Larval A. bahia were supplied from in-house cultures maintained by the contract testing laboratory using filtered natural seawater. Larval M. beryllina were purchased from Aquatic Biosystems, shipped overnight to the testing laboratory and held a minimum of 2 d before testing. Culture and holding conditions for both species were 258C and 20 parts per thousand salinity.

The static acute toxicity test methods followed U.S. EPA Test Method 821/R-02-012 [7], with slight modifications as described (Table 1). Americamysis bahia were 24 to 48 h old and M. beryllina 11 or 14 d old at test initiation. All organisms for a given exposure were within 24 h of the same age. Three replicates were conducted for each exposure concentration. Test organisms were randomly assigned across exposure and control treatments, with each replicate receiving 10 animals, for a total of 30 animals per treatment level. One-liter beakers containing 1 L test solution were maintained in 258C temperature-controlled water baths under a photoperiod of 16:8 h light:dark. All test vessels were aerated continuously (100 bubbles/min). The duration of the acute tests was 48 h for A. bahia and 96 h for M. beryllina. Temperature was monitored continuously using maximum–minimum thermometers; salinity and dissolved oxygen were measured once a day. All tests were conducted under contract with Smithers-Viscient Laboratory and performed in compliance with the Good Laboratory Practice regulations as provided in U.S. EPA 40 CFR 160 [8].

Nonweathered LSC oil, lot no. WP 681, was purchased from RT Corporation in 500-ml amber bottles and shipped directly to the testing laboratory. Selection of eight commercially available oil dispersants for testing was based on listing in the National Contingency Plan Product Schedule, product availability, and adequate production capacity. Liquid concentrates of Corexit 9500A (Nalco), Dispersit SPC 1000 (U.S. Polychemical), JD-2000 (GlobeMark Resources), Nokomis 3-AA, and Nokomis 3-F4 (Mar-Len Supply), Saf-Ron Gold (Sustainable Environmental Technologies), Sea Brat #4 (Alabaster), and ZI-400 (Z.I. Chemicals) were shipped directly from each manufacturer to the contract testing laboratory. They were logged into their test material center and maintained according to good laboratory practice and chain-of-custody requirements.

Dispersant-only tests

Exposure concentrations for use in acute toxicity tests with dispersants alone were prepared, with slight modification as listed in Table 1, following the requirements specified in U.S. EPA 62 FR 15576, Appendix C of Part 300 [4]. In brief, stock solutions were prepared by adding 1.1 ml dispersant to 110 ml seawater. The solutions were mixed using a top stirrer (TAMCO) equipped with a stainless steel blade at speeds providing a 70% vortex. An appropriate aliquot of stock was removed from the area between the mixing vessel wall and edge of the vortex and placed directly in the dilution water within an exposure beaker. Each exposure solution was mixed by stirring before introducing test organisms. The exposure concentration range for each dispersant was chosen to bracket the estimated median lethal concentration (LC50) values reported in the NCP Product Schedule (Table 2).

LSC and dispersant–LSC tests

Assessment of oil-only and dispersant–oil toxicity was determined using water-accommodated fractions (WAFs) of LSC or chemically enhanced water-accommodated fractions (CE-WAFs) of dispersant–LSC as described later. South Louisiana sweet crude WAFs were prepared following the methods of the Chemical Response to Oil Spills: Ecological Effects Research Forum [9] with the variable dilution modification described by Barron and Ka'aihue [10]. In brief, glass aspirator bottles with hose bibs at the base were each fitted with a length of silicone tubing and a hose clamp. Each bottle was filled with 19 L seawater, leaving a 20% headspace above the liquid, placed on a magnetic stir plate, and a stir bar was added. South Louisiana sweet crude was added at 25 g/L seawater, using a long tube attached to a glass funnel to reduce production of air bubbles in the surface slick. The stir plate was adjusted to obtain an oil vortex of 25% of the total volume of seawater, which provided a similar mixing energy in each WAF preparation. The

Table 1. List of modifications to test procedures specified under Appendix C of 62 CFR Part 300 Appendix C [4] for conducting acute toxicity tests with dispersants alone using the mysid shrimp, Americamysis bahia, and inland silverside, Menidia beryllina

Test parameter	Specified in SubPart J Appendix C	Method used in present study
Photoperiod and light intensity	Constant light conditions	16:8 h light:dark
	2,000 lumens/m^2	1,000 lumens/m^2
Glassware cleaning	Hexane immersion	Acetone rinse
Reference toxicant test	Two species simultaneously	Staggered tests
Rangefinder tests	Prior to definitive test	Use NCP data to define test concentrations
Age at test start:		
Mysid	5–7-d-old larvae	3–4 d old; all within 24 h same age
Menidia	7-d-old larvae	9–14 d old; all within 24 h same age
Stock solution preparation	Blender 10,000 rpm	Top stirring at 70% vortex
Mysid test solution mixing	Not specified	Short term gentle mixing following stock addition
Menidia test solution mixing	Test jars on shaker platform	Same procedure as for mysids
Loading of test organisms	Not specified	Impartial, two at a time
Dilution water	Natural seawater preferred	20-mm filtered natural seawater, salinity adjusted

Table 2. Nominal exposure concentrations of dispersant preparations used in acute toxicity tests with Americamysis bahia and Menidia beryllina

Dispersant	Definitive test concentrations (ml/L)	
	Mysid shrimp (Americamysis bahia)	Inland silverside (Menidia beryllina)
Corexit 9500A	100, 56, 32, 18, 10	320, 180, 100, 56, 32
Dispersit SPC 1000	56, 32, 18, 10, 5.6	10, 5.6, 3.2, 1.8, 1.0
JD-2000	3,200, 1,800, 1,000, 560, 320	5,600, 3,200, 1,800, 1,000, 560
Nokomis 3-AA	56, 32, 18, 10, 5.6	100, 56, 32, 18, 10
Nokomis 3-F4	100, 56, 32, 18, 10	100, 56, 32, 18, 10
Saf-Ron Gold	1,000, 560, 320, 180, 100	100, 56, 32, 18, 10
Sea Brat 4	320, 180, 100, 56, 32	100, 56, 32, 18, 10
ZI-400	320, 180, 100, 56, 32	100, 56, 32, 18, 10

bottles were securely covered; the solutions were mixed for 18 h then allowed to settle for 6 h. The WAF (aqueous phase) was removed from the bottom without disturbing the oil slick remaining on the surface. The WAF was remixed after removal, and 2 L WAF was used for analysis of TPH, with the remaining volume available to prepare the test solutions. In a secondary series of acute tests conducted only with M. beryllina, two WAFs using 50 and 100 g LSC/L seawater were prepared following the procedures described previously.

The method for preparing each of the eight dispersant/LSC CE-WAFs followed the LSC WAF procedure, with the addition of each dispersant at a ratio of 1:10 dispersant:oil (2.5 g/L) after the 25% oil vortex was established. Mixing and settling times followed the oil-only procedures described previously. Dispersant manufacturers have generally recommended application rates using dispersant-to-oil ratios between 1:50 to 1:10, depending on oil type and sea conditions. A ratio of 1:10 is the standard recommendation for dispersant toxicity testing, because it maximizes the effect of the dispersant on oil in the CE-WAF [10]. Two rounds of testing of the dispersant–oil mixtures were necessary to ensure that test concentrations bracketed the CE-WAF LC50 and met test condition requirements for both A. bahia and M. beryllina.

Separate oil-only WAFs were used to prepare test solutions for A. bahia and M. beryllina with LSC. For dispersant–oil testing, each CE-WAF was divided and used to prepare solutions for both A. bahia and M. beryllina tests. Natural filtered seawater adjusted to 20 parts per thousand with laboratory well water was used for all static acute tests. Larval A. bahia and M. beryllina were treated with dilutions of LCS WAF or dispersant/oil CE-WAF plus an untreated seawater control. Six concentrations (plus control) of the oil-only WAF were tested, with the highest exposure level being 100% WAF. Each of the dispersant/oil CE-WAF tests was performed with six to eight exposure concentrations plus an untreated seawater control treatment to bracket the median lethal concentration (Table 3).

Reference toxicity tests

Two sets of 48-h (A. bahia) and 96-h (M. beryllina) acute toxicity tests were conducted with the standard reference toxicant, sodium dodecyl sulfate (SDS; Sigma-Aldrich), to evaluate the relative sensitivity of the test organisms over the course of the two-phase study. The organisms tested with SDS were from the same populations and age range used in both phases of the toxicity tests conducted with dispersants, LSC, and dispersant–LSC mixtures.

Chemical analysis

A 1-L sample was collected from each WAF, and two replicate 1-L samples were collected from each CE-WAF for analysis of C9-C32 total petroleum hydrocarbons (TPH), the standard method for quantifying oil in water (Fig. S1). Each sample was extracted with methylene chloride, reduced to 1 ml, and analyzed by gas chromatography-flame ionization detection. Analysis was conducted on an HP5890 GC (Hewlett-Packard) following EPA SW-846, Method 8015B-DRO. Additional gas chromatography-flame ionization detection analyses were performed on the CE-WAFs of Corexit 9500A and JD-2000 to provide tentative identification of single high-level chemical peaks in the chromatograms. The peaks were identified as nonpetroleum hydrocarbon constituents in Corexit 9500A and JD-2000 consisting of ethoxylated mono- and trioleates and were removed from the calculation of their

Table 3. Measured concentrations, visual observations, and percent exposure concentrations of dispersant–oil chemically enhanced water accommodated fractions (CE-WAFs) and Louisiana sweet crude (LSC) WAF preparations used in acute toxicity tests with Americamysis bahia and Menidia beryllina

Dispersant-LSC CE-WAF or LSC-WAF	CE-WAF visual observations	Measured TPH in 100% CE-WAF or LSC-WAF (mg/L)	Definitive test concentrations (% CE-WAF)	
			Mysid shrimp (Americamysis bahia)	Inland silverside (Menidia beryllina)
9500A	Very dark brown	44.6	50, 25, 13, 6.3, 3.1, 1.6	100, 50, 25, 13, 6.3, 3.1
Dispersit SPC 1000	Cloudy beige	400	3.1, 1.6, 0.8, 0.4, 0.2 0.1, 0.05	6.3, 3.1, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05
JD-2000	Slightly cloudy with oil particulates	6.8	100, 50, 25, 13, 6.3, 3.1	100, 50, 25, 13, 6.3, 3.1
Nokomis 3-AA	Slightly cloudy beige	87	3.1, 1.6, 0.8, 0.4, 0.2 0.1, 0.05	6.3, 3.1, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05
Nokomis 3-F4	Dark cloudy brown	1,600	3.1, 1.6, 0.8, 0.4, 0.2 0.1, 0.05	3.1, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025
Saf-Ron Gold	Cloudy pearlescent white	57 (mysid) 63 (Menidia)	50, 25, 13, 6.3, 3.1, 1.6	6.3, 3.1, 1.6, 0.8, 0.4, 0.2
Sea Brat 4	Slightly cloudy, brown tint	86	6.3, 3.1, 1.6, 0.8, 0.4, 0.2	13, 6.3, 3.1, 1.6, 0.8, 0.4
ZI-400	Very cloudy brown	1,800	6.3, 3.1, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05	3.1, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025
LSC-Only	Clear	4.4 (mysid) 5.1 (Menidia)	100, 50, 25, 13, 6.3, 3.1	100, 50, 25, 13, 6.3, 3.1

respective TPH values. Final CE-WAF concentrations of TPH were determined as the average of the two replicate measured values and reported as milligrams TPH per liter.

Statistical analysis

The commercially available statistical software package CETIS (Tidepool Scientific Software) was used to calculate LC50 values, using an automated decision tree adapted from the U.S. EPA for selection of the appropriate statistical method [11,12]. The LC50 is defined as the concentration of a substance causing death in 50% of test organisms for a specified interval; in this case, 48 h for the A. bahia tests and 96 h for the M. beryllina tests. Procedures used to calculate LC50 values and 95% confidence intervals included linear regression methods, the nonparametric Spearman-Karber and Trimmed Spearman-Karber methods, and the binomial method. The LC50 values are reported as parts per million in microliters per liter for the dispersant-only tests and milligrams TPH per liter for tests with LSC and dispersant–LSC mixtures.

RESULTS

SDS reference toxicity

The LC50 values (and 95% confidence intervals) for the reference toxicant SDS were 23 mg/L (19–26 mg/L) and 18 mg/L (15–21 mg/L) for the 48-h A. bahia tests and 9.5 mg/L (8.7–10 mg/L) and 10 mg/L (8.6–12 mg/L) for the 96-h M. beryllina tests conducted during phases 1 and 2, respectively. During the last 24 h of the SDS test with M. beryllina conducted during phase 1, the temperature dropped to 228C, which was 28 below the acceptable criteria. However, no difference was seen in mortality counts between the 72-h and the 96-h observations, suggesting that the temperature change had no negative impact on the test or the final calculated LC50. For both species, the results of the reference toxicant testing were similar between phases 1 and 2, indicating similar sensitivity of test organisms over the course of the study.

Dispersant-only toxicity

Americamysis bahia. Control performance (without dispersant) met all criteria for an acceptable exposure in each A. bahia test (90% survival). All water quality parameters were within ranges specified in the protocol, with the exception of dissolved oxygen for the high test concentration (56 ml/L) in the Nokomis 3-AA exposure at 24 h, which was measured at 56% of saturation. Because dissolved oxygen levels were greater than 60% at other time points in the test and the toxicity was clearly dose related, the departure observed in the 56 ml/L concentration at 24 h was not considered to have had a negative impact on the exposure with Nokomis 3-AA. The LC50 values for dispersant acute tests with A. bahia ranged from 12 ml/L for Dispersit SPC 1000 to 788 ml/L for JD-2000 (Fig. 1; Table 4). Examples of representative concentration–response curves for exposures conducted with Corexit 9500A are presented in Figure 2. The U.S. EPA uses a five-step scale of toxicity categories to classify pesticides based on their acute toxicity to aquatic organisms (Table 5; http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis eco.htm#Ecotox). Using this toxicity classification, Corexit 9500A, Dispersit SPC 1000, Nokomis-3AA, Nokomis 3-F4, Sea Brat 4, and ZI-400 would be classified as slightly toxic, whereas JD-2000 and Saf-Ron Gold would be classified as practically nontoxic to A. bahia.

Menidia beryllina. All water quality parameters were within ranges specified in the test protocol, and M. beryllina control performance met all criteria for an acceptable exposure in each of the eight dispersant tests conducted (90% survival). The LC50 values for dispersant acute toxicity tests with M. beryllina ranged from 2.9 ml/L for Dispersit SPC 1000 to 130 ml/L for

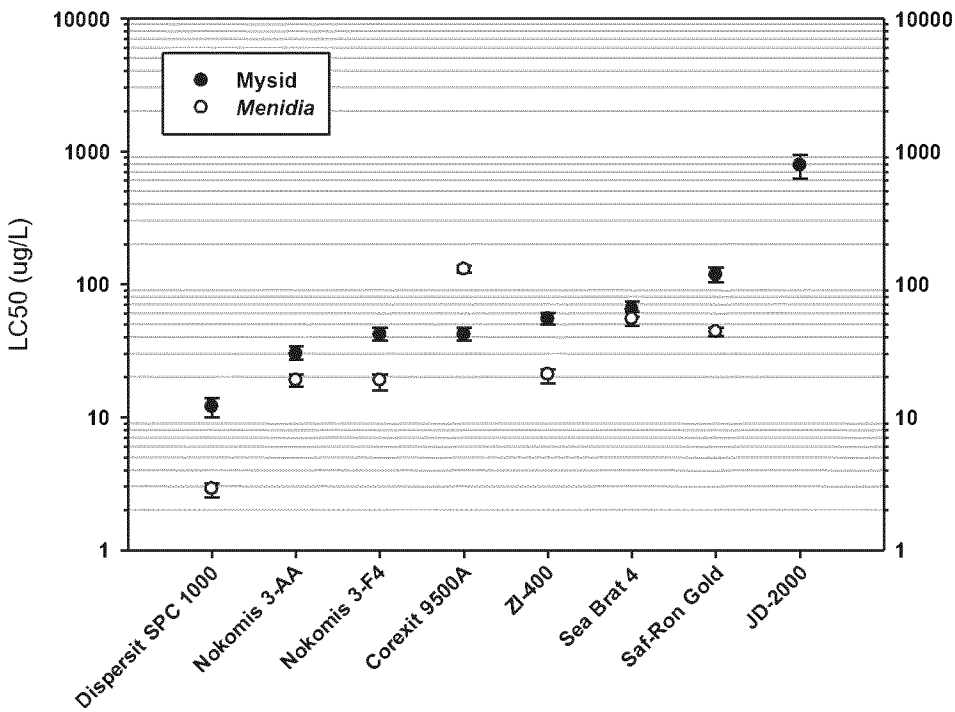

Fig. 1. Median lethal concentrations (LC50s) and 95% confidence intervals from Americamysis bahia 48-h and Menidia beryllina 96-h acute toxicity tests conducted with eight oil dispersants. Dispersants are listed in decreasing order of toxicity based on results with A. bahia. For M. beryllina, the LC50 for the dispersant JD-2000 exceeded the highest concentration tested of 5,600 ml/L (not shown).

Table 4. National Contingency Plan (NCP) Product Schedule listing of dispersants and results of Americamysis bahia 48-h and Menidia beryllina 96-h static acute toxicity tests with eight dispersants derived in the present study[a]

	Mysid shrimp (Americamysis bahia)		Inland silverside (Menidia beryllina)	
Dispersant	NCP Product Schedule LC50 (ml/L) [95% CI]	Present study LC50 (ml/L) [95% CI]	NCP Product Schedule LC50 (ml/L) [95% CI]	Present study LC50 (ml/L)[b] [95% CI]
Corexit 9500A	32.2 [26.5–39.2]	42[c] [38–47]	25.2 [13.6–46.6]	130 [122–138]
Dispersit SPC 1000	16.6 [14.1–19.6]	12[d] [10–14]	3.5 [3.1–4.0]	2.9 [2.5–3.2]
JD-2000	90.5 [76.1–108]	788[d] [627–946]	407 [330–501]	>5,600 [NA]
Nokomis 3-AA	20.2 [17.4–22.8]	30[b] [27–34]	34.2 [29.2–37.95]	18 [16–21]
Nokomis 3-F4	32.2 [28.4–36.5]	42[c] [38–47]	29.8 [24.0–35.4]	19 [16–21]
Saf-Ron Gold	63.0 [52.9–75.1]	118[b] [104–133]	29.4 [25.2–34.3]	44 [41–47]
Sea Brat 4	14.0 [┐10.4]	65[d] [57–74]	30.0 [┐16.2]	55 [49–62]
ZI-400	21.0 [17.9–24.5]	55[b] [50–61]	31.8 [28.7–35.1]	21 [18–23]

[a] Median lethal concentration (LC50, ml/L) values and 95% confidence intervals in brackets.
[b] Estimated by Spearman-Karber method.
[c] Estimated by binomial method.
[d] Estimated by linear regression method.

Corexit 9500A (Fig. 1). The LC50 for JD-2000 exceeded the highest test concentration of 5,600ml/L and was not retested. Using the U.S. EPA toxicity classification, Dispersit SPC 1000 would be considered moderately toxic, whereas Nokomis-3AA, Nokomis 3-F4, Saf-Ron Gold, Sea Brat 4, and ZI-400 would be classified as slightly toxic, and Corexit 9500A and JD-2000 as practically nontoxic to M. beryllina. Based on comparison of LC50 values and 95% confidence intervals, the rank order toxicity (most to least toxic) of the dispersants to M. beryllina was Dispersit SPC 1000 > Nokomis 3-AA, Nokomis 3-F4, ZI-400 >Saf-Ron Gold > Sea Brat 4 > Corexit 9500A > JD-2000.

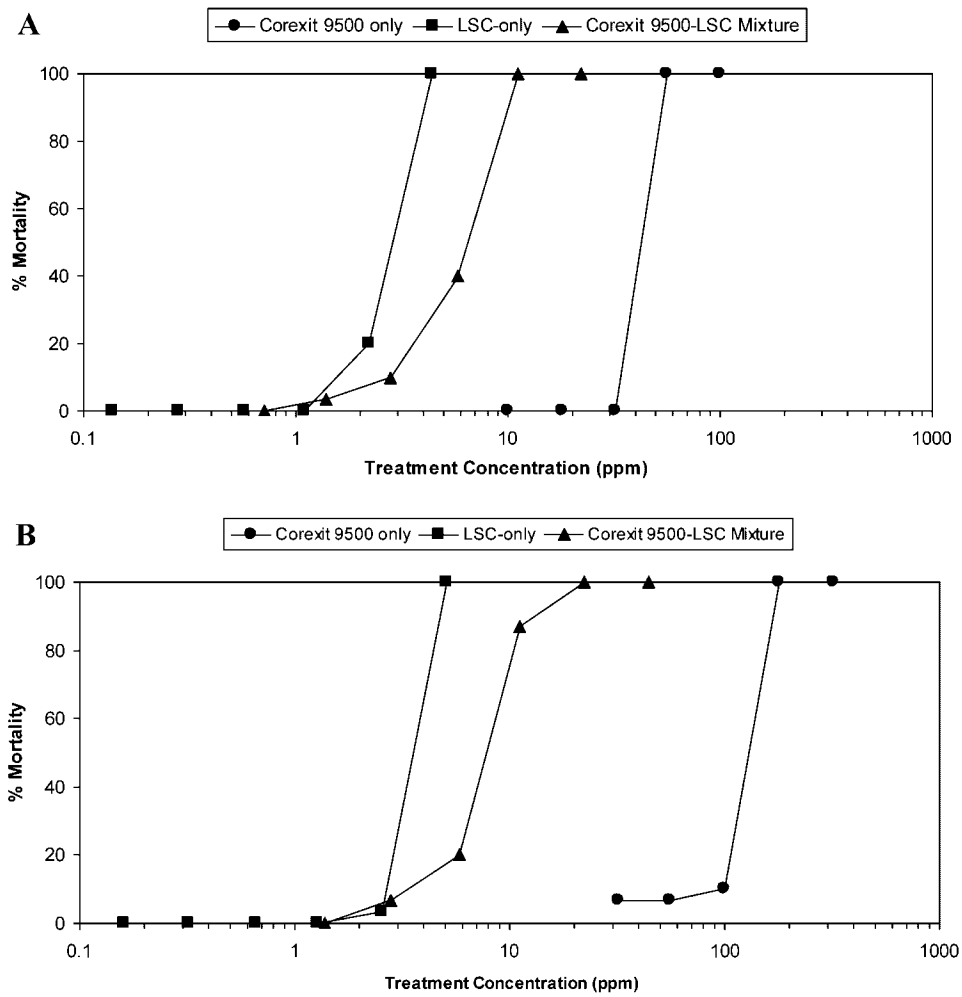

Fig. 2. Concentration–response curves for mysid shrimp, Americamysis bahia (A), and inland silversides, Menidia beryllina (B), exposed to Corexit 9500A-only, Louisiana sweet crude (LSC) water-accommodated fractions (WAF), and Corexit 9500A–LSC chemically enhanced WAFs.

Table 5. U.S. Environmental Protection Agency five-step scale of toxicity categories used to classify chemicals based on their acute toxicity

LC50 (ml/L or mg/L)	Toxicity classification
> 100	Practically nontoxic
>10 to 100	Slightly toxic
>1 to 10	Moderately toxic
0.1 to 1.0	Highly toxic
<0.1	Very highly toxic

LC50 ¼ median lethal concentration.

LSC-only toxicity

Americamysis bahia and M. beryllina. Control performance met all criteria for an acceptable exposure in each test (90% survival) for both A. bahia and M. beryllina exposures. All water quality parameters in all treatments were within ranges specified in the protocol for each species. The measured TPH concentration in the LSC WAF used for the acute mysid test was 4.4 mg/L, resulting in a calculated LC50 value of 2.7 mg/L and corresponding 95% confidence interval of 2.5 to 3.0 mg/L (Fig. 3). The measured TPH concentration in LSC WAF used for the acute test with M. beryllina was 2.9 mg/L where mortality did not exceed 7% in the highest concentration tested, consisting of 100% WAF. When M. beryllina was retested under identical conditions, no mortality occurred in the 100% WAF having a measured TPH of 2.3 mg/L. A secondary series of acute tests conducted using WAFs prepared with loading rates of 50 g and 100 g LSC/L seawater resulted in higher TPH concentrations of 5.4 and 5.1 mg/L, respectively. Using these WAF preparations, calculated LC50s and 95% confidence intervals of 3.5 mg/L (3.4–3.7 mg/L) and 4.05 mg/L (3.8–4.3 mg/L) were determined for M. beryllina exposed to LSC (Fig. 3). Using the U.S. EPA toxicity classification, LSC oil would be classified as moderately toxic to both A. bahia and M. beryllina.

Dispersant–LSC toxicity

The measured TPH in 100% CE-WAF of the dispersant–LSC mixtures used for both A. bahia and M. beryllina acute tests ranged from 6.8 to 1,800 mg/L (Table 3). Visual inspection of the CE-WAFs reflected the range of aqueous phase TPH in these mixtures.

Americamysis bahia. Control performance met all criteria for an acceptable exposure (90% survival) in all tests with A. bahia. A definitive LC50 was determined for the initial A. bahia test with Saf-Ron Gold CE-WAF, and all water quality parameters were within ranges specified in the protocol for all treatments. Initial mysid tests with CE-WAFs of Dispersit SPC 1000, Nokomis 3-AA, Nokomis 3-F4, and ZI-400 had LC50s less than the lowest concentration tested, which required a second round of testing. A second round of testing was also required for Corexit 9500A, JD-2000, and Sea Brat 4 because of temperature or dissolved oxygen deviations from protocols. All water quality parameters were within ranges specified in the test protocol for A. bahia, and definitive LC50 values were determined for each of the seven dispersant–oil CE-WAFs tested. The LC50 values for dispersant–oil acute toxicity tests with A. bahia ranged from 0.39 mg TPH/L for Nokomis 3-AA CE-WAF to 9.7 mg TPH/L for the ZI-400 CE-WAF (Fig. 3; Table 6). Using the U.S. EPA toxicity classification, oil–dispersant mixtures using Nokomis 3-AA would be considered highly toxic, whereas Corexit 9500A, Dispersit SPC 1000, JD-2000, Nokomis 3-F4, Saf-Ron Gold, Sea Brat 4, and ZI-400 would be classified as moderately toxic to A. bahia.

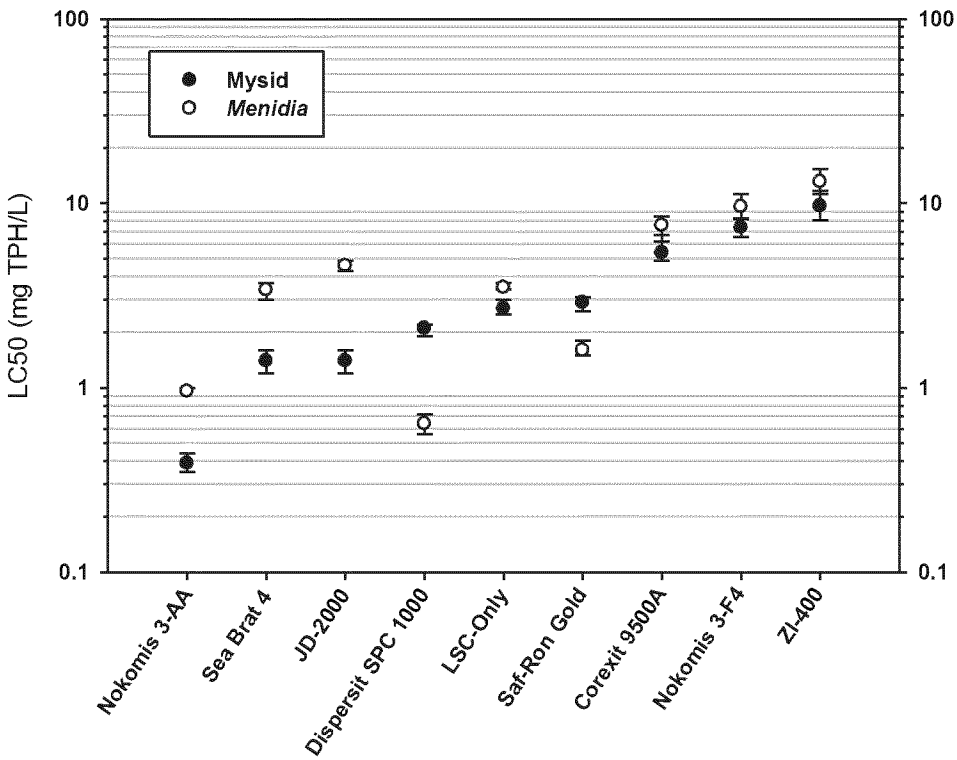

Fig. 3. Median lethal concentrations (LC50s) and 95% confidence intervals from mysid shrimp, Americamysis bahia, 48-h and inland silversides, Menidia beryllina, 96-h acute toxicity tests conducted with eight dispersant–oil chemically enhanced water-accommodated fractions (CE-WAFs) and Louisiana sweet crude (LSC)-only WAFs. Dispersant–oil mixtures and LSC are listed in decreasing order of toxicity based on results with A. bahia. TPH ¼ total petroleum hydrocarbon.

Table 6. Results of Americamysis bahia 48-hr and Menidia beryllina 96-h static acute toxicity tests with eight dispersant-oil mixture chemically enhanced-water accommodated fractions (CE-WAFs) and Louisiana sweet crude oil-only WAF derived in the present study[a]

	LC50 (mg TPH/L)[b] [95% CI]	
Dispersant CE-WAF	Mysid shrimp (Americamysis bahia)	Inland silverside (Menidia beryllina)
Corexit 9500A	5.4 [4.9–6.7]	7.6 [6.2–8.5]
Dispersit SPC 1000	2.1 [1.9–2.2]	0.64 [0.56–0.72]
JD-2000	1.4 [1.2–1.6]	4.6 [4.3–4.9]
Nokomis 3-AA	0.39 [0.35–0.44]	0.96 [0.96–1.0]
Nokomis 3-F4	7.4 [6.6–8.3]	9.6 [8.2–11.2]
Saf-Ron Gold	2.9 [2.6–3.1]	1.6 [1.5–1.8]
Sea Brat 4	1.4 [1.2–1.6]	3.4 [3.0–3.7]
ZI-400	9.7 [8.1–11.7]	13.1 [11.2–15.3]
Louisiana sweet crude oil-only WAF	2.7 [2.5–3.0]	3.5 [3.4–3.7]

[a] Median lethal concentration (LC50) values and 95% confidence intervals (CI) in brackets calculated using Spearman-Karber method.
[b] Measured mg total petroleum hydrocarbons/L.

Menidia beryllina. Control performance met all criteria for an acceptable exposure (90% survival) in all tests with M. beryllina. Initial tests with CE-WAFs of Dispersit SPC 1000, Nokomis 3-AA, Nokomis 3-F4, Saf-Ron Gold, and ZI-400 had LC50s less than the lowest concentration tested, which required a second round of tests to be conducted. Second-round testing was also required for Corexit 9500A, JD-2000, and Sea Brat 4 because of temperature or dissolved oxygen deviations from protocols. The LC50 values for dispersant–oil acute toxicity tests with M. beryllina ranged from 0.64 for Dispersit SPC 1000 to 13.1 mg TPH/L for ZI-400 (Fig. 3). Using the U.S. EPA toxicity classification, oil–dispersant mixtures using Dispersit SPC 1000 or Nokomis 3-AA would be considered highly toxic, whereas Corexit 9500A, JD-2000, Nokomis 3-F4, Saf-Ron Gold, and Sea Brat 4 would be classified as moderately toxic, with ZI-400 as slightly toxic to M. beryllina.

DISCUSSION

Companies that manufacture dispersants are required to submit information on the toxicity and percentage effectiveness of their products to the U.S. EPA contingent to listing their products on the NCP Product Schedule. As a result, the toxicity information from these tests was generated by a variety of commercial testing laboratories over an extended period. To ensure the most accurate data were available to make informed decisions on the least toxic, most efficient dispersants to combat the DWH spill, U.S. EPA's Office of Research and Development conducted independent toxicity studies using both cell-based in vitro assays and whole animal in vivo tests. The in vitro assays were based on U.S. EPA's ToxCAST program and focused on the toxicity and potential endocrine effects of the dispersants alone [13]. The in vivo acute tests presented here included dispersants, LSC, and dispersant–LSC mixtures conducted by a single contract laboratory under U.S. EPA oversight. Of the 15 dispersants on the NCP product schedule, eight were chosen for testing based on known toxicity and efficacy information, product availability for immediate testing, and availability of sufficient dispersant quantities to respond to the spill. In these studies, LSC was selected as the reference oil, because it was considered more relevant and representative of conditions occurring in the Gulf of Mexico than testing no. 2 fuel oil as specified by the NCP. Thus, our approach provided an independent, consistent, and quantitative assessment of acute toxicities of dispersants, LSC oil, and eight dispersant–LSC mixtures to two aquatic species inhabiting Gulf of Mexico waters.

An important component of the present study included assessing the accuracy of information on dispersant toxicity the manufacturers provided to the NCP. A qualitative comparison was made between LC50 values for the eight dispersants tested as well as with those available in the NCP Product Schedule. The reproducibility of static acute tests among laboratories using the same species/toxicant combination has been reported to fall generally within a factor of 3.5 among laboratories when using nominal concentrations (unmeasured treatment concentrations) for both freshwater and marine species [14]. Given the use of whole organisms in these tests, some variation in response attributable to differences in parameters such as culture and acclimation conditions, stock populations, or variable water quality is expected and acceptable. Factor ratios were used to compare LC50s derived for the same species/dispersant combination from different laboratories. The factor ratios between dispersant LC50 values determined in the present study and NCP-reported LC50 values were calculated as a ratio by dividing the higher of the two LC50 values by the lower LC50 value for each of the eight dispersants, respectively (Table 4). As an example, using information for A. bahia from Table 4, the factor ratio for Corexit 9500A was determined as 42/32.2¼ 1.3. For A. bahia, the factor ratios calculated for Corexit 9500A, Dispersit SPC1000, Nokomis 3-AA, Nokomis 3-F4, Saf-Ron Gold, and ZI-400 were less than or equal to 2.6, which was considered within normal interlaboratory variability [14]. Results for JD-2000 and Sea Brat 4 showed lower toxicities (i.e., higher LC50s), with factor ratios of 8.7 and 4.6, respectively, compared with their reported NCP LC50 values. With M. beryllina, the factor ratios calculated for Dispersit SPC 1000, Nokomis 3-AA, Nokomis 3-F4, Saf-Ron Gold, Sea Brat 4, and ZI-400 were less than or equal to 1.83, which was considered within normal interlaboratory variability. The factor ratios of 5.2 and 13.8 for Corexit 9500A and JD-2000 indicate that the LC50 values reported for these dispersants in the NCP Product Schedule would be considered different from (that is, lower than) the LC50 values for M. beryllina determined in the present study. Possible explanations for the 13.8-fold difference between the reported NCP LC50 for JD-2000 and the highest exposure concentration tested for M. beryllina in the present study may be attributable to batch-to-batch variability in the manufacturing process, instability of the stored product over time, or a change in the product formulation.

Corexit 9500A was the predominant dispersant used in response to the DWH event, with an estimated 6,974,621 liters used in the Gulf of Mexico in both surface and subsurface applications (www.restorethegulf.gov). Initially, small amounts of a predecessor dispersant, Corexit 9527, were applied during early response efforts until locally available stocks had been depleted. Previous studies reported by Singer et al. [6] indicate no significant difference in acute toxicity to marine organisms between the two Corexit formulations. This suggests the possibility that unique toxicological effects occurring through exposure to Corexit 9527 during the early period of the spill were probably negligible. In experiments with dispersants alone, we found the toxicity of Corexit 9500A to be essentially equivalent to most of the dispersants tested. The exceptions were the increased sensitivity of M. beryllina exposed to Dispersit SPC 1000 and the lack of toxicity observed in both species exposed to JD-2000. Without specific information on product formulations, surmising why the acute toxicity of these two dispersants, especially JD-2000, differed from the other dispersants by approximately an order of magnitude was difficult. With the exception of Corexit 9500A, little or no toxicity information with aquatic species is available for the remaining seven dispersants. Inspection of LC50 values for Corexit 9500 referenced in George-Ares and Clark [15] for the A. bahia and M. beryllina are from unpublished laboratory toxicity reports and identical to the values listed on the NCP Product Schedule, suggesting that these values were submitted to the U.S. EPA NCP. However, a comparative toxicity study conducted with Corexit 9500 and A. bahia, M. beryllina, and the sheepshead minnow (Cyprinodon variegatus) using 96-h acute static-renewal tests reported similar LC50s of 32, 79, and 180 mg/L, respectively [16]. In other studies using static-renewal exposures, 96-h LC50s (95% CL) were reported for Corexit 9500A of 21 mg/L (18.6–23.5 mg/L) for A. bahia, 79.3 mg/L (70.5–81.1 mg/L) for M. beryllina [17], and 35.9 mg/L (32.2–41.3 mg/L) for A. bahia [18], which are consistent with the results of the present study. Our results, together with reported values, suggest that A. bahia are marginally more sensitive than M. beryllina to Corexit 9500A, although the dispersant alone is considered only slightly toxic to both species using U.S. EPA toxicity categories.

Second-generation dispersants such as Corexit 9500A are typically less toxic than oil alone or dispersed oil [5,15], suggesting that dispersant toxicity during a spill response is of secondary concern to the inherent toxicity of the oil or dispersed oil. This observation was substantiated in the present study, in which LSC showed higher toxicity (lower LC50s) to mysids than the eight dispersants tested alone, with similar effects observed for M. beryllina. Anderson et al. [19] reported comparable 96-h LC50s (95% CL) for static tests conducted with adult M. beryllina of 5.5 mg/L (3.3–9.4 mg/L) and adult Americamysis (Mysidopsis) almyra, a sympatric species with A. bahia, of 8.7 mg/L (7.2–10.7 mg/L), suggesting little difference in sensitivity to LSC between larval and adult life stages of these species. Further, most of the dispersant–LSC mixtures we tested were within the same order of magnitude of toxicity as LSC alone, indicating a lack of additive or synergistic effect of the dispersants on LSC toxicity. Therefore, the principal concern associated with the use of dispersants is not the toxicity associated with a dispersant alone, but rather with dispersed oil and the increased bioavailability of toxic oil constituents dissipated over a larger spatial area of the water column.

Measured TPH concentrations were found to vary over a wide range for the eight 100% CE-WAF stock solutions used in preparation of the test concentrations (Table 3). Interestingly, the two dispersant–LSC stock solutions demonstrating the highest measured TPH, ZI-400 (1800 mg TPH/L) and Nokomis 3-F4 (1600 mg TPH/L), generated the lowest overall acute toxicity values for both A. bahia and M. beryllina. However, this trend did not extend to the other six dispersant/LSC mixtures tested, and whether dispersant-specific constituents were responsible, or whether the occurrence was a function of the low dilution factors used in preparation of the ZI-400 and Nokomis 3-F4 exposure concentrations, is unclear.

Some speculation has been made on whether the A. bahia and M. beryllina specified by the NCP for use in the acute toxicity studies of dispersants and dispersed oil serve as adequate surrogates capable of protecting the biological and geographical diversity of species potentially impacted by an oil spill event. Both species were selected for inclusion in the U.S. EPA acute and chronic test methods based on their ease of culture, availability of life stages, and relative sensitivity to a wide range of chemical contaminants. Inspection of reported values for a variety of dispersants, oils, and chemically dispersed oils indicate that both A. bahia and M. beryllina are generally representative of most aquatic vertebrate and invertebrate species tested [5,15]. However, only a small number of species of limited diversity have been routinely used in testing. Expansion of test species for organisms at risk to oil exposures should be considered based on habitat and geographic location to ensure that adequate information is available for hazard assessment should dispersant application become an option. Unique to the DWH spill was the first-time use of deepwater dispersant injection into the oil plume 1,544 meters below the surface of the Gulf of Mexico. Because of our limited knowledge of life histories for most deep-water pelagic and benthic animals, little is known of the impact on these organisms caused by the DWH event. Unfortunately, because of biological and logistical constraints such as animal availability and extreme environmental conditions, the routine use of deep-water organisms for dispersant and oil toxicity studies are not logistically or economically feasible. These difficulties coupled with the increasing pressure for deep-water oil exploration will force our continued reliance on surrogate test species.

Short-term acute toxicity tests using consistent methodologies and test organisms provided important and fundamental information to the U.S. EPA, allowing the continued use of Corexit 9500A during the DWH disaster. The comparative toxicity analysis of dispersants, sweet crude oil, and dispersant–sweet crude oil mixtures on standard aquatic test species provides an improved understanding of acute toxicological effects associated with dispersant use and helps inform future decision making.

SUPPLEMENTAL DATA

Fig. S1. Representative spectra from gas chromatography-flame ionization detection analysis. (48 KB PDF).

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AN INTRODUCTION

The Gulf of Mexico Research Initiative (GoMRI) is a 10-year research program that aims to reduce the impacts of oil spills on the marine environment and public health. The program is focused on improving society's understanding of oil spill issues. GoMRI provides support to the Sea Grant programs of the Gulf of Mexico (Florida, Mississippi-Alabama, Louisiana and Texas) for an extension and outreach effort to increase the use of oil spill science by people whose livelihoods depend on a healthy Gulf. This is a new model of a private entity engaging with the four Sea Grant programs in the Gulf of Mexico to develop a regional outreach program.

Program Goals

1. Share emerging oil spill science with target audiences.
2. Collect input from target audience to inform the Sea Grant outreach program, and deliver input to the GoMRI Research Board to assist in setting priorities.

The oil spill science outreach program allows Sea Grant specialists to find out what types of information target audiences want and develop tailor-made products for those audiences. The outreach specialists produce a variety of materials, such as fact sheets, focused on meeting stakeholder information needs. The specialists also gather input from target audiences through workshops and work with researchers to share oil spill research results at science seminars that are facilitated by the specialists. The program's outreach materials and events offerinformation about numerous published GoMRI research findingson such topics as dispersants, fisheriesissues and tar balls.

Target Audiences

The program has oil spill science outreach specialists who work with:

- Elected officials
- Emergency responders or managers
- Environmental non-profitstaffmembers
- Fishers
- Natural resource managers
- GoMRI outreach specialists
- Port and harbor employees
- Public health officials
- Tourism specialists
- University and college researchers

The specialists work as a team to implement outreach programs at a state and regional level. In addition, the program engages with the 29 other Sea Grant college programs throughout the nation to provide the latest oil spill science information.

Oil Spill Topics

The target audiences have expressed an interest in a variety of topics including, but not limited to:

- Seafood safety
- Human health
- Fisheries impacts
- Dispersants
- Impact to natural environment
- Oil spill response

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ABOUT SEA GRANT

The mission of Sea Grant is to enhance the practical use and conservation of coastal, marine and Great Lakes resources in order to create a sustainable economy and environment. There are 33 university-based Sea Grant programs throughout the coastal U.S. states and its territories. These programs are primarily supported by the National Oceanic and Atmospheric Administration (NOAA) and the states in which the programs are located. For more information about the four Sea Grant programs that border the Gulf of Mexico, visit *gulfseagrant.org.*

GULF OF MEXICO RESEARCH INITIATIVE

ABOUT GOMRI

The Gulf of Mexico Research Initiative is led by an independent and academic 20-member Research Board, which guides the research focus and the funding decisions to ensure the intellectual quality, effectivenessand academic independence of the GoMRI research. GoMRI was established using funds that are not associated with any oil spill penalties. For more information, visit *gulfresearchinitiative.org.*

Learn more about this outreach effortat *gulfseagrant.org/oilspilloutreach.*

Contacts

The Sea Grant oil spill science outreach team works together on multiple oil spill related research topics. Although each is based in a different state, they work regionally throughout the Gulf of Mexico.

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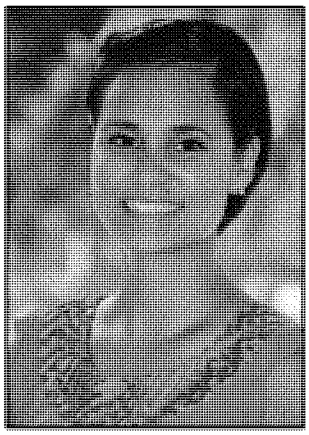

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Learn more about this effort at *gulfseagrant.org/oilspilloutreach*.

GULF OF MEXICO RESEARCH INITIATIVE

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MASGC-16-002

Emerging surfactants & sorbents for oil spill clean up

Emily S. Maung-Douglass, Larissa Graham, Christine Hale,
Stephen Sempier, LaDon Swann, Monica Wilson

The use of the oil dispersants Corexit 9527A and 9500A in response to the 2010 Deepwater Horizon oil spill was controversial due to potential impacts to aquatic life. This outreach publication examines alternative methods for oil spill response. This includes using cutting-edge technology and harnessing the power of naturally occurring chemicals and fibers.

Current approaches

The Deepwater Horizon (DWH) oil spill released 172 million gallons of oil into the ecologically sensitive and valuable habitats of the Gulf of Mexico. Responders used nearly 1.8 million gallons of chemical **dispersants** (**Corexit 9527A** and **9500A**) to combat the spill. These dispersants were applied to reduce the oiling of aquatic life and shoreline and to enhance the natural breakdown of oil by microbes. Tests conducted by the US Environmental Protection Agency (US EPA) show that Corexit 9500A minimally impacts aquatic life and that those effects are similar to other commercially available chemical dispersants.[Hemmer(2011)] Lingering concerns over human and aquatic health lead community members to ask what other approaches might be available for oil spill response.[Sempier et al. (2015)]

Emergency workers also used **sorbents** to soak up oil during Deepwater Horizon oil spill. Sorbents are useful because they can be removed from the water along with any absorbed oil.[Teli(2013)] Traditional sorbents are made from a variety of substances. These include man-made materials like plastic and natural materials like peat moss, straw, clay, and felt made from wool (felted wool).[EPA website, Zhu(2013)]

Emerging solutions

Microbial-based and inspired surfactants

Surfactants are one of several types of compounds used in chemical dispersants. Chemical forces naturally draw individual water molecules together. These strong forces prevent oil and water from mixing. Surfactants reduce the strength of attraction between individual water molecules. This leads to the formation of small oil droplets distributed throughout the water. These small droplets are easier for oil-degrading microbes to consume than oil slicks. The number of these microbes increase following an oil spill and in environments rich in nitrogen.[e.g.,Kasai(2002),Bookstaver (2015)] Scientists found that multiple species of microbes produce surfactants that help them consume the oil.[e.g.,Qiao(2010),Li(2013),Gutierrez(2013)] Laboratory tests show some strains of microbes can degrade as much as 65 percent of oil in the water in a little more than one week.[Li(2013)]

The makeup of surfactants produced by microbes varies. Some consist of combinations of compounds including sugars, amino acids, and **lipids**.[e.g., Gutierrez(2013),Marti(2014),Kegel(2016)] These surfactants have promise for use in oil spill response because they are effective, breakdown easily in the environment, and tend to have low toxicity. Scientists are mimicking these types of surfactants in the laboratory and searching for the genes that produce these surfactants.[e.g., Jackson(2015),Kegel(2016)] Surfactin is one type of surfactant produced by some microbes. It does not mix well with water, but it is effective at breaking up oil slicks. Scientists found that they can genetically modify the microbes that produce surfactin. These special microbes produce surfactants that are effective at both breaking down oil and mixing with water.[Marti(2014)]

Everyday materials – Finding treasure in unexpected places

Scientists show that everyday items can be useful during oil spill response too.

Soy lecithin is a natural surfactant extracted from soybeans. Its common uses include creating a smooth, creamy textures in processed foods. It attracts both water and oily, fatty substances. Using soy lecithin alone creates large oil droplets. Large oil droplets tend to pull together and create new oil slicks. Large oil droplets are also hard for the microbes to break down. Scientists experimented with mixing soy lecithin with other surfactants and chemically modified soy lecithin.[Athas(2014),Riehm(2015),Nyankson(2016)] These approaches are effective and create small oil droplets each with a coating.[Nyankson(2016)] The coating prevents oil droplets from reforming an oil slick. This stability is important since it will give microbes a stable meal of small oil droplets to feast upon. For comparison, soy lecithin-surfactant mixtures produced better oil droplets for microbes to consume than Corexit 9500A (Figure 1).[Athas(2014)]

Using plastic for oil spill clean-up combines old and new technologies and combats both oil and plastic pollution. Scientists discovered that they could create a thin, oil-absorbing film by melting together different types of plastics.[Saleem(2015)] The combination of plastics creates a gooey, gel-like material that later will be chemically treated and stretched out to dry. The finished product is a thin film with many microscopic holes that can absorb oil.

Jute is a fiber produced from the stalks of jute plants. It is commonly used to create burlap sacks and rug backings (Figure 2). The remains of sugarcane stalks after processing are tough and full of fibers called bagasse (Figure 3). Both of these natural fibers are biodegradable and can serve as sorbents. But it is not as simple as placing these fibers directly on an oil slick. Both materials absorb water. This reduces the fibers ability to absorb oil. Scientists found ways to modify the fibers to repel water and absorb more oil.[Sun(2003),Teli(2003)] These modified natural fibers are more effective than sorbents made of plastic fibers.[Teli(2013)] Bagasse modified in this way can absorb almost twice as much oil as traditional plastic-based sorbents.[Sun(2003)]

The sorbents previously mentioned work by soaking up oil. Sorbents can also work by getting oil and oil-based compounds to stick to them. This is how the sorbent carbon black (CB) works. Carbon black can be produced a number of ways, including charring wood, tar and bone. Potentially toxic oil-based compounds adhere to carbon black.[Saha(201S)] This reduces the amount of these oil-based compounds freely floating in the water and coming in contact with aquatic animals. Addition of CB particles to oil spills also creates stable oil-water mixtures that allows microbes to breakdown oil.

The shells of shrimp, crabs, and other crustaceans can enhance the ability of Corexit to work as a dispersant. The protein from these shells can be chemically modified into a compound called chitosan. Scientists found that chitosan works well with Corexit to treat oil. Chitosan reduces the amount of Corexit needed.[Venkataraman(2013)] It also helps create a gel-like substance that stops the movement of oil droplets in water. This leads to easier physical removal of oil from water. The addition of chemically-modified chitosan can also prevent oil droplets from gathering together over time to re-form slicks.[Benner(2015)]

Nantotechnology – Tiny materials with big impact

Nanomaterials are engineered materials made up of pieces that are up to 100,000 times smaller than the diameter of a human hair.[nano.gov] These tiny materials are used in everything from computers and cellular phones to workout clothing. Scientists are testing if their next application could be in oil spill clean-up.

Some nanomaterials are able to absorb and repel oil. **Carbon nanotube (CNT)** sponges are oneexample. They are a type of sorbent that looks like their name suggests. CNT sponges resemble large, household sponges and can be as much as 10 inches in length (Figure 4). However, the fibers making up the sponge are actually carbon nanotubes (tiny tubes made of carbon). Many CNTs are joined together to form a sponge that repels water. This sponge absorbs up to 23 times its weight in oil in only 15 minutes. Traditional sorbent materials like plastic fibers and felt made from wool can only take in eight to nine times their weight in oil (Figure 5). [ZHU(2013)]

The use of nano-sized materials in oil spill clean-up is innovative. How can responders remove these nano-materials from the environment after they have soaked up unwanted oil? Magnets may be the answer. Highly-engineered, magnetic CNTs effectively isolate and remove oil-based compounds from water in the laboratory.[Zhao2011] One potential drawback is cost. CNTs can be expensive. Scientists have been able to create relatively inexpensive, magnetic, iron-based nanoparticles in the lab. They create tiny, magnetized particles that can be added to coconut oil to create a **suspension** called a **nanofluid**. Magnetic coconut oil nanofluids are 91 percent effective at removing motor oil from water in laboratory tests. [Nabeel Rashin2014] During oil spill response, the coconut oil in the nanofluid would attract and attach to the oil in the environment. The mixture of magnetic particles, oil, and coconut oil can then be removed from of the water with a magnet (Figure 6).

Nanotechnology can be used to do more than absorb oil. It can be used to efficiently carry and apply oil-fighting chemicals directly to individual oil droplets too.

Scientists are also investigating how nanomaterials might work together with surfactants to improve oil spill clean-up. They found that nano-sized particles of silica work well with certain surfactants to create stable oil-water mixtures in the lab.[Worthen(2014)] The oil droplets were small and well-distributed in these mixtures. This creates excellent conditions for the natural breakdown of oil by microbes. **Halloysite nanotubes** (**HNTs**) are another nanomaterial scientists combine with surfactants to improve oil spill clean-up.[Owoseni(2014)]HNTs are made from microscopic sheets of a naturally occurring clay called halloysite. These sheets are rolled into tubes only nanometers in size called halloysite nanotubes (HNTs). Scientists filleach HNT with **DOSS**.[Owoseni(2014)] DOSS is the primary surfactant in the dispersants currently used by emergency responders (Corexit 9500A and 9527A). The modified HNTs work to stabilize oil-water mixtures in the lab and act as a delivery system for DOSS (Figure 7). The stability of thesemixtures improves as more HNTs are added. This stability means the oil droplets in the emulsion are less likely to recombine to form an oil slick. The number of small oil droplets formed increases as the amount of DOSS loaded on the nanotubes increases. This system of combining old (DOSS) with new technology (HNTs) make the dispersant more efficient and increases the ability for microbes to consume the oil.

The Gulf of Mexico Research Initiative (GoMRI) and others continue to explore new possibilities in the realm of oil spill clean-up. To learn more about this and the research being conducted on the Deepwater Horizon spill, visit the Gulf of Mexico Research Initiative website at www.gulfresearchinitiative.org. Visit the Gulf Sea Grant program website at http://gulfseagrant.org/oilspilloutreach to view other publications that provide additional information on the dispersants and the impact of oil and dispersant mixtures on aquatic animals. Learn about the legal framework governing the use of dispersants by visiting the Mississippi-Alabama Sea Grant site at http://masglp.olemiss.edu/publications/index.html and clicking on the keyword 'oil spills'.

Glossary

Carbon nanotubes (CNTs) – a nano-scale (1-100 nm) cylinder of carbon molecules. Has novel properties that make them potentially useful in a wide variety of applications in electronics, optics and other fields of materials science, as well as oil spill response.

Corexit – a modern dispersant approved for use in US waters that was used to minimize the presence of surface oil slicks during the Deepwater Horizon oil spill.

Corexit 9527A and 9500A – dispersants approved for use in US waters and those that were used to minimize the presence of surface oil slicks during the Deepwater Horizon oil spill.

Dioctyl sodium sulfosuccinate (DOSS) – a primary component of both dispersant formulas used in the Deepwater Horizon oil spill. It increases the attraction between oil and water molecules and hinders the formation of large oil slicks on the surface of the ocean. DOSS can also be found in consumer products such as detergents, cosmetics, and laxatives and, therefore, can be found in coastal waters.

Dispersants – chemicals that are used during oil spill response efforts to break up oil slicks and can limit floating oil from impacting sensitive ecosystems such as coastal habitats

Halloysite nanotubes (HNTs) – a nano-scale (1-100 nm) cylinder constructed from a naturally occurring aluminosilicate (halloysite). Has properties that make them potentially useful in a wide variety of applications including drug delivery, technology, and oil spill response.

Lipids – a group of compounds that does not dissolve in water. Includes natural oils, waxes, and fats.
Nanomaterials – engineered materials of which a single unit is sized between 1 and 1000 nanometers, but is usually 1—100 nm.

Sorbents – materials used to absorb oil during oil spill clean-up operations.

Surfactant – compounds that work to break up oil. Dispersants contain surfactants that break the oil slick into smaller droplets that can more easily mix into the water column.

Suspension – a mixture in which particles are dispersed throughout a bulk of fluid.

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Figures

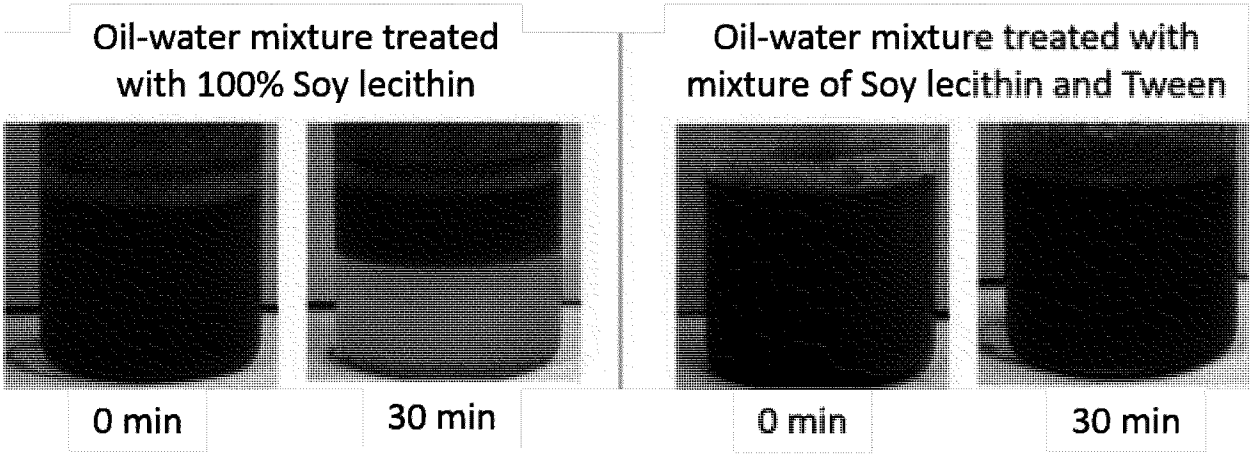

Figure 1. While soy lecithin disperses oil, it forms large oil droplets which re-form oil slicks over time (left-most images). A 60%-40% mixture of soy lecithin with the surfactant "Tween" creates tiny oil droplets that stay evenly distributed in water over time (right-most images). Images from: Athas et al. (2014).

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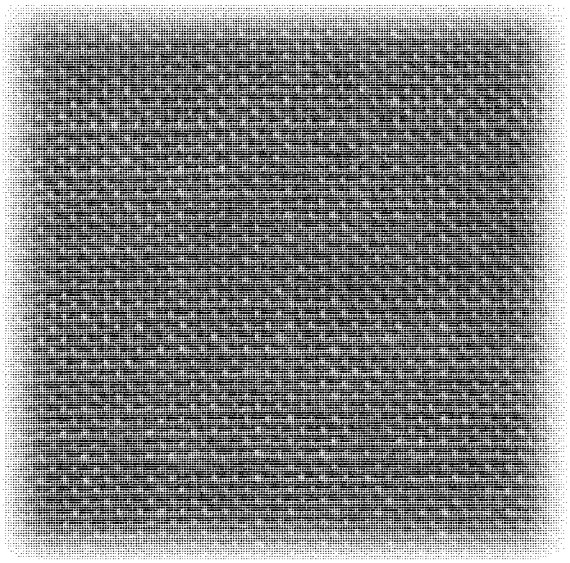

Figure 2. Jute fiber being extracted from the stem of jute plants (left). Jute fiber fabric (right). Images: Susantab (left), Luigi Chiesa (right)

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Figure 3. Sugarcane and Bagasse. Images: LSU AgCenter (top),Rufino Uribe (bottom left), Anna Frodesiak (bottom right)

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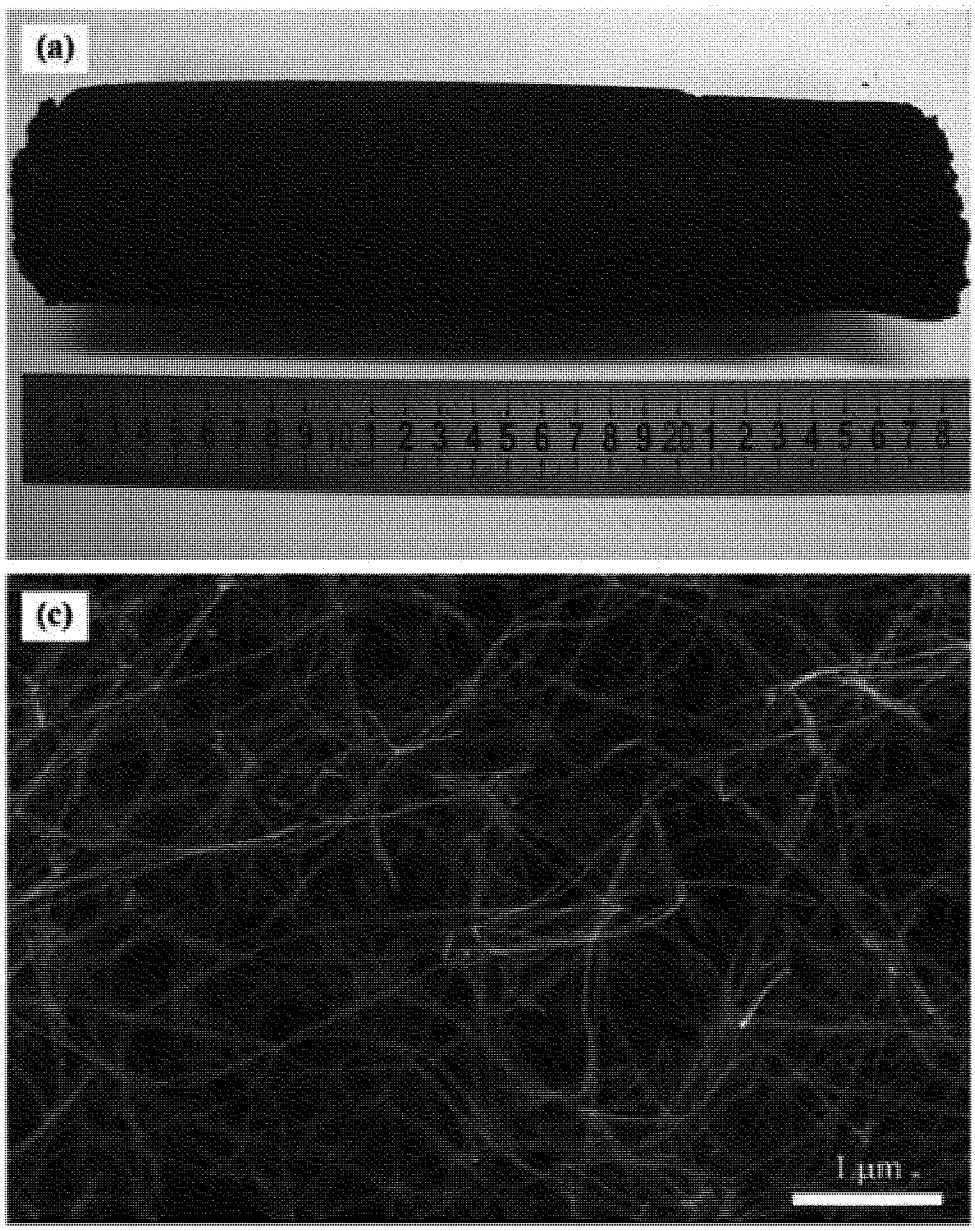

Figure 4. Carbon nanotube (CNT) sponges resemble ordinary, household sponges (top panel). However, the nano-sized tubes of carbon making up the sponge are evident when the sponge is viewed from a high-powered microscope (bottom panel). Image from: Zhu et al. (2013)

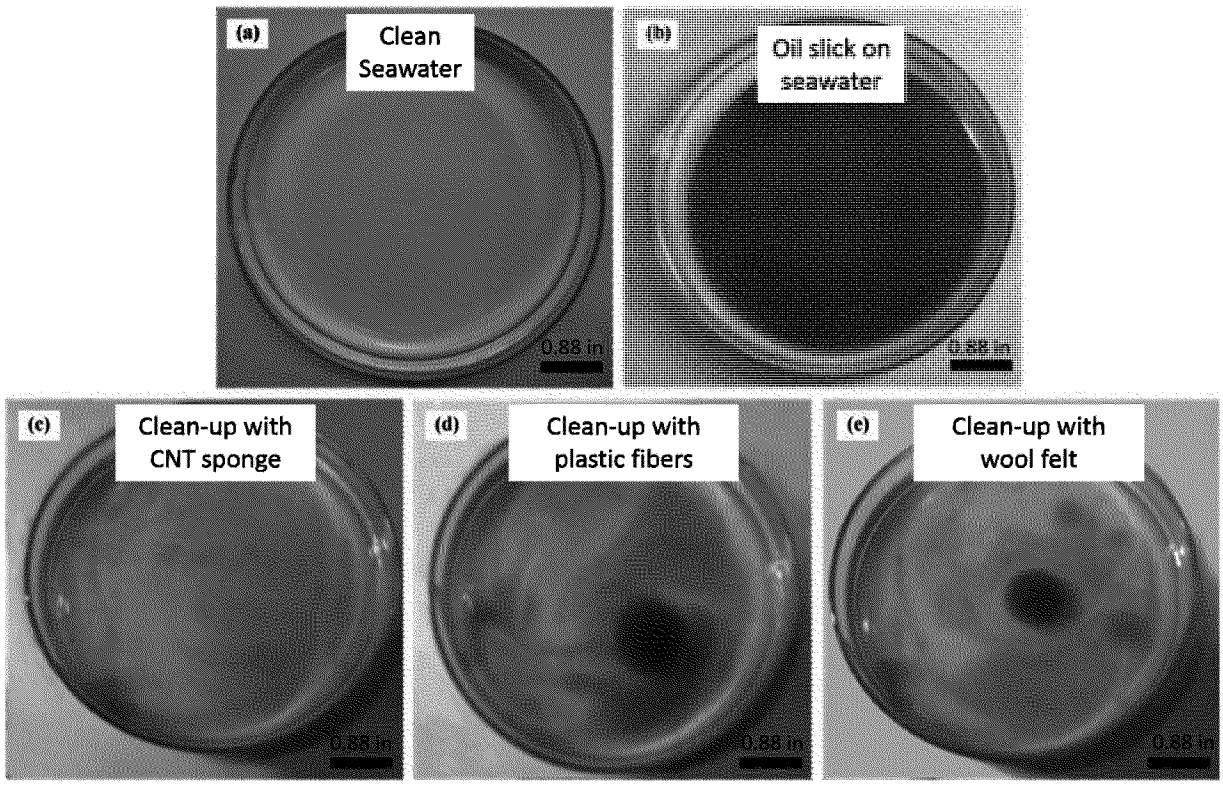

Figure 5. Laboratory testing shows that the effectiveness of sorbents varies by material. The top panels show clean seawater (a) and seawater with an oil slick (b). The bottom panels (c-e) show the effectiveness of three sorbents at soaking up an oil slick (each the one shown in panel b) for 15 minutes. Modified image from: Zhu et al. (2013)

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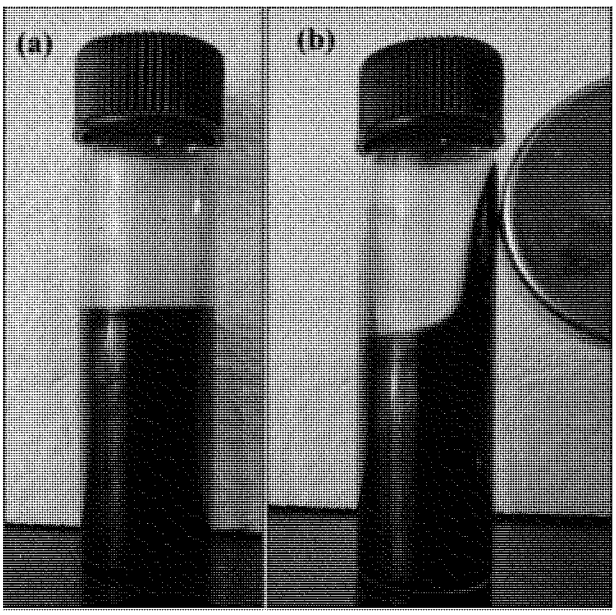

Figure 6. Magnetite–coconut oil nanofluid mixed with seawater and crude oil (a) without and (b) with a magnet. Images: Nabeel Rashin et al. (2014)

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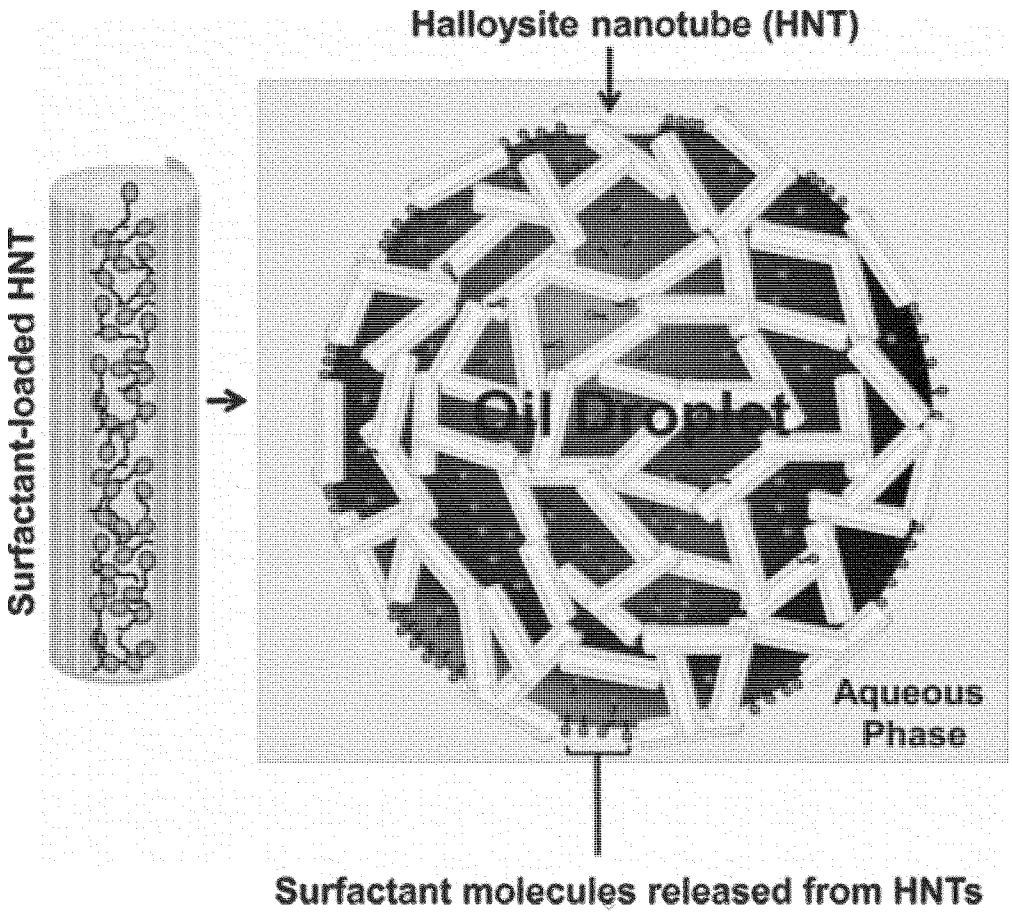

Figure 7. Nanotechnology has many applications, including in oil spill response. Pictured are nanotubes (HNTs) filled with surfactant. They coat oil droplets and release surfactant on the droplet's surface. Image: Owoseni et al. (2014)

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Michel

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I am pleased to inform you that the manuscript "Impact of mixing time and energy on the dispersion effectiveness and droplets size of oil" (Dr. Michel Boufadel) has now been accepted by the editor for publication.

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Impact of mixing time and energy on the dispersion effectiveness and droplets size of oil

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Abstract

The effects of mixing time and energy on Alaska Northern Slope (ANS) and diluted bitumen Cold Lake Blend (CLB) were investigated using EPA baffled flask test. Dispersion effectiveness and droplet size distribution were measured after 5-120 min. A modeling method to predict the mean droplet size was introduced for the first time to tentatively elucidate the droplet size breakup mechanism. The ANS dispersion effectiveness greatly increased with dispersant and mixing energy. However, little CLB dispersion was noted at small energy input (ε = 0.02 Watt/kg). With dispersant, the ANS droplet size distribution reached quasi-equilibrium within 10 min, but that of CLB seems to reach quasi-equilibrium after 120 min. Dispersants are assumed ineffective on high viscosity oils because dispersants do not penetrate them. We provide an alternative explanation based on the elongation time of the droplets and its residence in high intensity zones. When mixing energy is small, CLB did not disperse after 120 min, long enough to allow the surfactant penetration. Our findings suggest that dispersants may disperse high viscosity oils at a rougher sea state and a longer time. The latter could determine how far offshore one can intervene for effective responses to a high viscosity oil spill offshore.

Keywords: dispersion effectiveness, droplet size distribution, mixing time, mixing energy, viscosity

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1. Introduction

Due to increased offshore oil production, gas exploration, and crude oil transportation via marine tankers, several major oil spill accidents have occurred in the past few decades. Two worst oil spills in U.S. history, the Exxon Valdez spill in 1989 [1, 2] and the BP Deepwater Horizon (DWH) spill in 2010, brought about disastrous environmental consequences [3-5]. The Exxon Valdez spill released ~42 million liters of crude oil into Prince William Sound [6], while the BP DWH spill released ~780 million liters of crude oil into Gulf of Mexico [3, 7-9].

Oil spilled onto the water spreads initially as a surface slick [10], and could become dispersed in form of droplets due to mixing energy from waves. As oil is generally lighter than water, large oil droplets tend to return to the surface, especially provided that the mixing energy is small. The size of the droplets depends also on the interfacial tension (IFT) of the oil-water. Chemical dispersants contain surfactants that reduce the IFT of the oil-water, resulting in the formation of small droplets that tend to stay longer in the water column. The size distribution of the oil droplets affects when these droplets resurface or remain dispersed in the water column [11-13]. As the droplets get smaller, their specific interfacial area (surface area per unit volume) increases, which would facilitate oil dissolution [14] and biodegradation [15-17]. Hence, estimation of the droplets size distribution is essential for responding to oil spills and their long term behavior.

Thus, knowledge of the dispersion effectiveness is a key parameter [18]. Many factors influence dispersant performance, including oil composition and properties,

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mixing time, mixing energy, state of oil weathering, fluid dynamics, the type and amount of dispersants applied, temperature, and salinity of the water [19-23]. Studies on the dispersibility of Alaskan North Slope (ANS) and South Louisiana Crude oil under laboratory conditions were reported earlier [18, 24]. Relative to mixing energy, mixing time was reported to exert an insignificant effect on the dispersion effectiveness [20, 25]. However, the impacts of mixing energy and mixing time on the droplet size distribution has not been well documented. Additionally, with the interest in the diluted bitumen transport [26], understanding the effect of these parameters on a diluted bitumen garners people's attention. For this reason, we elected to conduct experiments on Cold Lake Blend (CLB), a bitumen blend combined with condensate [27]. Furthermore, the Weber number theory was employed for modeling the droplet size in the US Environmental Protection Agency (EPA) baffled flask dispersion effectiveness testing for the first time to characterize the droplet size distribution patterns and explain the droplet breakup mechanism.

The overall objective of this work was to evaluate the impact of mixing time and energy on the dispersion effectiveness and droplet size distribution of two oils: ANS and CLB at two rotation speeds (125 and 250 rpm). The behaviors of the two oils will be compared to better understand the physics of each and to provide additional guidelines for responders to oil spills. The objective was accomplished using a controlled laboratory experiment using the Environmental Protection Agency (EPA) Baffled Flask Test (BFT) [28, 29].

2. Materials and Methods

2.1 Experiment

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The EPA proposed protocol-BFT [24] was adopted to evaluate the dispersion effectiveness. The BFT protocol relies on using a 150-mL screw-cap trypsinizing flask with four baffles equally distributed on the side to allow for better mixing. The BF was equipped with a glass stopcock near its bottom so that a subsurface sample could be collected without disturbing the surface oil layer. Two mixing speeds were considered: 125 and 250 rpm, corresponding to the energy dissipation rate of 0.02 and 4.05 watt/kg, respectively [30]. Under these two mixing speeds, Zhao et al [30] and Kaku et al [31, 32] reported that there exists a region with –(5/3) slope in the energy spectra, which demonstrate the existence of the inertial subrange and the presence of turbulent flow. Delvigne and Sweeney [33] reported that the energy dissipation rate varies between 10^{-3} and 10^{-2} W/kg for the surface layer and between 1 and 10 W/kg for breaking waves. Terray et al [34] conducted velocity measurements in the top 2 m of Lake Ontario and found the energy dissipation rate to vary between 10^{-5} and 10^{-2}. The significant wave height in that study was about 0.25 m. For the mixing speeds we used, the low speed (125 rpm) may present the mixing energy in the surface layer, while the high speed (250rpm) for the breaking wave condition.

A volume of 120 mL synthetic seawater (see Supporting Information (SI) for details) was placed into the BF, followed by adding 100 μL of ANS or CLB using a 1-mL gas-tight syringe. The oil was then dispensed onto the center of the water surface gently. The mass of added oil was derived from the weight difference of the syringe before and after dispensing the oil. Subsequently, 4 μL of Corexit 9500 (Nalco Energy Service, L.P. Sugar Land, TX) was added to the center of the oil slick in the BF using a 1-

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10 μL Brinkmann Eppendorf repeater plus micropipette (Fisher Scientific, Pittsburgh, PA), giving the dispersant-to-oil volumetric ratio (DOR) of 1:25 based on the EPA protocol [35]. The dispersant was released as close as possible to the surface of the oil slick without actually touching it.

Following the addition of oil and dispersant, the BF was placed on the orbital shaker (Lab-Line Instruments Inc., Melrose Park, IL), with a variable speed control unit (40–400 rpm) and an orbital diameter of 2 cm, to provide designed mixing energy to the solutions in the flasks. The experiments were performed in the Thermo Scientific Precision™ refrigerated incubator at 15 ± 1 °C based the EPA protocol [35]. After each mixing time (5, 10, 60, and 120 min), the flask was removed from the shaker and kept stationary for 10 min. Subsequently, the first 5 mL of sample was drained from the stopcock and discarded [25, 28], followed by collecting 40 mL of sample into a 50 mL graduated cylinder. A subsample of 30 mL was taken for oil concentration measurement. Another 1 mL of subsample from the remaining 10 mL was used to measure the droplet size distribution in the water column. For each mixing time and mixing energy, duplicate experiments (two BFTs) were performed simultaneously to ensure that they are under the same conditions.

The information regarding the oil properties and droplet size distribution measurement is available in SI.

2.1. Measuring oil concentration

The oil concentration was measured through the absorbance of the oil solution in dichloromethane (DCM) using a Ultraviolet (UV)-VIS spectrophotometer (Cole Parmer

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4802 Scanning Double Beam UV/Visible Spectrophotometer, IL, USA) at the wavelengths 340, 370, and 400 nm. A blank extraction of synthetic seawater alone was also conducted. The extract and DCM was used as a blank solution initially, followed by the analysis of oil standards and samples. The absorbance was plotted against the wavelength and the area under the curve was calculated using the trapezoidal rule [21, 24, 35-37] :

$$\text{Area} = \frac{(\text{Abs}_{340} + \text{Abs}_{370}) \times 30}{2} + \frac{(\text{Abs}_{370} + \text{Abs}_{400}) \times 30}{2} \quad (1)$$

where, Abs_{340}, Abs_{370}, and Abs_{400} are absorbance at 340, 370, and 400 nm, respectively. The area in Eq. 1 was used to calculate the total oil dispersed in the water column following the equation:

$$\text{Total oil dispersed (g)} = \frac{\text{Area}}{\text{Calibration Curve Slope}} \times V_{\text{DCM}} \times \frac{V_{\text{tw}}}{V_{\text{ew}}} \quad (2)$$

Dispersion effectiveness (DE) is defined as the amount of total oil dispersed in the water column divided by the total amount of oil added:

$$\text{DE} = \frac{\text{Total oil dispersed}}{\text{m}_{\text{oil}}} \times 100 \quad (3)$$

where V_{DCM} is the volume of DCM extract; V_{tw} is the total volume of seawater in the BF; V_{ew} is the total volume of seawater extracted; m_{oil} (g) is the mass of the specific test oil added to test flask. Similar calculations were performed for the oil without dispersant.

2.2 Modeling Method

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A method based on the Weber number theory was used to predict the average equilibrium droplet size. The development of the method is mainly based on the work of Hinze [38] and Calabrese et al. [39]. In the BFT described above, the disruptive force associated with breaking up the oil droplets is the turbulent mixing energy introduced by the shaker, which is described by the energy dissipation rate per unit mass. The resistant forces to breakup include oil-water interfacial tension (F_s) and the shear forces within the droplet due to the droplet viscosity (F_v), where the dominant resistant force depends on the difference between the two forces. For the case of $F_s >> F_v$, the Sauter mean droplet diameter D_{32} at equilibrium can be estimated by [38-41]:

$$\frac{D_{32}}{D} = A\,We^{-3/5} \tag{4}$$

where, D is the diameter of the baffled flask measured physically, A is a system-dependent constant, and We is the Weber number given by:

$$We = \frac{\rho_c \overline{u'^2} D}{\sigma} \tag{5}$$

where, ρ_c is the density of the continuous phase (kg/m^3); σ is the oil-water interfacial tension (N/m); $\overline{u'^2}$ is the square of the average turbulent velocity of the baffled flask. In the inertial subrange of turbulence, the turbulence velocity can be expressed by [38, 42]:

$$\overline{u'^2} = C(\bar{\varepsilon} D)^{2/3} \tag{6}$$

where, $\bar{\varepsilon}$ is the average energy dissipation rate of the BF in a design rotational speed which is estimated using particle image velocimetry [30], and C is a constant.

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For high viscosity oil andwhen dispersants are used, the viscosity of the oil becomes the main resisting force for droplet breakup. Hinze [38] accounted for this fact using the a dimensionless viscosity group N_{vi}. This was further investigated by Calabrese et al. [39] and Wang and Calabrese [43] who derived a semi-empirical equation for estimating the droplet breakup in turbulent stirred-tank contactors, where both interfacial tension and viscous force of the droplet were important. Johansen et al. [44] also adopted their equation in a round turbulent jet for prediction of droplet breakup in subsea oil releases. The equation derived by Calabrese et al. [39] and Wang and Calabrese [43] is expressed as:

$$\frac{D_{32}}{D} = A\ We^{-3/5}\left[1 + B\ N_{vi}\right]^{3/5} \tag{7}$$

$$N_{vi} = \left(\frac{\rho_c}{\rho_d}\right)^{1/2} \frac{\mu_d \bar{\varepsilon}^{1/3} D_{32}^{1/3}}{\sigma} \tag{8}$$

where ρ_d is the density of the dispersed phase (kg/m^3); μ_d is the viscosity of the dispersed phase (pa·s). Eq. 7 is often referred to as the modified Weber number. Combining Eqs. 5-8, yields:

$$\frac{D_{32}}{D} = A\left(\frac{\rho_c\left(\bar{\varepsilon}D\right)^{2/3} D}{\sigma}\right)^{-3/5}\left[1 + B\left(\frac{\rho_c}{\rho_d}\right)^{1/2} \frac{\mu_d \bar{\varepsilon}^{1/3} D_{32}^{1/3}}{\sigma}\right]^{3/5} \tag{9}$$

where, the constant C in Eq. 6 is incorporated in the constant A. For the experimental data, the Sauter mean diameter was calculated from:

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$$D_{32} = \frac{\sum_{i=1}^{m} n_i d_i^3}{\sum_{i=1}^{m} n_i d_i^2} \quad (10)$$

where, m is the measured size bins, here it is 32 bins obtained from the Laser In-Situ Scattering and Transmissometry (LISST) measurement; n_i is the number concentration (#/m^3); d_i is the droplet diameter at each bin size (m).

3. Results

The density of ANS and CLB at 15 °C were measured at 0.878 and 0.924 g/mL, respectively (Table 1). The corresponding viscosities were determined as 18.9 and 237.3 mPa·s, respectively (Table 1). The IFT of ANS and CLB without dispersant is as 25.6 mN/m [45] and 16.3 mN/m [46], respectively (Table 1).

Table 1. Physical properties of oils

Oil	Condition	Temperature (°C)	Density[b] (g/mL)	Viscosity[b] (mPa·s)	IFT (seawater, mN/m)
Alaska North Slope (ANS)	Weathered[a]	15	0.878	18.9	25.6[c]
Cold Lake Blend (CLB)	Unweathered[a]	15	0.924	237.3	16.3[d]

Note: [a]More information about oil weather is available at http://www.itopf.com/knowledge-resources/documents-guides/fate-of-oil-spills/weathering/; [b] determined in this study; [c] from [45]; [d] from [46].

3.1 Dispersion Effectiveness

Figure 1 shows the dispersion effectiveness of ANS crude oil without and with dispersant after 5, 10, 60, and 120 min of mixing at 0.02 and 4.05 watt/kg. The case of no

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dispersantat 0.02 watt/kg provided none or little dispersion, with a slight increase with mixing time to reach ≅ 2% at 120 min. The case of no dispersant at 4.05 watt/kg showed larger values than that of no dispersant at 0.02 watt/kg, but the values remain small, reaching a maximum of ≅ 8% at 60 and 120 min.

In the presence of dispersant and at 0.02 watt/kg, it showed a steady increase with increasing mixing time, reaching around 40% at 120 min. The case with dispersant at 4.05 watt/kg practically reached the maximum dispersion effectiveness (around 50%) within 10 min. Slight increases were noted at 60 and 120 min, but these were within the experimental error. However, as the mixing time increased, while both dispersion effectiveness increased, the difference between them was small. For example, at 60 min, the dispersion effectiveness for 0.02 and 4.05 watt/kg were 29% and 50%, respectively; while at 120 min, the dispersion effectiveness for 0.02 and 4.05 watt/kg were 32% and 55%, respectively.

The dispersion effectiveness in the absence of dispersant was at least an order of magnitude smaller than that with dispersant for the corresponding mixing time and mixing energy. For example, the dispersion effectiveness at 0.02 watt/kg without dispersant was ≅ 2% at 120 min, while it was ≅ 32% when dispersant was used. At mixing energy of 4.05 watt/kg and mixing time of 120 min, the dispersion effectiveness without dispersant was ≅ 8%, while it was ≅ 55% with dispersant.

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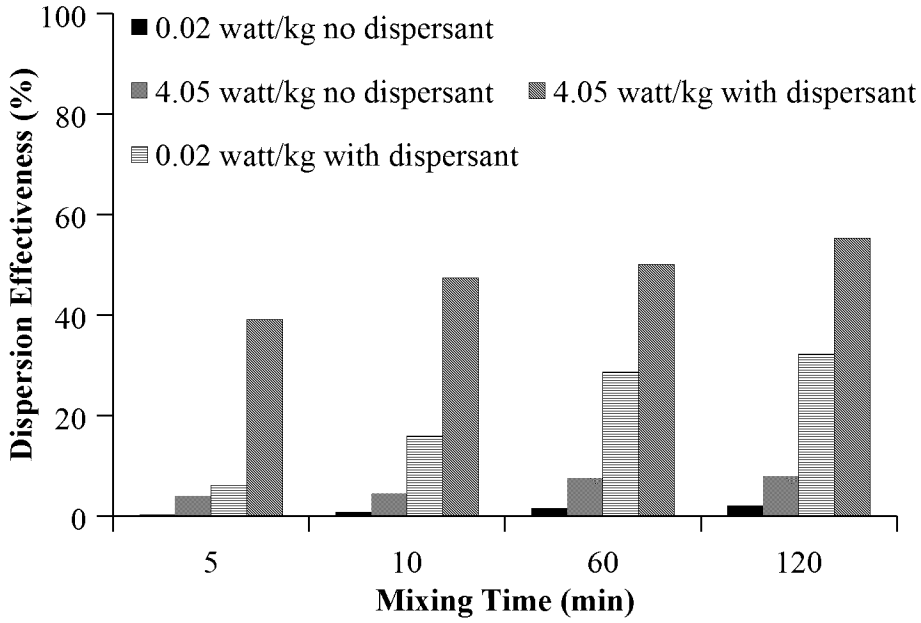

Figure 1. ANS dispersion effectiveness versus mixing time without and with dispersant at 0.02 and 4.05 watt/kg at 15 °C. The error bars represent the standard deviation based on duplicate experiments.

Figure 2 reports the dispersion effectiveness of CLB oil without and with dispersant after 5, 10, 60, and 120 min of mixing at 0.02 and 4.05 watt/kg. For the no dispersant case at 0.02 watt/kg, the dispersion effectiveness was essentially zero. The no dispersant at 4.05 watt/kg case showed essentially a constant value of less than 10% at 5-120 min. The dispersion effectiveness decreased at 60 and 120 min, which is due to sticking of the oil at the walls of the BF.

For the 0.02 watt/kg with dispersant, the dispersion effectiveness remained less than 5%. Some dispersion is only found for the 4.05 watt/kg with dispersant; with the dispersion effectiveness of around 20% at 5 and 10 min, and around 15% at 60 and 120 min. The decrease was due to the attachment of the CLB to the walls of the BF.

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In comparison with ANS, CLB showed much lower dispersion effectiveness for all cases, which is understandable as the CLB has a much higher viscosity, which would resist the breakup of oil droplets [29]. However, CLB showed a higher sensitivity to the mixing energy where the dispersion effectiveness increased tremendously between the 0.02 and 4.05 watt/kg.

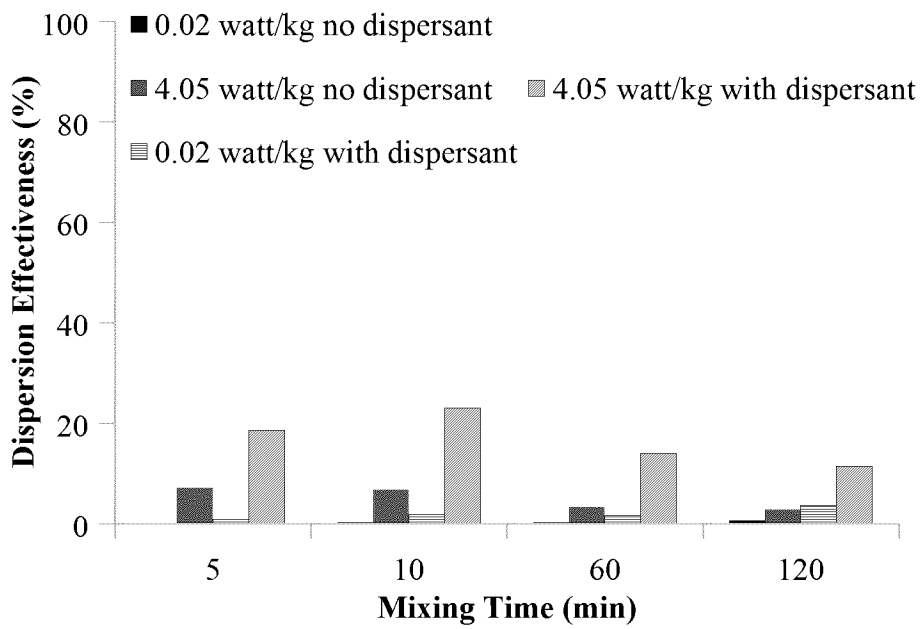

Figure 2. CLB dispersion effectiveness versus time without and with dispersant at 0.02 and 4.05 watt/kg at 15 °C. The error bars represent the standard deviation based on duplicate experiments.

3.2 Oil droplet size distribution

Figure 3a reports the droplet size distribution of ANS over time without dispersant at 0.02 watt/kg. The droplet size distribution was unimodal at 5 min, with the mode (i.e., peak) located at ≅ 330 μm, while they were bimodal at 10, 60, and 120 min, with the modes located at 32 and 390 μm, 63 and 390 μm, and 63 and 280 μm,

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respectively. The volume median diameter d_{32} took the following values 364, 399, 340, and 293 μm at 5, 10, 60, and 120 min, respectively. The oil droplets produced by the mixing energy were typically >100 μm and appeared to ascend and coalesce at the surface, reflected by slight changes in d_{32} as increasing time.

Figure 3b shows droplet size distribution of ANS over mixing time with dispersant at 0.02 watt/kg. Bimodal droplet size distribution was observed for this case, with modes located at 53 and 390 μm. The findings reached a close agreement with earlier studies that indicated that oil droplet sizes in the presence of dispersant under wave conditions span in the range of 2.5-70 μm in a distinctive, bimodal or multimodal droplet size distribution [27, 47]. The d_{32} was determined as 36, 17, 19, and 6 μm after 5, 10, 60, and 120 min of mixing, respectively. Also the amount of small oil droplets (<100 μm) increased with mixing time in the presence of dispersant. For example, Figure 3b shows that ≅ 50% of the volume was in droplet sizes <100 μm at 5 min, while the same group occupied more than ≅ 60% at 10 min, and around 80% at 60 min.

Figure 3c shows the droplet size distribution of ANS without dispersant at 4.05 watt/kg. Multimodal droplet size distribution was noted at 5 min, with modes located at 33, 170, 331, and 390 μm, while unimodal droplet size distribution was observed at other times with the mode located at about 120 μm. The values of d_{32} decreased as 122, 28, 22, and 16 μm after 5, 10, 60, and 120 min, respectively. The 4.05 watt/kg without dispersant generally generated droplet sizes <70 μm, with the exception of 5 min case (d_{32} = 122 μm). The oil droplet size decreased steadily with mixing time. In comparison with the 0.02 watt/kg case, the absence of dispersant at 4.05 watt/kg resulted in improved ANS oil

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dispersion, reflected by at least a few folds smaller values of d_{32} at 4.05 watt/kg than those at 0.02 watt/kg at the corresponding mixing time.

Figure 3d reports the droplet size distribution of ANS with dispersant at 4.05 watt/kg. After 5 min of shaking, more than 80% of the volume was in droplets <100 μm, and 50% of the volume was in sizes <3.0 μm. The distribution at 60 min and 120 min were the same for droplets >50 μm. However, there is marked difference for smaller diameters, and this could be due to the fact that formation of the small droplets is due to tip-streaming [48], whereby the release of micron size droplets from larger ones is determined by "sloughing" from the large droplets rather than by breakage due to hydrodynamic force. Thus, for ANS with dispersant, doubling the mixing energy resulted in a much larger volume fraction <50 μm. Additionally, the droplet size distribution at 120 min was very different with the 4.05 watt/kg providing droplets whose maximum sizes are <3 μm.

Figure 3e shows the droplet size distribution of CLB without dispersant at 0.02 watt/kg. The droplet size distribution was bimodal at each time, with the modes located at around 24 and 390 μm. One notes that d_{32} reached about >350 μm after 5-120 min of mixing. A negligible change in the concentration of CLB droplets at 10 and 60 min was observed. As the mixing time proceeded to 120 min, a slight increase in the volume of CLB droplets < 400 μm was noted.

Figure 3f shows the droplet size distribution of CLB with dispersant at 0.02 watt/kg. Around 90% of the oil volume remained in sizes >300 μm at 5 min. The distribution was unimodal at 5 min with a peak around 350 μm, but it became bimodal at

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10 min with modes at about 50 and 350 μm. The volume fraction of droplets <100 μm increased from almost zero at 5 min to more than 30% at 10 min. It continued to increase until reaching more than 60% at 120 min. The volume fraction >400 μm decreased from around 80% at 5 min to around 50% at 10 min and eventually to less than 10% at 120 min. The 60 min curve shows a uniform droplet size distribution between 50 and 350 μm. However, the droplet size distribution shows an increase in the volume fraction of droplets around 150 μm. The values of d_{32} = 399, 67, 33, and 412 μm after 5, 10, 60, and 120 min of mixing, respectively.

Figure 3g shows the CLB particle distribution without dispersant at 4.05 watt/kg. At 5 and 10 min, the distributions were bimodal at ≅ 70 and ≅ 400 μm with most of the CLB volume >400 μm. The droplet size distribution switched to multimodal at 60 and 120 min, with weakly-defined modes; the mode of the 10 min at 400 μm seems to have decreased to ≅ 350 μm at 60 min and to ≅ 320 μm at 120 min. There was a large uncertainty in sizes <10 μm due to adhesion of the oil on the BF and the LISST walls. The droplet size distribution had d_{32} = 375, 344, 62, and 61 μm after 5, 10, 60, and 120 min of mixing, respectively. But in essence, only a small fraction of the volume (< 40%) was in sizes <100 μm.

Figure 3h shows the droplet size distribution of CLB with dispersant at 4.05 watt/kg. At 5 min, the droplet size distribution is bimodal with peaks at around 30 and 350 μm. The distribution remained biomodal until 60 min; the larger peak shifted down to around 300 μm while the smaller peak remained at 30 μm. The distribution at 120 min shows a clear peak at around 5 μm, and around 85% of the volume was in droplets <20

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μm. The 4.05 watt/kg CLB with dispersant showed poorer dispersion relative to ANS dispersion under the same condition, while it gave more effective dispersion as opposed to 4.05 watt/kg CLB with no dispersant and 0.02 watt/kg CLB with dispersant.

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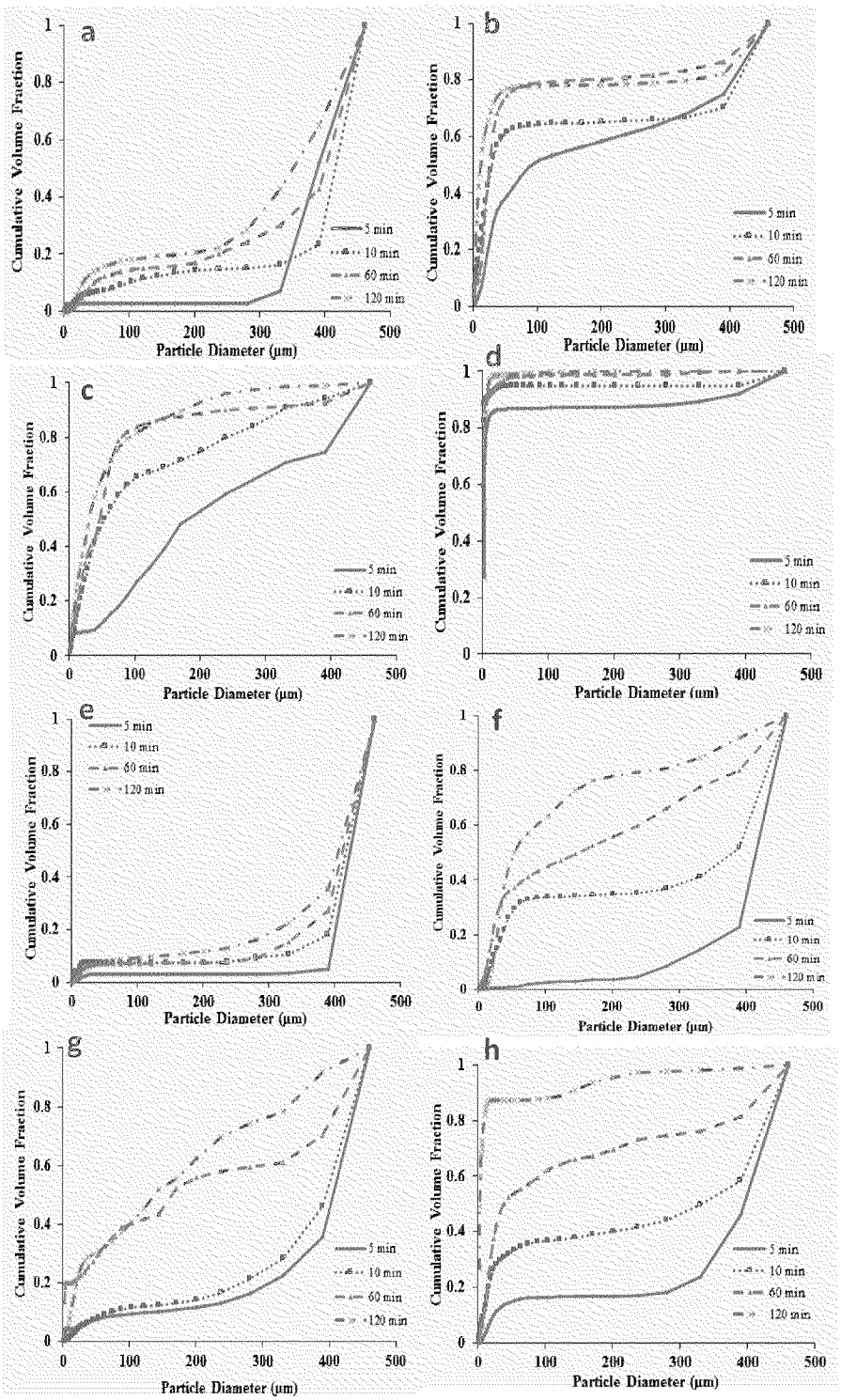

Figure 3. Evolution of the droplet size distribution with time. **a)** ANS without dispersant at 0.02 watt/kg; **b)** ANS with dispersant at 0.02 watt/kg; **c)** ANS without dispersant at 4.05 watt/kg; **d**) ANS with dispersant at 4.05 watt/kg; **e)** CLB without dispersant at 0.02 watt/kg; **f)** CLB with dispersant at 0.02 watt/kg; **g)** CLB without dispersant at 4.05 watt/kg; **h)** CLB with dispersant at 4.05 watt/kg.

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3.3 Droplet size modeling

Figure 4 shows the measured and calculated Sauter mean droplet diameter by Eq. 9 with the best fit coefficients A = 0.01 and B = 1.65. The overall predicted mean droplet sizes agree closely with the measured data, having the coefficient of determination (R^2) of 0.84. The cases in the absence of dispersants are more scattered than the ones with dispersants. This may be due to the droplets re-coalescence during the course of settling time after the shaking was stopped, introducing more uncertainties in the measurements for the cases without the addition of dispersants.

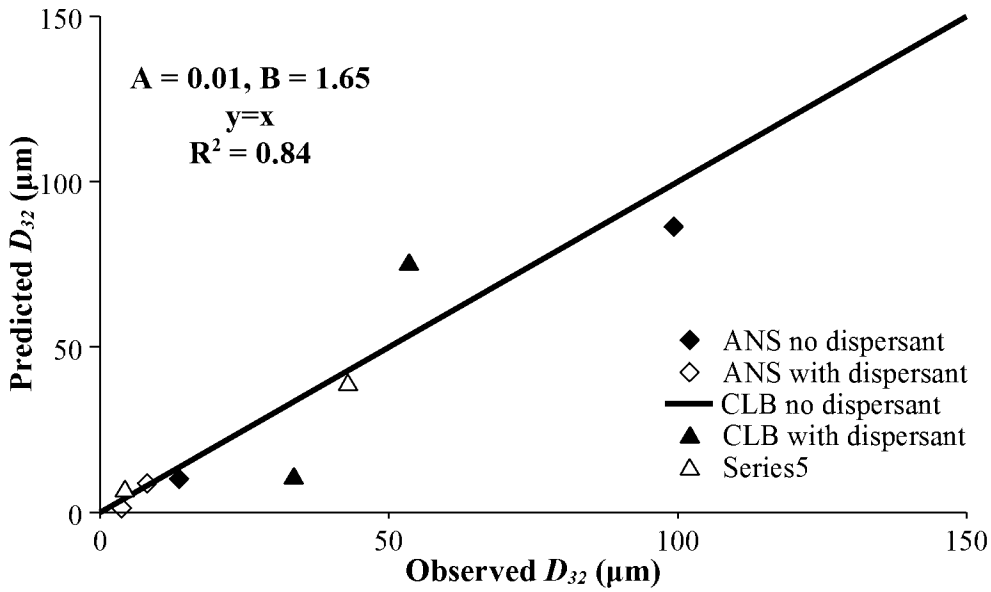

Figure 4. The measured Sauter mean droplet diameter from the baffled flask against the calculated ones from the modified Weber number (Eq. 9). The measured mean droplet diameter were computed from Eq. 10. The predicted values were determined with best fit coefficients A = 0.01 and B = 1.65.

Comparisons of the predictions by the Weber number (Eq. 4) and the modified Weber number involving viscous force (Eq. 9) are presented in Figure 5. For ANS whose

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viscosity is relatively low (σ = 18.9 mPa·s, Table 1), Figure 5a shows the differences between the results from the two equations are minimum, even for the cases with dispersants, demonstrating that the dominant resistance force to breakup for ANS is the interfacial tension. The predicted D_{32} is close to the measured D_{32} with higher differences for the cases without dispersants as indicated in Figure 4. For the relatively high viscosity oil of CLB (σ = 237.3 mPa·s, Table 1), Figure 5b shows the differences are apparent for all cases, indicating both interfacial tension and viscous force of droplets become important for the breakup of CLB. Similar to ANS oil, the predicted D_{32} has larger differences from the measurements for the dispersant cases.

Figure 5 also shows that Eq. 9 predicted larger D_{32} values, which is due to the fact that both interfacial tension and viscosity are accounted for in resisting the breakup of the droplets. Also, the difference seems to be largest with dispersants, particularly for CLB at 4.05 watt/kg. This is the first attempt to use Weber number theory to characterize the droplet size in the EPA BFT, which shows promising predictions for both low and high viscosity oils, and cases with and without dispersants. Further experiments are needed to test the equation and optimize the coefficients.

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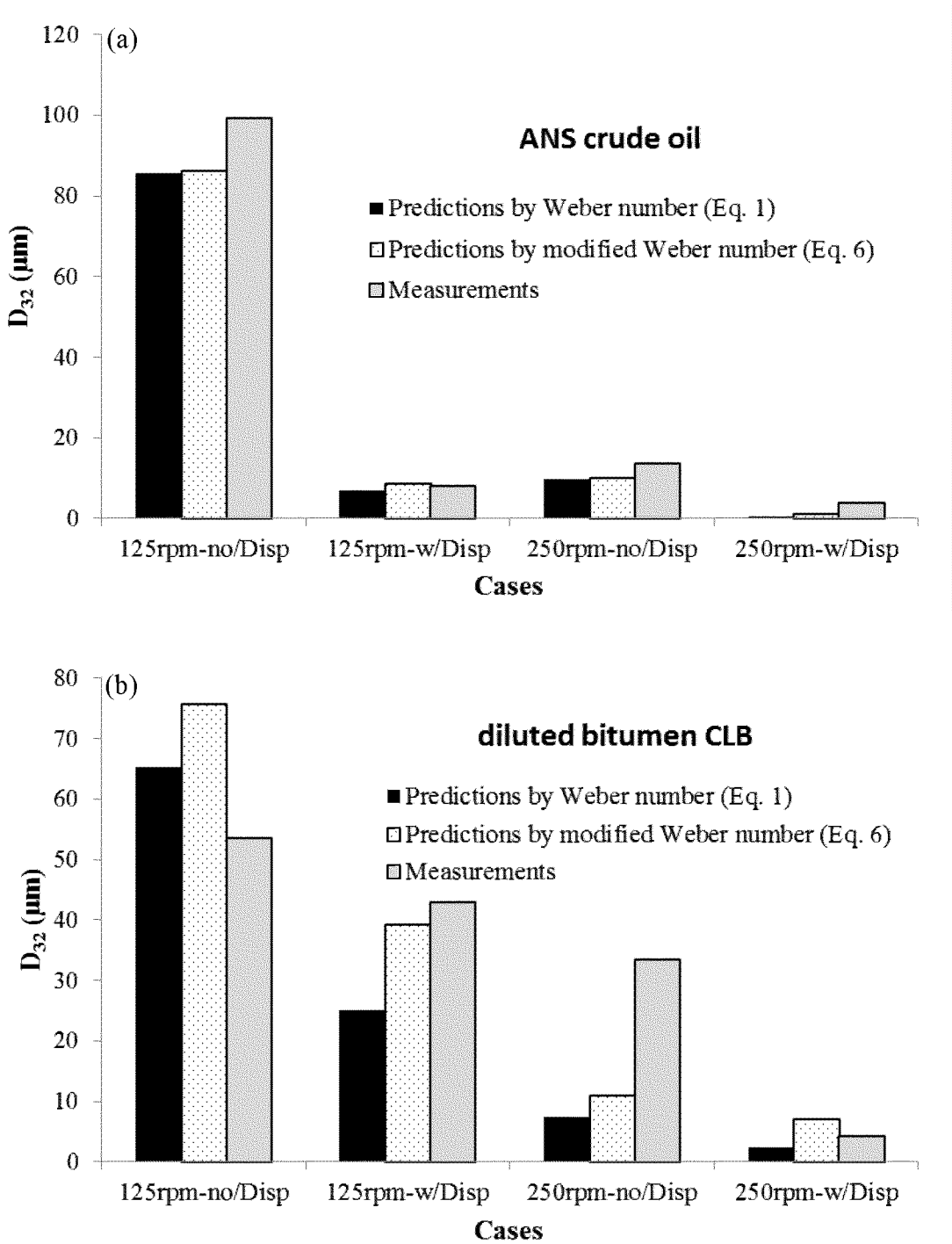

Figure 5. Comparison of the predictions by the Weber number (Eq. 4) and the modified Weber number (Eq. 9): a) ANS; and b) CLB

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4. Discussion

When evaluating the dispersion effectiveness of a dispersant-oil system, it is common to use the EPA BFT [24], which relies on evaluating the dispersion effectiveness (Eq. 3) after 10 min of mixing at 200 rpm. In this regard we evaluated the dispersion effectiveness of the CLB with Corexit 9500 due to recent interests in diluted bitumen [26, 49, 50]. The dispersion effectiveness of CLB was around 20% (Figure 2) relative to 50% for ANS (Figure 1). The dispersion effectiveness with dispersant does not change much with time, further providing credence to the EPA BFT (i.e., for selecting the 10 min mixing time).

The droplet size distribution provided richer information than the dispersion effectiveness, because although the dispersion effectiveness did not change much with time after 10 min (with dispersant), the droplet size distribution exhibited major changes herein. This gives major implications on the submergence of droplets in the presence of waves, as it has been observed that droplets <100 μm spread into the water column following wave breakup while larger ones return to the surface.

It has been well-established that most dispersant do not work on high-viscosity oils. One reason is that the dispersant does not penetrate into the thick oil slick [51], and thus, the effectiveness of a dispersant decreases as increasing oil viscosity [51-53]. This argument might explain the final droplet size distribution (e.g., at 120 min), but not their evolution with time. In particular, with dispersant, the droplet size distribution of ANS at 4.05 watt/kg reached steady state within (essentially) 10 min (Figure 3d), while that of

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the CLB evolved until 120 min (Figure 3h). In addition, the dispersion effectiveness of the CLB at 0.02 watt/kg is much smaller than that at 4.05 watt/kg, which was not the case for the ANS. Had the penetration of dispersant into the oil slick been the major factor for oil dispersion, then the difference between the 0.02 and 4.05 watt/kg for the CLB would have been smaller, essentially similar to the small difference between 0.02 and 4.05 watt/kg for the ANS. Furthermore, for the CLB at 4.05 watt/kg, it is expected that the dispersant was able to penetrate the oil after 10 to 30 min of mixing. But the evolution from 60 to 120 min cannot be explained based on the penetration, and for this reason, we offer below an argument based on the hydrodynamics.

Since the seminal work by Hinze [38], the breakup of oil droplets has been correlated to the Weber number, which represents the ratio of destructive forces due to turbulence to resisting forces due to interfacial tension for low viscosity oils. For high viscosity oils, Hinze altered the traditional Weber number by introducing a modified Weber number that accounts for the viscosity of the oil in resisting breakup [38]. This approach was validated [39, 43] in reactors at steady state (after 120 min of mixing). However, the role of high viscosity when the droplet size distribution is evolving has not been thoroughly investigated. Based broadly on our recent work [29], we argue that turbulence is highly heterogeneous, and thus while one uses average quantities to quantify mixing (the average energy dissipate rate or the average shear rate G), the breakup of oil droplets depend on the local scale and not only on the flask-averaged quantities. This could be clearly noticed in Figure 6, where the energy dissipation rate varies greatly. Thus, when an oil droplet enters a high turbulence intensity zone, it gets

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elongated with the droplet viscosity and interfacial tension resisting the elongation. If the droplet's elongation exceeds a maximum elongation, the droplet breaks otherwise it would "survive". Assuming that droplets of diameter d_0 break when the ratio of their maximum elongation x_b to d_0 exceeds a particular value, typically 2.0 [54], and assuming a uniform elongation time (a needed approximation herein), one finds that the elongation time to reach the maximum elongation x_b is:

$$T_E = \frac{\mu_d \ln\left(\frac{x_b}{d_0}\right)}{\left[2.3\varepsilon^{2/3} d_o^{2/3} - C_\sigma \frac{\sigma}{d_0}\right]} \tag{11}$$

where μ_d is the dynamic viscosity of the dispersed phase (i.e., oil), ε is the energy dissipation per unit mass, C_σ is an empirical coefficient taken as equal to 0.4, and σ is the IFT between the dispersed phase and the continuous phase. The left term in the denominator represents the dynamic pressure that would break the droplet.

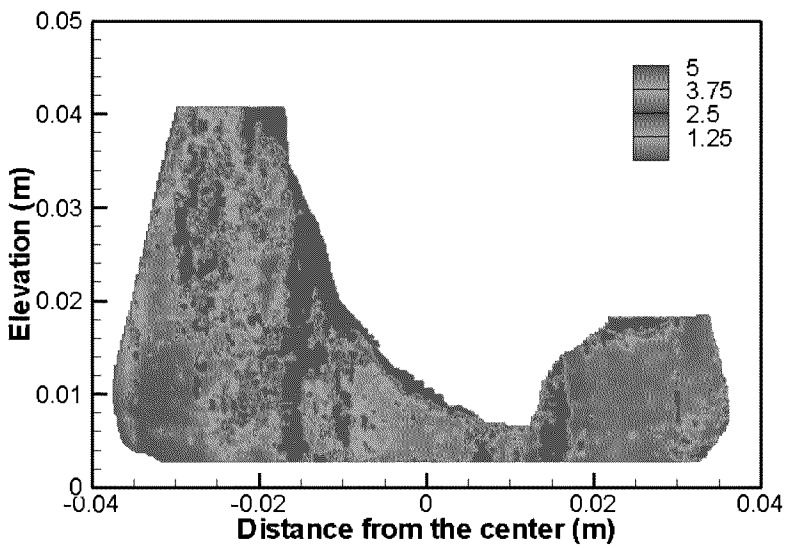

Figure 6. Contour plots of the energy dissipation rate (ε) in the BF for 250 rpm. Labels

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represent values of ε (W/kg). Note how ε varies in space. From [30].

One notes from Eq. 11 that the increase in the oil viscosity increases the elongation time of the droplet, and thus the survival of the droplets. The decrease in the IFT (due to the addition of dispersant) increases the denominator and thus decreases the turnover time. However, the dynamic pressure could be large, and thus, a decrease of an already-small IFT does not play a major role.

To better understand the effect of mixing time and mixing energy on dispersant effectiveness, we report in Figure 7 the measurements of IFT between oil and seawater over time in the presence of dispersant. One notes that the IFT between CLB and seawater is a few folds larger than that between ANS and seawater, which is due to the fact that the dispersant did not penetrate into the CLB within the tensiometer. More importantly in Figure 7 is that the IFT decreases with increasing time and rotation speed. This might sound counter-intuitive as one expects the oil in the BF to "scavenge" the dispersant. Two possible explanations are: 1) As oils contain a trace amount of natural surfactants, it is possible that these natural surfactants were released and accumulated at the oil/water interface and thus further reduced the IFT of oil-water [55]; 2) It is possible that the mixing pulverized the dispersant causing it to be more effective.

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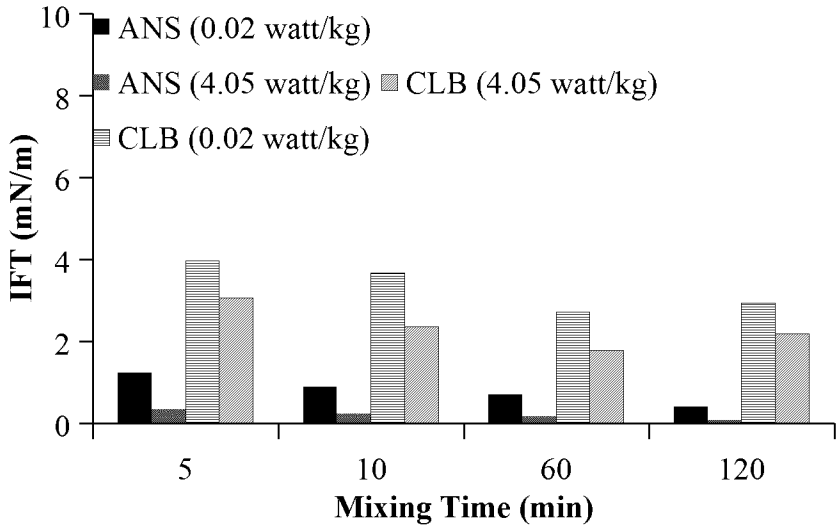

Figure 7. IFT of oil-water with dispersant (Corexit 9500, DOR = 1:10) versus time at 0.02 and 4.05 watt/kg. The error bars represent the standard deviation based on duplicate measurements.

Acknowledgements

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To: Barron, Mace[Barron.Mace@epa.gov]; Holder, Edith[holder.edith@epa.gov]; Conmy, Robyn[Conmy.Robyn@epa.gov]
Cc: Craig Watts[craig@hydrosphere.net]; Charlie Jarman[cjarman@hydrosphere.net]
From: Peter Meyer
Sent: Mon 6/27/2016 5:51:24 PM
Subject: Question regarding Corexit EC9500A

Mace/Robin/Edith,

Where can I find the "self-dispersibility limit" for Corexit EC9500A?

If you don't know, that's fine. I can look into it myself.

Thanks,

Peter

3.3 *Preparation of Exposure Concentrations*

3.3.1 *Concentration Selection.*
Preliminary rangefinder tests may be necessary using a series of logarithmic concentrations (*e.g.* 0.1, 1, 10, 100 µl dispersant product/L or mg TPH/L) to determine the appropriate exposure concentration range necessary to determine LC_{50} values and 95% confidence intervals. For definitive tests, conduct a minimum of five test concentrations using a geometric ratio between 1.5 and 2.0 (*e.g.* 2, 4, 8, 16, and 32). Note that when testing only the dispersant product, the highest test concentration must not exceed the dispersant's self-dispersibility limit.

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To: Conmy, Robyn[Conmy.Robyn@epa.gov]
From: McClellan, Kim
Sent: Thur 8/18/2016 3:36:42 PM
Subject: Comments from STICS Entry
E12PG00037_Draft Final Report_June 2016_clean copy_abg.docx

Hi Robyn,

Please address the comments and then email me a revised version so that I can attach it to your record and resubmit for approval and clearance.

Alice Gilliland's Comments:

Excellent report! Given the topic, will be routed for STICS review at the Laboratory level. Prior to my approval, I am returning this to the author with my review comments ("E12PG00037_Draft Final Report_June 2016_clean copy_abg.docx).	Alice Gilliland	08/18/2016 8:32 AM

Thanks,

Kim

To: Conmy, Robyn[Conmy.Robyn@epa.gov]
Cc: Grosser, Robert[Grosser.Robert@epa.gov]; Sundaravadivelu, Devi[sundaravadivelu.devi@epa.gov]
From: Holder, Edith
Sent: Wed 9/7/2016 5:29:52 PM
Subject: to keep you updated

Robyn,

We hope you are feeling better and ready to go tomorrow.

We did an experiment in your absence yesterday. You had said earlier that you were not going to do EEMS on every analytical combination, but we understood that completely characterizing ANS was a definite goal. So yesterday, we used the prepared flasks and analyzed IFO120. Devi operated the ECO as well as the LISST. The tests were

IFO120 75ppt Corexit

50ppt Corexit

50ppt Accell

We have already tested IFO120 @ 35ppt measuring EEMS and have 40ppt, 60ppt, 100ppt, and 125 ppt hypersalinities remaining for further EEMS measurements.

Tomorrow, we plan to test ANS @ 125ppt against Corexit, Accel, and none.

Next week while you are gone, we can continue to test IFO120 @ 75ppt against Accell and SPC1000 and 50ppt against SPC 1000.

Then on Sept. 15 when you are back, we will run the tests originally scheduled for yesterday

ANS 75ppt none

50ppt none

50ppt Finasol

The week of Sep 19-22, we will test at 150 rpm using ANS, 35, 60 and 100 ppm against Corexit and Accell. That should give us a nice little data set for comparison.

I hope this is satisfactory.

Edie

Edith L. Holder

Pegasus Technical Services, Inc.

On-Site Contractor to the U.S. EPA

ORD/NRMRL/LRPCD

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Cincinnati, OH 45268

Phone: 513-569-7178

Email: holder.edith@epa.gov

To: Conmy, Robyn[Conmy.Robyn@epa.gov]
From: Mike Fulton - NOAA Federal
Sent: Tue 4/12/2016 4:59:05 PM
Subject: Re: purchase fo finasol and corexit

Good luck. You may have more leverage than we did. At one point, I thought we were very close to getting some, but their attorneys changed their mind.

On Tue, Apr 12, 2016 at 8:56 AM, Conmy, Robyn <Conmy.Robyn@epa.gov> wrote:

Thanks Mike. This is our same contact, so I guess I will just keep trying to reach her. EPA is looking to procure a few gallons and will be doing toxicity testing. We will ask nicely and point out that the testing will be used to help with finalizing the NCP Subpart J language. If they still won't sell, we will look into if there is regulatory language that exists to help convince them to waive the waiver requirements. As you know, access to Nalco dispersants is quite a challenge for research.

Cheers,

Robyn

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Robyn N. Conmy, Ph.D.

Research Ecologist

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727-692-5333 (Personal mobile)

conmy.robyn@epa.gov

From: Mike Fulton - NOAA Federal [mailto:mike.fulton@noaa.gov]
Sent: Tuesday, April 12, 2016 8:41 AM

To: Conmy, Robyn <Conmy.Robyn@epa.gov>
Subject: Re: purchase fo finasol and corexit

Hi Robyn,

See below contact info for NALCO. You may have more success if you can sign a waiver that you won't use for toxicity testing. Good luck.

Mike

Debby.Theriot@nalco.com

281 263 7709

On Tue, Apr 12, 2016 at 7:58 AM, Conmy, Robyn <Conmy.Robyn@epa.gov> wrote:

> Thanks Mike. I spoke to a contact at Total yesterday and we are getting some Finasol. Nalco hasn't responded to a voicemail as of yet, so your contact at Nalco could be a huge help.

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conmy.robyn@epa.gov

From: Mike Fulton - NOAA Federal [mailto:mike.fulton@noaa.gov]
Sent: Monday, April 11, 2016 5:00 PM
To: Conmy, Robyn <Conmy.Robyn@epa.gov>
Subject: Re: purchase fo finasol and corexit

Hi Robyn, Ultimately, We weren't able to get Corexit from Nalco, but I'll get you the contact info for both manufacturers.

On Monday, April 11, 2016, Conmy, Robyn <Conmy.Robyn@epa.gov> wrote:

Hi Mike,

Awhile back we had discussed NOAA's possible procurement of Corexit and Finasol for your toxicity work. Would you mind sharing the POC for Nalco and Total that were contacted in your hunt to procure the dispersants?

Thanks,

Robyn

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To: Conmy, Robyn[Conmy.Robyn@epa.gov]; raghuraman.venkatapathy@ptsied.com[raghuraman.venkatapathy@ptsied.com]; Holder, Edith[holder.edith@epa.gov]; Deshpars[deshpars@mail.uc.edu]; Zhang4y5[zhang4y5@mail.uc.edu]
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From: Devi Sundaravadivelu
Sent: Wed 2/24/2016 9:54:07 PM
Subject: Meeting Summary
Meeting Summary.pdf

Hi All,

Please find attached the summary from today's meeting. I will send another email summarizing details regarding PCR samples (and include prioritized sample list) in which I will include Jorge and Mike.

Thanks,

Devi

--
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