Data Evaluation Report on the Toxicity of 2,4-DB DMA to Rainbow Trout (*Oncorhynchus mykiss*), Early Life Cycle

DP Barcode: 439488 MRID No.: 50179901

Data Requirement:	EPA PC Codes	030801 & 030819
	EPA DP Barcode	439488
	EPA MRID	50179901
	EPA Guideline	850.1400

Test Material: 2,4-DB DMA

Purity(%): 500 g/L 2,4-DB DMA (43.8% w/w 2,4-DB)

Primary Reviewer: Rebecca L. Bryan
Staff Scientist, CDM/CSS-Dynamac JV

Signature: Rebecca L. Bryan
Date: 8/23/17

Secondary Reviewer: Teri Myers
Senior Scientist, CDM/CSS-Dynamac JV

Signature:
Date: 09/28/17

Primary Reviewer: Cameron Douglass, Ph.D.
Biologist, USEPA/OCSPP/OPP/EFED/ERBIV

Signature: Cameron Douglass 2018.10.11 14:51:34 -04'00'
Date: 10/11/18

CITATION: Minderhout, T. *et al*. 2017. 2,4-DB: An Early Life-Stage Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*). Unpublished study performed by EAG Laboratories (formerly Wildlife International), Easton, Maryland. Laboratory Study No. 467A-104A. Study sponsored by 2,4-DB Task Force c/o Data Group Management, Raleigh, North Carolina. Study initiated April 28, 2016 and completed February 7, 2017.

EXECUTIVE SUMMARY:

In a 87-day chronic toxicity study under flow-through conditions, rainbow trout (*Oncorhynchus mykiss*) embryos were exposed to 2,4-DB DMA (500 g/L, 43.8% w/w 2,4-DB) at nominal concentrations of 0 (negative and solvent controls), 0.26, 0.64, 1.6, 4, and 10 mg a.e./L. Mean-measured concentrations were <0.2 (negative and solvent controls), 0.27, 0.67, 1.66, 4.19, and 10.8 mg a.e./L.

Hatching success (-34%) and larval survival at swim-up on Day 42 (-97%) were significantly ($p<0.05$) reduced at the 10.8 mg a.e./L treatment level. At test termination (study Day 87, or 61 days following hatching), survival was significantly ($p < 0.05$) reduced 17 and 98%, respectively, at the 4.19 and 10.8 mg a.e./L treatment levels. Total length of surviving rainbow trout juveniles was significantly ($p < 0.05$) reduced 14% at 4.19 mg a.e./L treatment levels, and mean wet and dry weights were reduced 31-34% at the 4.19 mg a.e./L (note that the 10.8 mg a.e./L treatment level was excluded from statistical analysis of growth data due to 98% mortality of individuals in this group). Therefore, based on reduced post-hatch survival, length and weight, the 87-day NOAEC and LOAEC values were 1.66 and 4.19 mg a.e./L, respectively, using mean-measured concentrations.

This study is **scientifically sound** and is classified as **acceptable**.

Results Synopsis

Test Organism Size/Age: Embryos, <24 hours old
Test Type: Flow-through

Time to hatch

NOAEC: 10.8 mg a.e./L LOAEC: >10.8 mg a.e./L

Hatching success
NOAEC: 4.19 mg a.e./L LOAEC: 10.8 mg a.e./L

Larval survival (i.e. at swim-up, D42)
NOAEC: 4.19 mg a.e./L LOAEC: 10.8 mg a.e./L

Post-hatch survival (i.e., at test termination, D87)
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

Total length
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

Wet weight
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

Dry weight
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

Endpoint(s) affected: Hatching success, larval survival, post-hatch survival, total length, wet and dry weight
Most sensitive endpoint(s): Post-hatch survival, total length, wet and dry weight

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: This study was conducted following guidelines outlined in the U.S. EPA *Ecological Effects Test Guidelines* OPPTS 850.1400: Fish Early-Life Stage Toxicity Test (April 1996).

No deviations from OCSPP 850.1400* were observed.

** Note that the updated (October 2016) OCSPP guideline was used in the evaluation of this study, with consideration made of differences in the 1996 OPPTS 850.1400 and the 2016 OCSPP 850.1400 Guidelines.*

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality claims statements were provided. This study was conducted in accordance with U.S. EPA GLP Standards as published in 40 CFR Parts 160 and 792 with the following exceptions: routine water screening analyses were not performed in accordance with the stated GLP, but were performed using a certified laboratory and standard US EPA analytical methods; and the stability of the reference substance under the conditions of storage at the test site was not determined in accordance with GLP.

A. MATERIALS:

1. Test Material: 2,4-DB DMA

Description: Light brown liquid
Lot No./Batch No.: JPB/596/046
Purity: 500 g/L 2,4-DB DMA (43.8% w/w 2,4-DB)
Density: 1.142 g/mL

Stability of compound under test conditions: The mean measured concentrations were 102 to 108% of nominal levels for the 87-day test.

Storage conditions of test chemicals: Ambient

2. Test organism:
Species: Rainbow Trout (*Oncorhynchus mykiss*)

EPA recommends rainbow trout (Oncorhynchus mykiss), bluegill sunfish (Lepomis macrochirus) or fathead minnow (Pimephales promelas) for freshwater, and sheepshead minnow (Cyprinodon variegatus) for estuarine/marine. OECD recommends rainbow trout, fathead minnows, zebra fish, and ricefish but does not exclude the use of other species.

Age/embryonic stage at test initiation:
Embryos, <24 hours post-fertilization

EPA recommends fish embryo age at test initiation is as soon as possible after the eggs have been fertilized.

Method of collection of the fertilized eggs: Not reported

Source: Unfertilized eggs and sperm were obtained from Troutlodge, Inc. (Sumner, Washington).

B. STUDY DESIGN:

1. Experimental Conditions

a. Range-finding study: The concentrations for the definitive study were selected in consultation with the sponsor, and were based on exploratory range-finding toxicity data. The range-finding test was conducted at nominal concentrations of 0.024, 0.081, 0.27, 0.9, and 3.0 mg a.e./L with negative and solvent (DMF, 0.1 mL/L) controls. Hatching success was 100% for all treatment groups (including controls). The mean percent larvae surviving on Day 24 was 97-100% for all treatment groups (including controls). The mean percent larvae reaching swim-up was 90-95% for all treatment groups (including controls). Mean wet weights were 120.7 and 119.8 mg, respectively for the negative and solvent controls, compared to 116.7, 120.6, 121.2, 118.9, 116.3 mg in the nominal 0.024, 0.081, 0.27, 0.90, and 3.0 mg a.e./L groups, respectively.

b. Definitive study:

Table 1: Experimental Parameters

Parameter	**Details**	**Remarks** ***Criteria***
Parental acclimation, if any Period: Conditions (same as test or not): Feeding (type, source, amount given, frequency): Health: (any mortality observed)	Details of parental cultures were not provided.	N/A *EPA recommends embryos be immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage.*
Number of fertilized eggs/embryos in each treatment at test initiation	120 embryos/treatment level, divided into 30 embryos/chamber, and 4 replicate chambers/treatment	The 30 embryos per replicate were divided into two incubation cups (15/cup) and larvae thinned to 15 per replicate on Day 42. *Each treatment should include a minimum of 60 embryos, divided equally between at least 2 replicate test chambers. OECD also recommends at least 60 eggs, divided between at least 2 replicates.*
Concentration of test material Nominal: Mean-measured:	0 (negative and solvent controls), 0.26, 0.64, 1.6, 4, and 10 mg a.e./L <0.2 (negative and solvent controls), 0.27, 0.66, 1.66, 4.19, and 10.8 mg a.e./L	Concentrations reported in terms of 2,4-DB acid equivalents (*i.e.* mg ae/L). LOQ = 0.2 mg a.e./L *A minimum of 5 concentrations, spaced by a constant factor not exceeding 3.2, and a control, all replicated, plus solvent control if appropriate should be used.* *- Toxicant concentration should be measured at least 5 times at regular intervals, and at least once per week for studies longer than one month.* *- Concentrations of test substance in solution should be within ± 20% of the mean measured values during study.* *- One concentration should adversely affect a life stage and one concentration should not affect any life stage.*

Parameter	**Details**	**Remarks** ***Criteria***
		OECD also recommends that 5 concentrations be spaced by a constant factor not exceeding 3.2 and concentrations be within ± 20% of the mean measured values.
Solvent (type, percentage, if used)	Dimethylformamide (DMF), 0.1 mL/L	None *The solvent should not exceed 0.1 ml/L in a flow-through system. Recommended solvents include dimethylformamide, triethylene glycol, methanol, acetone, and ethanol.* *OECD recommends that the solvent not have an effect on survival nor produce any other adverse effects; concentration should not be greater than 0.1 ml/L.*
<u>Number of replicates</u> Negative control: Solvent control: Treatments:	 4 4 4/level	None *At least 2 replicates per test concentration and control.* *A solvent control should be used in conjunction with a solubilizing agent.*
<u>Test condition</u> Static renewal/flow-through: Type of dilution system for flow through method: Flow rate: Renewal rate for static renewal:	 Flow-through Continuous-flow diluter *ca.* 10 volume additions per day N/A	Flow splitting accuracy was within ±10%. *Flow-through systems are generally recommended, including a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, or saturator system). EPA recommends that flow rate equal at least 5 test chamber volumes per 24 hours, and not vary by more than 10% throughout the test (OECD also recommends 5 test chamber volumes/24 hours). For flow through tests, biomass loading rate should not exceed 0.5 g/L/24 h and 5 g/L solution at any time. For static-renewal, EPA recommends 2 renewal procedures; either transfer eggs and larvae to new, clean vessels or retain organisms in vessels and change at least 2/3 test water.*

Parameter	**Details**	**Remarks** ***Criteria***
Aeration, if any.	No aeration was reported.	N/A *Aeration is not recommended.*
Duration of the test:	87 days: 26-day hatching period and 61-day post-hatch period	None *EPA recommended test duration is 32 days (or 28 days post-hatch) for fathead minnow and sheepshead minnow; 30 days post-hatch for zebra fish and ricefish; 2 weeks after controls are free-feeding (or 60 days post-hatch) for rainbow trout; and 28 to 60 days for other species. OECD recommendations for test duration are species specific and range from 28-60 days.*
<u>Embryo cups, if used</u> Type/material: Size: fill volume:	 Glass cylinders 50 mm diameter with nylon screen mesh (425 µm) Not reported	Embryo cups were oscillated vertically via a rocker arm at 2 rpm.
<u>Test vessel</u> Type/material: Size: Fill volume:	 Glass 9-L *ca.* 7 L (16.0 cm depth)	None *EPA recommends any glass, stainless steel, or other chemically inert vessels can be used.*
Source of dilution water: <u>Quality of dilution water</u> Particulate matter: TOC: Un-ionized ammonia: Residual chlorine: Total organophosphorus pesticides:	Moderately hard freshwater from on-site well was filtered (25- and 0.45-µm), aerated, and UV sterilized. Not reported <1 mg C/L (4 weeks before test) Not reported Not reported Not detected	Results from the analysis of the well water from a sample taken on December 7, 2015 were provided. *Any water with test species control survival meeting % hatching success and % post-hatch success in guideline is suitable as a test water. Maximum allowable concentrations for water quality parameters are provided in EPA's 850.1400 guideline (http://www.epa.gov/ocspp/pubs/frs/home/draftguidelines.htm). EPA also recommends testing for heavy*

Parameter	**Details**	**Remarks** ***Criteria***
Total organochlorine pesticides + PCBs: Organic chlorine:	 Not detected Not reported	*metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, sulfate), and suspended solids.* *OECD accepts any water in which the test species show control survival at least as good as presented in SEP.*

Parameter	**Details**	**Remarks** ***Criteria***
<u>Water quality during testing</u> Hardness: pH: Dissolved oxygen: Temperature(s): Salinity (for marine or estuarine species): <u>Other measurements</u> Photoperiod: Interval of water quality measurements:	 128 to 156 mg/L as $CaCO_3$ 8.0 to 8.3 7.7-10.5 mg/L (≥71% saturation) Daily: 11.2 to 12.1°C Continuous: 11.24 to 12.50°C N/A Alkalinity: 162-182 mg/L $CaCO_3$; Conductivity: 303-364 µS/cm 16 hour light/8 hour dark with 30-minute transition periods. Light intensity was 102 lux. Temperature was measured in all replicates at initiation, weekly during the test, and at termination. Additionally, temperature was continuously recorded in one negative control replicate. DO and pH were measured in alternating replicates from all levels at initiation, weekly during the test, and at termination. Hardness, alkalinity, and specific conductance were measured in alternating replicates of the negative control and highest concentration level at initiation, weekly during the test, and at termination.	 *<u>EPA Recommendations:</u>* *Dissolved Oxygen: 60 - 100% saturation. OECD recommends that DO concentration be between 60 - 90% saturation.* *Temperature for fathead minnow, zebra fish and sheepshead minnow: 25±2°C; rainbow trout: 10-12±2°C, depending on life stage; and ricefish: 23-24±1-2°C, depending on life stage. Temperature should not deviate by more than ±1.5 °C between test chambers or between successive days during test.* *Salinity for sheepshead minnow: 15-30 ‰ ±2%; salinity for silverside: 20 ‰. Recommended photoperiod: 12-16 hours, depending on species.* *EPA recommends DO, salinity (if relevant), and temperature be measured weekly, and pH and hardness be measured at the beginning and end of the test. Temperature should preferably be monitored continuously in at least one test vessel.* *OECD also recommends at a minimum DO, salinity (if relevant) and temperature should be measured weekly, and pH and hardness at the beginning and end of the test.*

Parameter	**Details**	**Remarks** ***Criteria***
Post-hatch details When the post-hatch period began: Number of hatched eggs (alevins)/treatment released to the test chamber: Day on which alevins were released from the incubation cups to the test chamber:	 Days 23 All hatched larvae were released Day 26	Hatching success was 81-83% in the controls. *Hatching success in each control should be ≥ 66% for rainbow trout and fathead minnow; >75% for sheepshead minnow; and >66 or >80% for other species as shown.* *Post-Hatch success in each control should be ≥ 70% in rainbow trout, fathead minnow and zebra fish; ≥ 80% in ricefish and sheepshead minnow; and ≥ 60 to ≥ 80% for other species as shown.*
Post-hatch Feeding Start date: Type/source and frequency of feed:	 Day 42 (end of swim-up stage) From Day 0 to 7 post-hatch, swim-up larvae were fed three times daily with salmon-starter mash. Thereafter, larvae were fed salmon-starter mash three times daily on weekdays and twice daily on weekends and holidays. Fish were not fed for 55 hours prior to termination.	*EPA's 850.1400 guidelines for feeding and handling recommendations for brood and test animals of recommended species and other species are at (https://www.regulations.gov/document?D=EPA-HQ-OPPT-2009-0154-0033).*
Stability of chemical in the test system	The mean measured concentrations were consistent throughout the test (range of 102-108% of nominal).	N/A
Recovery of chemical: Frequency of measurement: LOD: LOQ:	102 to 108% of nominal Days 0, 7, 13, 20, 27, 34, 41, 48, 55, 62, 69, 76, 83, and 87 Not reported 0.200 mg a.e./L	Recoveries are based on mean measured concentrations, and reported in terms of 2,4-DB acid equivalents.

Parameter	**Details**	**Remarks** ***Criteria***
Positive control:	N/A	N/A
Fertilization success study, if any: Number of eggs used: Day on which the eggs were removed to check the embryonic development:	 120 embryos Day 11	Four incubation cups with 30 embryos each.
Other parameters, if any:	Biomass loading at test termination was 0.25 g/L/day, and instantaneous loading at test termination was 2.57 g fish/L.	

2. Observations:

Table 2: Observations

Parameters	**Details**	**Remarks** ***Criteria***
Parameters measured including the sublethal effects/toxicity symptoms	- Hatching success - Time to hatch - Post-hatch survival (at swim-up prior to thinning on Day 42; from thinning to termination on Day 87) - Larval swim-up - Measurement of growth (length, wet weight, and dry weight) - Clinical signs of toxicity or abnormal behavior	None *Recommended parameters measured include:* *- Number of embryos hatched;* *- Time to hatch;* *- Mortality of embryos, larvae, and juveniles;* *- Time to swim-up (if appropriate);* *- Measurement of growth (length and weight); dry weight (24 hours at 60°C) is preferred to wet weight;* *- Incidence and description of morphological abnormalities and behavioral effects;* *- Observations of other effects or clinical signs.*

Parameters	**Details**	**Remarks** ***Criteria***
Observation intervals/dates for: Egg mortality: No. eggs hatched: Fry (alevin) mortality: Swim-up behavior: Growth measurements: Embryonic development: Other sublethal effects:	 Daily Daily Daily Daily up to Day 42 Day 87 (Day 61 post-hatch) Day 11 Daily	*Observations on hatching and survival should be made at least once daily; length and weight at end of test; and morphological abnormalities and behavioral effects at adequate intervals, depending on duration of test.*
Water quality was acceptable (Yes/No)	Yes	
Were raw data included?	Yes	
Other observations, if any	N/A	

II. RESULTS AND DISCUSSION

A. EXPOSURE:

The study author reported that method validation tests were conducted with the study medium alongside the definitive toxicity test samples (*i.e.*, for Days -3, 0, 7, 14, 17, 20, 27, and 35) with fortification concentrations of 0.2 and 12 mg a.e./L, and yielded mean recoveries of 98-101% of nominal concentrations.

Residues of the test item in the negative control solutions were never above the minimum quantifiable level (MQL) of 0.2 mg a.e./L. Overall mean-measured (0-87 d) concentrations (± SD) of the test item were <0.2, 0.27 ± 0.01, 0.67 ± 0.02, 1.66 ± 0.06, 4.19 ± 0.1, and 10.8 ± 0.78 mg a.e./L in the 0 (negative and solvent controls), 0.26, 0.64, 1.6, 4, and 10 mg a.e./L treatment levels (nominal), respectively. Mean-measured concentrations represented recoveries (relative to nominal concentrations) of 102, 104, 104, 105, and 108%, respectively.

The study author noted that the test solutions in mixing and test chambers appeared clear and colorless during the test for all treatment levels, with no evidence of precipitation observed in any control or treatment solution.

B. BIOLOGICAL EFFECTS:

According to the study author, on Day 11, mean egg viability in the four fertilization control replicates was 88%.

Time to hatch and hatching success: Embryo hatching began on study Day 23 (both in control and treatment groups), and by Day 26 ≥90% of surviving embryos had hatched. Remaining unhatched embryos were kept until they hatched, or died by Day 31 of the test. Because the majority of the embryos in both control and treatment groups hatched on the same day, the time to hatch endpoint was not statistically assessed by the study author. The study author reported that a "visual assessment" of the embryo hatch data indicated no apparent differences in time to hatch between control groups and treatment groups.

Mean hatching success was 83, 81, 79, 78, 90, 78, and 55%, respectively, in the negative control, solvent control, 0.27, 0.67, 1.66, 4.19 and 10.8 mg a.e./L groups (see **Table 3**).

Time to Swim Up: Rainbow trout fry began swimming up from the bottom of the test chambers on Day 37 of the study (or Day 11 post-hatch) in control and treatment groups. By Day 42 of the test (or Day 16 post-hatch), 93, 91, 88, 80, 79, 63, and 0%, respectively, of larvae in the negative control, solvent control, 0.27, 0.67, 1.66, 4.19 and 10.8 mg a.e./L groups had swum up.

Survival: Rainbow trout survival from Day 26 of the study (*i.e.*, beginning of the post-hatch period) to Day 42 of the test (*i.e.*, thinning), was 94, 94, 91, 96, 97, 89 and 2%, respectively, in the negative control, solvent control, 0.27, 0.67, 1.66, 4.19 and 10.8 mg a.e./L groups. Survival of rainbow trout fry from Day 42 of the test (*i.e.*, thinning) to Day 87 of the study (*i.e.*, test termination) was 100, 100, 100, 100, 100, 83 and 0%, respectively, in the negative control, solvent control, 0.27, 0.67, 1.66, 4.19 and 10.8 mg a.e./L groups.

Growth: Total lengths, wet and dry weight measurements were determined for all surviving fish at study termination (see **Table 4**). Due to significantly ($p < 0.05$) reduced rainbow trout survival in the 4.19 and 10.8 mg a.e./L treatment groups at test termination, growth data from these treatment groups were excluded from statistical analysis of growth endpoints.

Mean total lengths were 51.7-51.9 mm in the controls, compared to 51.6, 51.3, 50.2 and 44.5 mm in the 0.27, 0.67, 1.66, and 4.19 mg a.e./L treatment groups, respectively. Mean wet weights were 1.2-1.22 g in the controls, compared to 1.18, 1.22, 1.15 and 0.83 g in the 0.27, 0.67, 1.66, and 4.19 mg a.e./L treatment groups, respectively. Mean dry weights were 0.23-0.24 g in the controls, compared to 0.23, 0.24, 0.22 and 0.15 g in the 0.27, 0.67, 1.66, and 4.19 mg a.e./L treatment groups, respectively.

Clinical signs of toxicity: According to the study author, the majority of surviving larvae in the negative control, solvent control, and 0.27, 0.67, and 1.66 mg a.e./L groups generally appeared normal throughout the study. Apparently treatment-related sublethal effects were noted in the 4.19 and 10.8 mg a.e./L treatment groups during the study, including: small, pale, weak or morphologically deformed fish; and fish displaying abnormal behavior (*e.g.*, lying at the bottom of the test chamber).

Table 3: Study author-reported data on Rainbow Trout (*Oncorhynchus mykiss*) hatching success, larval survival and swim-up in a 87-d chronic toxicity test with 2,4-DB DMA (43.8% 2,4-DB).

Mean-measured Concentrations (mg a.e./L)	No. Eggs at D0	Hatching Success (%)	Larval Swim-up (%)	Survival (%)	
				Larvae (D0 – Thinning)	Juveniles (Thinning – Termination)
Negative Control [a]	120	83	93	94	100
Solvent Control [a]	120	81	91	94	100
0.27	120	79	88	91	100
0.67	120	78	80*	96	100
1.66	120	90	79*	97	100
4.19	120	78	63*	89	83*
10.8	120	55*	0*	3*	0*

[a] LOQ = 0.2 mg a.e./L
* Per the study author, test item treatment mean is significantly ($p < 0.05$) different from the pooled control according to Fisher's Exact test.

Table 4: Study author-reported data on Rainbow Trout (*Oncorhynchus mykiss*) growth (total length, wet and dry weight) in a 87-d chronic toxicity test with 2,4-DB DMA (43.8% 2,4-DB).

Mean-measured Concentrations (mg a.e./L)	Total length (mm)	Wet weight (g)	Dry weight (g)
Negative Control [a]	51.7 ± 0.2	1.20 ± 0.02	0.23 ± 0.01
Solvent Control [a]	51.9 ± 0.5	1.22 ± 0.02	0.24 ± 0.01
0.27	51.6 ± 0.5	1.18 ± 0.05	0.23 ± 0.01
0.67	51.3 ± 0.3*	1.22 ± 0.03	0.24 ± 0.01
1.66	50.2 ± 0.2*	1.15 ± 0.03*	0.22 ± 0.01*
4.19	44.5 ± 1.3†	0.83 ± 0.06†	0.15 ± 0.02†
10.8	---¶	---¶	---¶

* Per the study author, test item treatment mean is significantly ($p < 0.05$) different from the pooled control according to Dunnett's one-tailed test.
† Due to significant survival effects (*i.e.*, 83% juvenile survival), this treatment level was excluded from further statistical analyses.
¶ No data available due to 100% mortality of individuals in this treatment group.

C. REPORTED STATISTICS:

Statistical analyses were performed on hatching success, larval survival, time to swim-up, and growth (total length, wet weight, and dry weight) data; data on time to hatch were evaluated by visual

interpretation of the data. No significant differences were detected between the negative and solvent control group treatment means, and so control data were pooled for comparison to treatment groups.

Discrete data (*i.e.* hatching success, number of larvae reached swim-up, number of live larvae at swim-up prior to thinning and number of live larvae from thinning to test end) were analyzed using Chi-square and Fischer's Exact test (α=0.05). Continuous data (*i.e.* total length, wet weight, and dry weight) were evaluated first for normality using the Shapiro-Wilk's test, and for homogeneity of variance using Bartlett's test (α = 0.01 for both tests). All data passed the assumption tests for normality and homoscedasticity, and, therefore, analysis of variance (ANOVA) and Dunnett's one-tailed test (α = 0.05) were used to compare treatment means with the pooled controls. The study author also estimated regression-based endpoints (*i.e.*, EC_{10} and EC_{20} values), but the reviewer did not include these results in this DER as the focus of this study type is the determination of hypothesis-based results.

All statistical tests were performed using mean-measured concentrations as acid equivalents (mg a.e./L), and TOXSTAT (Ver. 3.5) or SAS (Ver. 8.2) statistical software.

Hatching success
NOAEC: 4.19 mg a.e./L LOAEC: 10.8 mg a.e./L

Larval survival (i.e. at swim-up, D42)
NOAEC: 4.19 mg a.e./L LOAEC: 10.8 mg a.e./L

Post-hatch survival (i.e., at test termination, D87)
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

Total length
NOAEC: 0.27 mg a.e./L LOAEC: 0.67 mg a.e./L

Wet weight
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

Dry weight
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

D. VERIFICATION OF STATISTICAL RESULTS:

Reviewers verified that the data in **Tables 3 and 4** accurately match the results reported by the study author; in order to clarify the interpretation of CETIS™ test results the reviewer re-presented the study results based on percent inhibition relative to the negative control (see **Table 5**).

Table 5: Reviewer-Calculated Effects of 2,4-DB DMA (43.8% 2,4-DB) on Rainbow Trout (*Oncorhynchus mykiss*) Growth and Reproductive Success

Mean-measured Concentrations (mg a.e./L)	Hatching Success	Time to Hatch	Larval Survival	Post-Hatch Survival	Length	Wet Weight	Dry Weight
	% Inhibition (Relative to Negative Control)						
0.27	5	-2	4.4	0	0.3	1.7	2.8
0.67	6	-3.1	-1.7	0	0.8	-1.2	-2

1.66	-8	-2	-3.3	0	2.9[1]	4.8	4.7
4.19	6	0	5.4	16.7€	14†	31.3*	34*
10.8	34*	3.1	97.3*	98.3€	---¶	---¶	---¶

* Treatment mean is significantly different from the negative control treatment mean (Dunnett's test, $p \leq 0.05$).
† Treatment mean is significantly different from the negative control treatment mean (William's test, $p \leq 0.05$).
€ Treatment mean is significantly different from the negative control treatment mean (Jonckheere's Terpstra's test, $p \leq 0.05$).
¶ No data available due to 100% mortality of individuals in this treatment group.
[1] Treatment mean is significantly different from the negative control treatment mean (William's test, $p \leq 0.05$), but the magnitude of the adverse effect is not considered by the reviewer to be biologically meaningful. Therefore, this adverse effect is not accounted for in the determination of the toxicity endpoints for this study.

The toxicity effects of formulated 2,4-DB on *O. mykiss* were analyzed using CETIS™ statistical software (version 1.9.2.8 with database backend settings implemented by EFED on July 25, 2017) (see **Appendix I** for CETIS™ reports).

The negative and solvent control groups were compared using a two-sample t-Test assuming equal variances and no differences were detected ($p>0.05$); treated groups were compared to the negative control. All data besides post-hatch survival – except for post-hatch survival data – approximately met assumptions of normality and homoscedasticity and were therefore analyzed using parametric tests; post-hatch survival data did not meet either assumption for parametric tests, and so were analyzed using non-parametric tests. Post-hatch survival and length data represented a monotonically increasing trend, and so were analyzed using Jonckheere's Terpstra's and William's tests, respectively. All other data represented non-monotonically increasing trends and were analyzed using Dunnett's Multiple Comparison tests.

All analyses were based on mean-measured concentrations on the basis of 2,4-DB acid equivalents (a.e.), and used $\alpha = 0.05$ unless otherwise noted.

Note that statistical analyses indicated that there was a significant ($p < 0.05$) reduction in total length of 2.9% at the 1.66 mg a.e./L treatment level. While statistically significant, this adverse effect (0.4 mm based on mean measurements) was considered by the reviewer to not be a biologically meaningful adverse effect, and was not accounted for in the determination of the LOAEC value based on data for this parameter. The reviewer instead determined that the LOAEC value for this parameter would more appropriately be 4.19 mg a.e./L based on a 14% reduction (or a mean reduction of 1.5 mm) in total length at this treatment level.

Time to hatch
NOAEC: 10.8 mg a.e./L LOAEC: >10.8 mg a.e./L

Hatching success
NOAEC: 4.19 mg a.e./L LOAEC: 10.8 mg a.e./L

Larval survival (i.e. at swim-up, D42)
NOAEC: 4.19 mg a.e./L LOAEC: 10.8 mg a.e./L

Post-hatch survival (i.e., at test termination, D87)
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

Total length
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

Wet weight
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

Dry weight
NOAEC: 1.66 mg a.e./L LOAEC: 4.19 mg a.e./L

Endpoint(s) affected: Hatching success, larval survival, post-hatch survival, total length, wet and dry weight
Most sensitive endpoint(s): Post-hatch survival, total length, wet and dry weight

E. REVIEWER'S COMMENTS:

The experimental phase of the definitive test was conducted from May 20 to August 15, 2016. All core guideline validity requirements (OCSPP 850.1400) appear to have been met by this study. Specifically, dissolved oxygen content was maintained ≥ 60% saturation, water temperature did not differ by more than ± 1.5°C between test chambers or between successive days at any time during the test, hatching success was >75%, post-hatch larval survival was >75%, and there were no significant effects of the solvent on survival or development of the early life stages.

With the exception of the time to hatch endpoint, which the study author did not statistically analyze, the reviewer's and study author's results for all but one (*i.e.*, total length) of the remaining endpoints were identical; this despite the study author using a pooled control treatment mean and the reviewer using the negative control treatment mean only for statistical mean comparisons. For the total length endpoint, the study author's analyses (using a Dunnett's one-tailed test with comparison to the pooled control treatment mean [51.8 mm]) resulted in a LOAEC of 0.67 mg a.e./L, while the reviewer's analyses (using a Dunnett's Multiple Comparison test with a comparison to the negative control treatment mean [51.7 mm]) resulted in a LOAEC of 1.66 mg a.e./L. The reviewer's analyses indicated that there was a 0.8% inhibition of Rainbow trout body length at the 0.67 mg a.e./L.

The reviewer's conclusions are reported in the Executive Summary and Conclusions sections, and are based on mean-measured concentrations reported as 2,4-DB acid equivalents (a.e.).

F. CONCLUSIONS:

Hatching success (-34%) and larval survival at swim-up on Day 42 (-97%) were significantly ($p<0.05$) reduced at the 10.8 mg a.e./L treatment level. At test termination (study Day 87, or 61 days following hatching), survival was significantly ($p < 0.05$) reduced 17 and 98%, respectively, at the 4.19 and 10.8 mg a.e./L treatment levels. Total length of surviving Rainbow trout juveniles was significantly ($p < 0.05$) reduced 3 and 14%, respectively, at the 1.66 and 4.19 mg a.e./L treatment levels, and mean wet and dry weights were reduced 31-34% at the 4.19 mg a.e./L (note that the 10.8 mg a.e./L treatment level was excluded from statistical analysis of growth data due to 98% mortality of individuals in this group).

This study is **scientifically sound** and is classified as **acceptable**.

III. REFERENCES:

None; other than standard guidelines and methodologies.