UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

DATE: March 27, 2012

SUBJECT: 2, 4-D: Revised Evaluation of Data Identified in NRDC Petition and Associated Documents

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Petition No.: N/A	**Regulatory Action:** N/A
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This revision is to modify the previous document to incorporate subsequent analysis and discussion of the issues raised in the NRDC petition and the Endocrine Disruptor Review Team (TXR No. 0055461) assessment of the data cited by the Test Order recipient in response to the Agency's Endocrine Disruptor Screening Program (EDSP) Test Order. The purpose of both documents is to provide an overview of the data that were identified in the NRDC petition for 2, 4-D and subsequent related responses and comments. Additionally, this document describes whether the data have been utilized in an appropriate manner by NRDC and others in order to support the development of the Agency response document to the petition. These data were submitted by the following organizations: 2,4-D Task Force; Beyond Pesticides; Michael Newton (private citizen); from the New York State Departments of Environmental

Conservation and Health; and the National Council For Air and Stream Improvement, Inc.[1]

1 Detailed information available at docket EPA-HQ-OPP-2008-0877 at www.regulations.gov

1.0 Introduction

The NRDC petition focused on the regulation of 2, 4-dichlorophenoxyacetic acid (i.e., 2, 4-D) raised a number of technical issues. Subsequent responses and additional comments by the 2, 4-D task force and other impacted organizations also raised additional technical issues. The purpose of this document is to provide a technical basis for the Agency response to these submissions. It describes the screening and review process which has been completed by the Agency for the various citations which were used by NRDC and in the subsequent related comments. These documents referenced above cited a number of guideline and literature research studies as well as other sources of information. This process involved several steps that included (1) indentifying all pertinent information, (2) developing a framework for categorizing the information, referred to as "bins", (3) completing a screen of each citation, and (4) completing further review of a study based on the results of the screening process if necessary.

The process used in this document for categorizing or "binning" the cited studies and the criteria used for screening purposes is described in ***Section 2: Overview Of Binning And Review***. ***Section 3: Overview of Hazard Data*** presents a brief overview of the findings obtained by examining all data related to the hazard assessment and dose response characterization. This topic is very broad and includes issues related to mutagenicity, endocrine disruption, neurotoxicity, metabolism, and carcinogenesis. ***Section 4: Overview of Exposure/Epidemiological Data*** presents a brief overview of the findings obtained by examining all data related to exposure assessment. This topic is very broad and includes issues related to dietary exposure, epidemiological analysis, population based biological monitoring, and the use of planned exposure studies designed to elicit the impacts of specific parameters. ***Section 5: Overview of Pet Incident Data*** presents a brief overview of the findings obtained by examining all data related to pet incidents associated with 2, 4-D use. ***Section 6: Summary*** presents a brief overview of all findings obtained by examining the data. Several sources of data focused on ecological issues such as ecotoxicity. These have not been addressed herein but have been evaluated in a separate document unless they are pertinent to a human health related issue. [Note: Each study has also been reviewed to determine if it needs a review for compliance with ethical criteria for human research and are noted as such.]

Appendix A: Data Summary contains all citations with an associated code to describe the bins in which they have been placed for the purposes of this analysis and which indicates the type of review received (e.g., screen only). **Appendix B: Hazard Data Summaries** includes the reviews of the data prepared for those types of information. Similarly, **Appendix C: Exposure Data Summaries** includes the reviews of the data prepared for those types of information. **Appendix D: Pet Incident Summaries** includes the reviews of the studies focused on impacts for pets as a result of 2, 4-D exposure. A tiered approach was used to evaluate each study cited by NRDC and in the subsequent comments. In this process each study was screened which entailed developing a summary that included a description of the methods and results and a description of the credibility related to how it was used by each specific commenter or in the NRDC petition itself. If further analysis was required, a more extensive review was completed. In some cases, information was cited by various submitters that were not applicable to the groupings described above. Generally this type of information required no further summary outside of the screening exercise. The screening summaries for these types of documents are included in

Appendix E: Meta Information. This is the only mention of these papers in this document.

Overview Of Binning Process

A variety of data were cited in the NRDC petition and the associated, subsequent documents. For efficiency purposes each citation was categorized based on a coding system that delineated the data based on the type of content and the level of review required. The codes that were used to delineate the data based on type of content include: hazard data, exposure data, pet incident data, and ecological data. As indicated above, the category of hazard data is very broad and includes issues related to mutagenicity, endocrine disruption[2], neurotoxicity, metabolism, and carcinogenesis. Each of these subcategories has a unique code associated with it for descriptive purposes. The codes for the various types of hazard data include:

- **MUT:** For studies that address possible issues related to mutagenicity.
- **EN:** For studies that address possible endocrine disruption issues.
- **MET:** For studies that address issues related to metabolism.
- **NEU:** For studies that address possible neurotoxic effects.
- **DA:** For studies that address dermal absorption issues.
- **CAN:** For studies that address carcinogenesis.
- **IP/SUBQ:** For studies that involved interperitonel or subcutaneous dosing regimens.
- **NC:** Relates to a hazard issue but does not pertain directly to a category described above.

Along with varied types of hazard data, a number of different types of exposure data were cited. These categories were also assigned separate codes, which include:

- **EPI:** For studies that address epidemiological data.
- **PB:** For studies that address population-based biological monitoring or other sampling.
- **PL:** For studies that address planned exposure studies such as required by guideline.

Studies that address pet incidents were fairly limited and are denoted by the code **"PI".**
It should be noted there are several studies that address a variety of ecological issues (e.g., ecotoxicity). In these cases, for tracking purposes, the studies are denoted by the code **"ECO"**. They have been reviewed in this document only if their results relate to another category associated with human exposure or risk.[3] In these cases the study will also be coded with the other pertinent category code (e.g., **"ECO/EN"** for an eco study which may provide information on endocrine disruption issues). [Note: In some cases when books are cited, a document has already been addressed in some manner, or the material does not fit one of the criteria described above the code is "**NR**" (No Action Required) because a screening document or further reviews are not required.]

2 Note that separate documents have been developed as part of the 2,4-D screen as an endocrine disruptor. Please refer to them for more detailed information (TXR No. 0055461).
3 EFED created a separate review document focused on the eco-related studies that do not contain any human health related information. Please refer to it for more detailed information.

It should be noted that the Agency must comply with updated requirements for protecting human subjects involved in research (i.e., 40CFR26) and review, under certain circumstances by the Human Studies Review Board (see http://www.epa.gov/osa/hsrb/ for more detailed information). It is not clear if the studies cited in the petition and related documents would meet current Agency criteria that would allow for their use in a regulatory decision. All studies with possible issues have been noted with a "Yes" if they need further ethics review should they be relied upon by the Agency.

Overview of Hazard Data

A summary of the available hazard data is included below. In some cases specific papers were utilized in the NRDC petition itself and in one or more subsequent, related comments. In other cases citations were utilized only in single comments. For clarity, this section summarizes the actions associated with each citation for each code and the results are presented in alphabetical order based on the name of the primary author. Additionally, it is noted where each citation has been used.

Bin: Neurotoxicity

Bortolozzi, *et al.* (2003)
Asymmetrical Development of the Monoamine Systems in 2, 4-Dichlorophenoxy acetic acid (2,4-D) Treated Rats.
[Bin: Neurotoxicity]
[Cited: NYS DOH]
[Human Studies Considerations Apply: No]
The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. Study examined the effects of 2, 4-D on endogeneous monoamines and their metabolites [noradrenaline, dopamine, 3, 4-dihyrdoxyphenylacetic acid, homovanillic acid, serotonin, and 5-hydroxyindolacetic acid] in the brains of neonatal rats exposed during gestation [dietary exposure of pregnant rats from gestation day (GD) 16 to 23] and/or postnatally *via* the diet up to postnatal day (PND) 90. According to the authors, results support view that during the critical period in which monoamines can influence cell development, neonatal exposure to 2, 4-D produces alterations on these neurotransmitter systems, which they equated to previous findings expressed in spontaneous circling activity towards the right side by adult offspring of dams exposed to 2, 4-D, indicating a correlation between monoamines brain level alterations and this behavior. The study was cited by NYS DOH as an additional study that should be considered in any new assessment along with those identified in the NRDC petition. The cited study does not provide any dose-response information since only one dose level was tested. When there is only one toxic dose administered to test animals, the effects seen do not generally provide information useful for risk assessment as do studies conducted with multiple doses, including dose levels at which effects are not seen. Having information at dose levels above and below a level at which effects are seen provides useful information that can be considered in a risk assessment. Additionally, the dose level tested (70 mg/kg/day) in the cited study exceeds the rat's renal clearance mechanism; i.e., it overwhelms the body's ability to excrete 2, 4-D, which allows 2, 4-D to build up in the body and exert its toxic effects. By design, studies investigating possible mechanisms of toxicity use dose levels that will cause the effect (toxicity) being investigated. The cited study does not provide information regarding potential effects of 2, 4-D on neurotransmitters at dose levels below the saturation level for renal clearance. An extended 1-generation reproductive toxicity study (MRID 47972101) on 2,4-D (submitted to Agency in response to the data requirements identified in the 2005 RED) includes an assessment of developmental neurotoxicity (including

neurobehavioral tests) at dose levels that do not saturate metabolic processes. In the extended 1-generation reproductive toxicity study, 2, 4-D was administered to young adult rats for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation. The young rat was exposed during gestation, lactation *via* the milk, and directly *via* the diet following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which incorporated a functional observational battery (FOB), which included cage-side, hand-held, and open-field observations of behavior, and measurements of body weight, rectal temperature, grip strength, and landing foot splay; motor activity using an automated system for measuring motor activity; and an assessment of the startle response to auditory stimuli. On PND 60, a neuropathological exam was conducted on the brain (including the cerebrum, thalamus/hypothalamus, cerebellum, and medulla), spinal cord, dorsal root ganglia, dorsal and ventral roots, peripheral nerves, and skeletal muscle. The examination of the brain included assessment of brain weight and gross measurements, microscopic measurements (morphometrics), and brain myelin. Three dose levels were tested. There were no treatment-related adverse effects on any of the numerous parameters assessed across life stages, which included multiple neurotoxicity-related endpoints similar to those in the study cited by NRDC. The parental rats were assessed for clinical signs of toxicity and maternal behavior, among other parameters. Maternal behavior towards the offspring was comparable among the groups, and there were no clinical signs, such as circling to the right observed in the dams or offspring or signs of maternal neglect. Although monoamine levels were not monitored in this study, neonatal exposure was similar to that in the cited study, and the dose levels were adequate (approaching saturation of renal clearance).

The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Bortolozzi, *et al*. (1999)

Behavioral Alterations Induced in Rats by a Pre- and Postnatal Exposure to 2, 4-Dichlorophenoxyacetic Acid.

[Bin: Neurotoxicity]

[Cited: NYS DOH]

[Human Studies Considerations Apply: No]

Study examined the effects of 2,4-D on physical/sexual development [fur growth, eye opening, testes descent, vaginal opening); surface righting (considered a reflection of subcortical maturation); negative geotaxis (tests labyrinthine and cerebellar integration; hindlimb support (tests neuromuscular and locomotor development); open field tests (measures activity and habituation; # crosses, rearings, vertical head movements, grooming episodes); 5-HT syndrome behaviors [forepaw movements (treading, excessive placement or tapping), hunching (back arching), sprawling of limbs, backing, hindlimb abduction, mobility (ability for locomotion despite gait abnormalities, including paddling), abnormal head movements (vertical or lateral side-to-side repetitive movements), wet-dog shakes (including head shakes), lying on side, limb extension dystonic, and flat body posture; circling behavior; measurement of catalepsy] of neonatal rats exposed during gestation (dietary exposure of pregnant rats from GD 16 to 23) and/or postnatally *via* the diet up to PND 90. According to the investigators, 2, 4-D exposure at a dose producing no overt signs of toxicity may cause long-term alterations in functional state of rat CNS, detected through a neurobehavioral test battery; however, they indicated that exposure during pre- and postnatal development induced behavioral abnormalities that seem to disappear in adulthood after 2,4-D cessation. The study was cited by NYS DOH as an additional study that should be considered in any new 2, 4-D assessment, along with those identified in the NRDC petition. This study is one of several

studies by the same investigators performed to address the mechanism of 2,4-D neurotoxicity, and it does not provide any dose-response information. The only dose used (70 mg/kg/day) exceeds rat renal clearance levels and does not provide information regarding potential effects of 2, 4-D on neurotransmitters at dose levels below the saturation level for renal clearance. In the extended 1-generation reproductive toxicity study on 2,4-D (MRID 47972101), young adult rats were administered 2, 4-D for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation. The young rat was exposed during gestation, lactation, and directly following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR), similar parameters as assessed in the cited study. Additionally, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry, and a special stain was used to evaluate brain myelination. The extended 1-generation reproductive toxicity study on 2, 4-D was performed at three dose levels up to the saturation level for renal clearance. There was no evidence of developmental neurotoxicity in this study that investigated the same parameters as assessed in the cited study. The no effect dose is greater than the points of departure (PODs) utilized for the human health risk assessment supporting the 2, 4-D RED. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Bortolozzi, *et al.* (2004)

Effects of 2, 4-Dichlorophenoxyacetic Acid Exposure on Dopamine D2–Like Receptors in Rat Brain.

[Bin: Neurotoxicity]

[Cited: NYS DOH]

[Human Studies Considerations Apply: No]

Study examined the effects of 2, 4-D on neonatal rats exposed during gestation (dietary exposure of pregnant rats from GD 16 to 23) and/or postnatally *via* the diet up to PND 90. Neonates were sacrificed at several ages for membrane receptor binding assays employing [^{3}H] nemonapride. According to the investigators, regional increases in D2–type receptor density may explain certain behaviors, such as catalepsy and right-turning preference in 2, 4-D exposed rats observed in earlier studies by the same investigators. When 2, 4-D exposure stopped, DA D2–type receptor density apparently recovered and stabilized to control level, suggesting reversible vulnerability of D2–type receptors to 2, 4-D exposure. This study is one of several research studies by the same investigators performed to address the mechanism of 2, 4-D neurotoxicity, and it does not provide any dose-response information. The only dose used (70 mg/kg/day) exceeds rat renal clearance levels and does not provide information regarding potential effects of 2, 4-D on neurotransmitters at dose levels below the saturation level for renal clearance. The study was cited by NYS DOH as an additional study that should be considered in any new assessment along with those identified in the NRDC petition. An extended 1-generation reproductive toxicity study on 2,4-D, in which offspring were exposed to 2, 4-D during gestation, lactation *via* the milk, and directly *via* the diet through PND 22, 70, 139, includes an assessment of developmental neurotoxicity (including neurobehavioral tests) at dose levels that do not saturate metabolic processes. In the extended 1-generation reproductive toxicity study on 2, 4-D (MRID 47972101), young adult rats were administered 2, 4-D for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation. The young rat was exposed during gestation, lactation, and directly following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle

response (ASR), similar parameters as assessed in the cited study. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry, and a special stain was used to evaluate brain myelination The extended 1-generation reproductive toxicity study on 2,4-D was performed at three dose levels up to the saturation level for renal clearance. There was no evidence of developmental neurotoxicity. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Bortolozzi, *et al*. (2001)

Intracerebral Administration of 2, 4-Dichlorophenoxyacetic acid Induces Behavioral and Neurochemical Alterations in the Rat Brain.

[Bin: Neurotoxicity]

[Cited: NRDC petition; 2, 4-D Task Force]

[Human Studies Considerations Apply: No]

Study examined the effects of 2, 4-D in male rats following intrastriatal injection. Injection of 2,4-D into the striatum produced a marked depression in locomotor activity and elicited a moderate circling towards the right side post dose. These changes were accompanied by a decrease in serotonin and an increase in homovanillic acid levels. Administration into the nucleus accumbens induced similar behavioral and neurochemical patterns, although rats did not present right turning. The Agency notes that the route of exposure is not appropriate for human risk assessment; human exposure would not be by direct injection into the brain. Additionally, the relationship of the levels of 2, 4-D attained in the brain following intracerebral injection to those attained following oral ingestion is not known. NRDC cited the study, among others, as evidence that postnatal exposures to 2, 4-D during the critical period of development of the infant brain raise serious scientific concerns. The Task Force states that this study cited by NRDC was not a developmental neurotoxicity study but a very high dose adult toxicity study in which 2, 4-D was directly administered into the rat's brain, which is a route not relevant to human exposures. The Task Force pointed out that the estimated concentrations of 2, 4-D in each brain area were 2-4 mM, which was 40- to100-fold the concentration in brain after systemic treatment. As noted above, in the extended 1-generation reproductive toxicity study on 2,4-D (MRID 47972101), young rats were exposed during gestation, lactation (*via* the milk), and directly (*via* the diet) following weaning and were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). Additionally, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry, and a special stain was used to evaluate brain myelination. The extended 1-generation reproductive toxicity study on 2, 4-D was performed at three dose levels up to the saturation level for renal clearance. There was no evidence of developmental neurotoxicity. These no effect doses are greater than the points of departure (PODs) utilized for human health risk assessment supporting the RED. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Brusco, *et al*. (1997)

2, 4-Dichlorophenoxyacetic Acid Through Lactation Induces Astrogliosis in Rat Brain. Molecular and Chemical Neuropathology.

[Bin: Neurotoxicity]

[Cited: NYS DOH]
[Human Studies Considerations Apply: No]
The goal of the study was to detect the effect of 2,4-D on astrocytes in different CNS regions of 25-day old pups exposed to 2,4-D through mother's milk [dams exposed i.p. during lactation days (LD) 9-25] using 2 rat astroglial markers. The investigators point out that one of the most widely documented cellular reactions following nervous system damage has been hypertrophy of astrocytes, often referred to as a reactive gliosis or astrogliosis characterized by rapid synthesis of glial fibrillary acidic protein (GFAP) intermediate filament (a sensitive and early biomarker of neurotoxicity). A glial reaction was detected at the level of serotonergic nuclei and extreme astrogliosis in the hippocampus and cerebellum in 2, 4-D pups. There was a significant increase in the number, size, number of processes, and density of immunostaining. The investigators concluded that exposure during the first days of life modifies the astroglial cytoarchitecture in parallel with previous neuronal changes observed by these investigators. The study does not provide any dose-response information (discussed previously) and the dose to pups is unknown. The only dose tested (70 mg/kg/day) is one expected to exceed rat renal clearance levels. The study was cited by NYS DOH as an additional study that should be considered in any new assessment along with those identified in the NRDC petition. The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. As noted above, in the extended 1-generation reproductive toxicity study on 2,4-D (MRID 47972101), young rats were exposed during gestation, lactation (*via* the milk), and directly (via the diet) following weaning, and were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry, and a special stain was used to evaluate brain myelination. Myelination was also evaluated in the PND 22 pups. At the highest dose tested, which exceeded in males and was approaching the level of renal saturation in females, there was no evidence of developmental neurotoxicity/neuropathology. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results in the cited study do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Duffard, *et al.* (1995)
Developmental Neurotoxicity of the Herbicide 2,4-Dichlorophenoxyacetic Acid.
[Bin: Neurotoxicity; Endocrine]
[Cited: NRDC petition; 2, 4-D Task Force]
[Human Studies Considerations Apply: No]
The citation is only an abstract, which has few details. Based on the title of the citation, article was binned under neurotoxicity. Study examined the effects of 2, 4-D on neonatal rats exposed at a single dose level during gestation (*i. p.* exposure of pregnant rats from GD 16 to PND 21) and postnatally *via* the diet up to adult life. Vaginal smears were monitored, and prolactin and progesterone levels were measured. Authors stated that these preliminary results demonstrate a 2, 4-D adverse effect on the endocrine system. Study does not provide any dose-response information. The only dose used (70 mg/kg/day) is one expected to exceed rat renal clearance levels following oral exposure. The study does not provide information regarding potential effects of 2, 4-D on estrous cycle or hormone levels at dose levels below the saturation level for renal clearance. NRDC cited this study as demonstrating endocrine disrupting effects of 2,4-D. The Task Force cited it as one of a series of studies that NRDC contends provides evidence of 2, 4-D endocrine activity. The Task Force does not consider this study as providing compelling evidence of potential environmental or human endocrine health concerns, since it was

conducted at a high dose (well above saturation of renal clearance) and, therefore, is of limited value for risk assessment. The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. The cited study was considered by the Endocrine Disruptor Review Team (TXR No. 0055461). An extended 1-generation reproductive toxicity study on 2,4-D, in which offspring were exposed to 2, 4-D during gestation, lactation, and directly *via* the diet through PND 22, 70, 139, includes an assessment of the endocrine system; evaluation of potential effects of 2, 4-D on parental male and female reproductive function; potential effects in offspring on survival, growth, development of nervous, immune, and reproductive systems; endocrine and selected systemic toxicity parameters; includes estrous cycle evaluation (female adult rats and offspring), evaluation of sperm parameters, thyroid hormone assessment; anogenital distance, nipple retention and puberty onset. There were no treatment-related adverse effects on any of the numerous parameters evaluated to assess the endocrine system following 2, 4-D exposure at dose levels approaching saturation of renal clearance. The changes in estrous cycle noted in the cited study were not replicated in the extended 1-generation reproductive toxicity study. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Duffard, *et al.* (1996)

Central Nervous System Myelin Deficit in Rats Exposed to 2,4-Dichlorophenoxy acetic Acid Throughout Lactation.

[Bin: Neurotoxicity]

[Cited: NRDC petition; 2, 4-D Task Force; NCASI]

[Human Studies Considerations Apply: No]

Study examined the effects of 2, 4-D on 25-day old pups exposed to 2, 4-D through mother's milk (dams exposed *i.p.* during LD 9-25, 9-15, or 15-25). Myelin deficit was observed in pups nursed from PND 9-25 or PND 15-25; in latter group, body and brain weight were not affected, so deficit in myelin cannot be attributed to undernourishment. There was a clear deficit in myelin staining and myelin chemical markers. The Agency notes that the study does not provide any dose-response information. The only dose used (100 mg/kg/day) exceeds rat renal clearance levels and does not provide information regarding potential effects of 2, 4-D on myelination at dose levels below the saturation level for renal clearance. The cited study was previously considered by the Agency in its rationale for additional toxicology data (DNT) in the 2005 RED. NRDC cited this study and others that they conclude identify adverse effects in offspring after lactational exposure. One of several studies cited by NRDC as providing evidence that postnatal exposures to 2, 4-D during critical period for development of the infant brain raise serious scientific concerns. The Task Force noted that the study was conducted at an excessively high dose (level expected to exceed the threshold level for saturating renal clearance of 2, 4-D) and included the use of an unrepresentative route and mode of administration (intraperitoneal) that is not relevant for human health pesticide risk assessment. Although the dams were exposed *i.p.*, the Agency notes that the pups were exposed *via* maternal milk. The dose to the pups is not known. The Task Force also considered this paper and other NRDC cited papers from the same group of investigators to be methodologically flawed [litter was not used as the unit for statistical analyses (to avoid bias due to litter effects); the reporting of histopathological evaluation limited, etc.]. NCASI referenced this study as one of the studies cited in the NRDC petition as supporting the claim that infants are at risk; however, NCASI argues that doses used in the study are extremely high and likely exceed renal clearance levels and are in range known to produce frank maternal toxicity, which is inappropriate dose selection for developmental toxicity studies. NCASI considers the fact that high doses are needed to elicit the reported effects as support of previous EPA conclusion that 2, 4-D use according to label

direction does not pose a risk to humans. The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. In the extended 1-generation reproductive toxicity study on 2, 4-D (MRID 47972101), young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (*via* the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). An evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation was performed. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. The extended 1-generation reproductive toxicity study on 2, 4-D was performed at three dose levels up to the saturation level for renal clearance. At the highest dose tested, there was no evidence of developmental neurotoxicity, and there was no adverse effect on myelination. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Evangelista de Duffard, *et al*. (1995)

Altered Behavioral Responses in 2,4-Dichlorophenoxyacetic Acid Treated and Amphetamine Challenged Rats.

[Bin: Neurotoxicity]

[Cited: NRDC petition; 2, 4-D Task Force; NCASI]

[Human Studies Considerations Apply: No]

Study examined the effects of 2, 4-D following acute exposure (*i. p.*) to rats. Various components of the (5-HT) serotonin syndrome (behavioral response induced by stimulation of 5-HT receptors) were observed in rats after acute dose of 2, 4-D plus amphetamine, but not with 2, 4-D alone. The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. NRDC cited study as showing that 2, 4-D results in delays in brain development and abnormal behavior patterns, including apathy, decreased social interactions, repetitive movements, tremors, immobility, with females being more sensitive. The Task Force stated that the study does not assess brain development, and the findings could be non-specific effects rather than any selective neurological effect. NCASI stated that the NRDC petition fails to mention that dose levels used likely exceed renal clearance levels and the route of exposure is not appropriate. The dose levels used (50 and 100 mg/kg/day) are at or exceed rat renal clearance levels. An extended 1-generation reproductive toxicity study on 2, 4-D, in which offspring were exposed *via* an appropriate route to 2, 4-D during gestation, lactation, and directly through PND 22, 70, 139, includes an assessment of developmental neurotoxicity (including neurobehavioral tests) and neuropathology at dose levels that do not saturate metabolic processes. The young rats were exposed during gestation, lactation (*via* the milk), and directly (*via* the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). An evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation was performed. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. The extended 1-generation reproductive toxicity study on 2, 4-D was performed at three dose levels up to the saturation level for renal clearance. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on myelination. The cited study does not identify a

more sensitive endpoint for human health risk assessment than already identified by the Agency. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Ferri, *et al*. (2007)

Selective Oxidative Stress in Brain Areas of Neonate Rats Exposed to 2,4-Dichlorophenoxyacetic Acid Through Mother's Milk.

[Bin: Neurotoxicity]

[Cited: NRDC petition; 2, 4-D Task Force]

[Human Studies Considerations Apply: No]

Study examined the effects of 2,4-D on 25-day old pups exposed to 2,4-D through mother's milk (dams exposed i.p. during LD 9-25 to dose level of 100 mg/kg/day). Reactive oxygen species (ROS) levels increased in the prefrontal cortex, striatum, and midbrain; GSH content decreased in striatum and midbrain of 2, 4-D pups compared with basal levels of 25-day-old pups. The investigators concluded that the results suggest that the developing brain areas have different susceptibilities to the adverse effect of 2, 4-D, especially those areas related to the dopaminergic system, and that oxidative stress is one 2, 4-D mechanism of toxicity. One of several studies cited by NRDC regarding lactational exposure and the need to consider lactational exposure in the aggregate RA. The Task Force referenced this study as another publication cited by NRDC that was conducted at a dose well above pharmacokinetic nonlinearity and included the use of an unrepresentative route and mode of administration (intraperitoneal) that is not relevant for human health pesticide risk assessment, which they conclude would further amplify nonlinear pharmacokinetic performance. The Agency agrees that the route of exposure of the maternal rats is not relevant for human pesticide risk assessment; however, the pups were exposed *via* the maternal milk, which is relevant. NRDC is correct that lactational exposure was not addressed in the 2, 4-D dietary exposure assessment. The Agency acknowledges that there are remaining uncertainties associated with attempting to characterize exposures from breast milk. In order to address this uncertainty, EPA is seeking monitoring data for 2,4-D in human breast milk (e.g., EPA's National Children's Study). The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. In the extended 1-generation reproductive toxicity study on 2,4-D (MRID 47972101), young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (via the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). An evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation was performed. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. The extended 1-generation reproductive toxicity study on 2, 4-D was performed at three dose levels up to the saturation level for renal clearance. At the highest dose tested (82 mg/kg bw/day in males/59 mg/kg bw/day in females), there was no evidence of developmental neurotoxicity and no adverse effect on myelination. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Garcia, *et al*. (2004)

Study of Tyrosine Hydroxylase Immunoreactive Neurons in Neonate Rats Lactationally Exposed to 2,4-Dichlorophenoxyacetic Acid.

[Bin: Neurotoxicity]

[Cited: NRDC petition; 2, 4-D Task Force]
[Human Studies Considerations Apply: No]
Study examined the effects of 2, 4-D on 25-day old pups exposed to 2,4-D through mother's milk (dams exposed i.p. during LD 9-25 to dose levels of 70 or 100 mg/kg/day). The investigators concluded that tyrosine hydroxylase immunoreactivity is significantly decreased, probably as a consequence of an inhibitory serotonin (5-HT) effect. This 2, 4-D inhibitory effect on the dopaminergic neurons probably mediated by 5-HT may lead to biochemical and functional consequence like alterations in DA biosynthesis, storage, release, feedback control systems or receptor sensitivity. This study identifies effects on pup brain following lactational exposure. NRDC cited study as support for the need to consider lactational exposure to 2, 4-D in aggregate RA. NRDC is correct that lactational exposure was not addressed in the 2, 4-D dietary exposure assessment. The Agency acknowledges that there are remaining uncertainties associated with attempting to characterize exposures from breast milk. In order to address this uncertainty, EPA is seeking monitoring data for 2,4-D in human breast milk (e.g., EPA's National Children's Study). The Task Force referenced this study as another publication cited by NRDC that was conducted at a dose well above pharmacokinetic nonlinearity and included the use of an unrepresentative route and mode of administration (intraperitoneal) that is not relevant for human health pesticide risk assessment, which they state would further amplify nonlinear pharmacokinetic performance. The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. In the extended 1-generation reproductive toxicity study on 2,4-D (MRID 47972101), young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (*via* the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). An evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation was performed. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. The extended 1-generation reproductive toxicity study on 2,4-D was performed at three dose levels up to the saturation level for renal clearance. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on myelination. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Garcia, *et al.* (2006)
Dopamine-β-Hydroxylase immunohistochemical Study in the Locus Coeruleus of Neonate Rats Exposed to 2,4-Dichlorophenoxyacetic Acid Through Mother's Milk.
[Bin: Neurotoxicity]
[Cited: NRDC petition; 2, 4-D Task Force]
[Human Studies Considerations Apply: No]
Study examined the effects of 2,4-D on 22-day old pups exposed to 2,4-D through mother's milk (dams exposed i.p. during LD 9-22 to dose levels of 70 or 100 mg/kg/day). The investigators concluded that tyrosine hydroxylase immunoreactivity is significantly decreased, probably as a consequence of an inhibitory serotonin (5-HT) effect. This 2, 4-D inhibitory effect on the dopaminergic neurons probably mediated by 5-HT may lead to biochemical and functional consequence like alterations in DA biosynthesis, storage, release, feedback control systems or receptor sensitivity. This study identifies effects on pup brain following lactational exposure. NRDC cited study as showing potential effects on the brain of neonatal rats (alterations in brain enzymes and neurotransmitters) and as support for the

need to consider lactational exposure to 2, 4-D in aggregate RA. The Task Force referenced this study as another publication cited by NRDC that was conducted at a dose well above pharmacokinetic nonlinearity and included the use of an unrepresentative route and mode of administration (intraperitoneal) that is not relevant for human health pesticide risk assessment, which they state would further amplify nonlinear pharmacokinetic performance. The Agency agrees that the route of exposure of the maternal rats is not relevant for human pesticide risk assessment; however, the pups were exposed *via* the maternal milk, which is relevant. As noted previously, the cited study is one of several research studies from the same group of investigators looking into a possible mechanism of 2, 4-D neurotoxicity. All of these research studies incorporate a dose level that will elicit the response being investigated (in this case, alterations in neurotransmitters). In the extended 1-generation reproductive toxicity study on 2,4-D (MRID 47972101), young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (via the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). An evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation) was performed. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. The extended 1-generation reproductive toxicity study on 2, 4-D was performed at three dose levels up to the saturation level for renal clearance. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on myelination. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Giasson, *et al*. (2000)
New Link Between Pesticides and Parkinson's Disease.
[Bin: Neurotoxicity]
[Cited: 2, 4-D Task Force]
[Human Studies Considerations Apply: No]

This citation does not mention 2, 4-D. Paper discusses another paper [Betarbet et al., 2000; rats exposed chronically (*i.v.*) to rotenone], which reports that chronic administration of rotenone can induce the major features of Parkinson's disease (PD) in rats. The paper states that this "remarkable observation not only provides a new animal model for the study of PD, but will also reinvigorate interest in the possible role of exposure to pesticides and other toxins as a cause of human neurodegenerative disease." This paper was cited by Task Force in response to NRCD's use of the Ton, et al., 2006 zebrafish paper to support their (NRDC) claim that 2, 4-D is associated with developmental neurotoxicity in mammals, including decreased motor activity and Parkinson's-like tremors. Task Force states that the support for the Ton claim is a "news and view commentary" by Giasson and Lee, 2000, which discusses using the pyrethroid insecticide and pesticide rotenone as a model for Parkinson's disease. As discussed earlier, the extended 1-generation reproductive toxicity study on 2, 4-D addresses neurotoxicity endpoints including assessments of motor activity, functional operational battery (FOB), and myelination. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on motor activity or myelination. The no adverse effect levels observed in the extended 1-generation reproductive toxicity study are greater than the PODs utilized for human health risk assessment supporting the RED.

Konjuh, *et al*. (2008)

Neonatal hypomyelination by the herbicide 2,4-dichlorophenoxy acetic acid. Chemical and ultrastructural studies in rats.
[Bin: Neurotoxicity]
[Cited: NRDC petition; 2, 4-D Task Force; NCASI]
[Human Studies Considerations Apply: No]
Study examined the effects of 2, 4-D on myelination in 22-day old pups exposed to 2,4-D through mother's milk (dams exposed to 70 mg/kg/day i.p. during LD 9-22). Study referenced by NRDC as one that shows that 2, 4-D impairs normal deposition of myelin in developing brain. NRDC states that the effects of 2,4-D make it likely that fetuses, infants, and children will be more susceptible to long-term adverse health effects from exposure to 2. 4-D. Study does not provide any dose-response information (do not know dose to pups *via* the milk). The only dose used (70 mg/kg/day administered *i.p.* to the maternal animal exceeds rat renal clearance levels following oral exposure and does not provide information regarding potential effects of 2, 4-D on myelination at dose levels below the saturation level for renal clearance. Task Force pointed out the excessive dose and route of exposure were not appropriate among other study deficiencies. NCASI stated that the petition fails to mention that dose levels likely exceed renal clearance levels and route of exposure is not appropriate. The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. In the extended 1-generation reproductive toxicity study on 2, 4-D (MRID 47972101), young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (*via* the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR), An evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation) was performed. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. The extended 1-generation reproductive toxicity study on 2, 4-D was performed at three dose levels up to the saturation level for renal clearance. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on myelination at any dose level. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.
Rosso, *et al.* (2000)
2, 4-Dichlorophenoxyacetic Acid (2,4-D) in Developing Rats Alters Behavior, Myelination and Regions Brain Gangliosides Pattern.
[Bin: Neurotoxicity]
[Cited: NRDC petition; 2, 4-D Task Force; NCASI]
[Human Studies Considerations Apply: No]
Study examined the effects of 2, 4-D on neonatal rats following sc injections (70 mg/kg during PND 12-25 or 100 mg/kg during PND 7-25) every 48 hours. It was reported that early exposure of pups to high dose resulted in a deficit in myelin deposition and behavioral alterations together with GM ganglioside reduction, which could be reflecting a 2,4-D CNS injury, although a weak undernutrition could be involved also. When 2,4-D dose was lower, body and brain weight effects were not observed, but changes in GM1 contents and in myelin deposition, and alterations in muscular force and motor activity were still observed. The dose levels are above level for saturation of renal clearance mechanism (oral route) and the route of exposure (sc) of pups is not relevant to human exposure and risk assessment. However, the reported deficit on myelin deposition was also observed following pup exposure *via* milk

(Duffard, 1996). NRDC referenced the study as one that shows that 2,4-D appears to impair normal deposition of myelin in developing brain, and that effects of 2,4-D make it likely that fetuses, infants, and children will be more susceptible to long-term adverse health effects from exposure to 2, 4-D. Task Force commented that effect may be due to undernutrition. The Task Force noted that the study was conducted at an excessively high dose (level expected to exceed the threshold level for saturating renal clearance of 2, 4-D) and included the use of an unrepresentative route and mode of administration that is not relevant for human health pesticide risk assessment. The Task Force also considered this paper and other NRDC cited papers from the same group of investigators to be methodologically flawed [litter was not used as the unit for statistical analyses (to avoid bias due to litter effects); the reporting of histopathological evaluation limited, etc.]. NCASI stated that petition fails to mention that dose levels likely exceed renal clearance levels and route of exposure is not appropriate. The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. The extended 1-generation reproductive toxicity study on 2, 4-D, in which offspring were exposed to 2, 4-D during gestation, lactation *via* the milk, and directly through PND 22, 70, 139, includes an assessment of developmental neurotoxicity and neuropathology (including an evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation) at dose levels that do not saturate metabolic processes. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on myelination at any dose level. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Rosso, *et al*. (2000)

2,4-Dichlorophenoxyacetic acid disrupts the cytoskeleton and disorganizes the Golgi apparatus of cultured neurons.

[Bin: Neurotoxicity]

[Cited: NYS DOH]

[Human Studies Considerations Apply: No]

Study examined the effects of 2, 4-D in primary cultures of cerebellar granule cells (24-hr exposure). In this *in vitro* study (cultured neurons), there was a dose-dependent inhibition of neurite extension; paralleled by a significant reduction in cellular content of both dynamic and stable microtubules, a disorganization of Golgi apparatus, and inhibition in synthesis of complex gangliosides. The investigators concluded that the primary toxic effects of 2, 4-D on neurons are most probably due to inhibition of microtubule polymerization, and these observations raise the possibility that at least one mechanism underlying 2,4-D neurotoxicity involves an inhibition of microtubule assembly. This mechanistic study is not directly applicable to risk assessment. The relationship of the dose (mM) to levels attained in the brain *in vivo* is not known. The study was cited by NYS DOH as an additional study that should be considered in any new assessment along with those identified in the NRDC petition. This citation is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. In response to the required additional data, the registrant conducted an extended 1-generation reproductive toxicity study on 2, 4-D, in which offspring were exposed to 2, 4-D during gestation, lactation, and directly through PND 22, 70, 139, includes an assessment of developmental neurotoxicity and neuropathology (including an evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation) at dose levels that do not saturate metabolic processes. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on myelination at any dose level. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. The extended one-generation reproduction study confirms the protectiveness of the existing Points of

Departure as to neurotoxic effects.

Rosso, *et al*. (1997)

Effects of 2, 4-Dichloro phenoxyacetic Acid on Central Nervous System of Developmental Rats. Associated Changes in Ganglioside Pattern.

[Bin: Neurotoxicity]

[Cited: NYS DOH]

[Human Studies Considerations Apply: No]

Study examined the effects of 2, 4-D on neonatal rats following sc injections of 2, 4-D (70 mg/kg or 100 mg/kg) every 48 hours from PND 7- PND 17 or PND 7- PND 25. Results suggest a delay in CNS development when pups exposed to 100 mg/kg/day (sc); at lower dose, brain capable of triggering biochemical mechanisms producing a plasticity response, which is expressed as changes in ganglioside content and composition. The dose levels would be expected to exceed the threshold level for saturation of renal clearance mechanism; route of exposure (sc) of pups is not relevant to human pesticide exposure or risk assessment. The study was cited by NYS DOH as an additional study that should be considered in any new assessment along with those identified in the NRDC petition. The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. In the extended 1-generation reproductive toxicity study on 2, 4-D (MRID 47972101), young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (via the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). An evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation was performed. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. The extended 1-generation reproductive toxicity study on 2,4-D was performed at three dose levels up to the saturation level for renal clearance. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on myelination at any dose level. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Sturtz, *et al*. (2006)

Detection of 2, 4-dichlorophenoxy acetic acid in rat milk of dams exposed during lactation and milk analysis of their major components.

[Bin: Neurotoxicity]

[Cited: NRDC petition; 2, 4-D Task Force]

[Human Studies Considerations Apply: No]

Study examined the effects of 2, 4-D on the nutritional status of pups following exposure of the dams to 2, 4-D at dose levels of 15, 25, 50, or 70 mg/kg/day *via* the diet during PND 1 to PND 16. It was reported that maternal dietary exposure during lactation results in damage to pups nutritional status; 2,4-D produced a dose-related reduction of polyunsaturated fatty acids (PUFAs); significant changes in milk fatty acid composition indicating a disturbance in the supply of PUFAs to the neonate, which can alter normal development of the brain. The study provides data following oral doses to the maternal rat, which are below, at, and above renal clearance capacity. NRDC cited study as confirmation that offspring are exposed to 2, 4-D during lactation. Additionally, it was pointed out that this 16-day exposure at dose level of 15 mg/kg/day resulted in significant decreases in pup body weight gains, and 2, 4-D accumulated in maternal milk and altered the nutritional content of the milk. NRDC considered

the study especially relevant in light of EPA selecting a NOAEL of 25 mg/kg/day for short-term (1-30 days) oral exposure and states that EPA should re-do the short-term oral risk assessment using 15 mg/kg/day as a LOAEL rather than 25 mg/kg/day for the NOAEL. The Agency notes that the cited paper only provides pup body weight gain data (in the form of a graph), and data on pup body weights were not provided. The study does not make clear whether it was measuring decrements in body weight gain or absolute body weight. Body weight gain is a calculated value and can be misleading. For example, say the control and treated group both show a body weight of 50.9 grams on day X. The control group gains 1.1 grams and the treated group gains 0.8 gram. This is a 24% decrease in body weight gain for the treated group compared to the control. However, 52 grams vs 51.7 grams is a 1% difference in body weight. The cited study does not show a clear dose response effect. Although there is a greater effect on body weight (gain) comparing the lowest and highest doses, the body weight effects are essentially the same at 15 and 25 mg/kg/day (11.4% and 11.6%) and that same phenomena is seen at 50 and 70 mg/kg/day (17.8% and 16.7%). Additionally, the decrease in body weight gain was observed from the 6th day of lactation to the end of the experiment. 2, 4-D Task Force pointed out that these results were not duplicated in a recent rat dietary life-stage study (Marty, 2009), which demonstrated decreased pup body weight only when dams were treated with 2,4-D ≥800 ppm in diet (premating through PND 21 (52, 61, 71-117 mg/kg/day); no effects on body weight at 400 ppm (25, 28, 37-57 mg/kg/day); at 800 ppm, 2,4-D pharmacokinetic behavior in lactating dams was distinctly non-linear (Saghir, 2009); i.e., renal clearance mechanism saturated. Based on the toxicokinetic profile, the doses in the extended one-generation reproduction study were adjusted during the lactational period to prevent excessive dosing both to the maternal rat and to the pups during early lactation and due to a "double exposure" when pups are both nursing and starting to consume diet (as in the case on PND 16). Adjustments to the diet were also performed in the Sturtz study, although the procedures used were different and may, to some extent, explain the results in the Sturtz study compared to the extended one-generation reproduction study. At the highest dose tested in the extended 1-generation reproduction study, which exceeded renal clearance, decreased pup body weights were observed throughout lactation, and the no observed adverse effect level for pup body weight was established at 300 ppm (≈30 mg/kg/day) for the PND 22 offspring. This NOAEL is greater than the POD utilized for the human health risk assessment supporting the 2, 4-D RED. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to body weight effects.

Sturtz, *et al.* (2000)

Detection of 2, 4-Dichlorophenoxy Acetic Acid (2,4-D) Residues in Neonates Breast-Fed by 2,4-D Exposed Dams.

[Bin: Neurotoxicity]

[Cited: NRDC petition; 2, 4-D Task Force; NCASI]

[Human Studies Considerations Apply: No]

Study examined the effects of 2, 4-D on the neonatal rats during lactation (PND 1-9 or PND 1-15); dams were exposed *i.p.* (0, 50, 70, 100 mg/kg) every other day to maintain 2, 4-D neonates' exposure. Residues of 2,4-D were found in stomach content, blood, brain, kidneys of PND 4 pups and older; residues were dose- and exposure-time dependent; at 100 mg/kg/day, decreased pup BW and tissue weight and diminished stomach content were noted. The investigators concluded that the results demonstrate that 2,4-D is transferred to neonates and decreased body and organ weights could be due to a diminished milk intake or/and to the direct 2,4-D toxic effect. When 2, 4-D was withdrawn from dams, 2,4-D residues remained in stomach content of the pups for at least a week. NRDC cited study as evidence of exposure of pups to 2, 4-D through maternal milk, thereby resulting in potentially significant exposures to the nurseling. NRDC states that despite knowing this research and route of exposure, EPA failed to include any lactational exposure in its aggregate risk assessment. The Task

Force considers the study as limited in value for human risk assessment since the dose levels to the lactating rats were in excess of saturation of renal clearance of 2, 4-D and toxicity observed in the resulting non-linear pharmacokinetic range does not inform human risk assessment, because nonlinear doses are exceedingly unlikely to occur in humans. Additionally, the Task Force notes that EPA was aware, as a result of animal feeding studies using exaggerated doses, that 2, 4-D may be present in milk. There was a discussion of this issue in the 2, 4-D RED. Compared to the high doses used in these studies, the EPA estimates of the maximum dietary exposure from food to human females ages 13-50 is 0.01018 mg/kg/day and the average exposure is 0.000642 mg/kg/day (USEPA memorandum, 2002). These values range from 4900 to 1 million times lower than the values in the cited rat study. NCASI stated that the NRDC petition fails to mention that the dose levels likely exceed renal clearance levels and the route of exposure is not appropriate. Study provides dose-response information at dose levels at or above level for saturation of renal clearance mechanism. Although the dams were dosed *i.p.,* the pups were exposed *via* the milk; the dose to the pups is not known. However, this study, in conjunction with another Sturtz study and data from the extended 1-generation reproductive toxicity study, which determined the levels of 2, 4-D in the maternal rat's milk, may provide information for determining pup dose. In the extended 1-generation reproductive toxicity study on 2, 4-D (MRID 47972101), young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (*via* the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). An evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation was performed. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. The extended 1-generation reproductive toxicity study on 2, 4-D was performed at three dose levels up to the saturation level for renal clearance. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on myelination. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Sturtz, *et al*. (2008)

Effect of 2, 4-Dichlorophenoxyacetic acid (2,4-D) on rat maternal behavior.

[Bin: Neurotoxicity]

[Cited: NRDC petition supplement; Beyond Pesticides; NYS DOH]

[Human Studies Considerations Apply: No]

Study examined the effects of 2, 4-D on maternal behavior. Dams were administered 2,4-D *via* diet (0, 15, 25, 50 mg/kg/day) from PND 1-7; on PND 5 and PND 7, dams (12/dose group) were tested for maternal behavior [nursing behavior with retrieval, crouching, licking (of whole body of pup), anogenital licking, time spent out of nest and eating; latency and length of retrieval, latency of crouching]. Serum prolactin levels; endogenous monoamines (noradrenaline, dopamine, 3,4-dihyrdoxyphenylacetic acid, homovanillic acid, serotonin, and 5-hydroxyindolacetic acid) and their metabolites were determined. Maternal nesting behavior was not affected. Differences in mother-pup interactions were observed at all dose levels (latencies of retrieval; latencies of crouching; increased time for retrieval of 8 pups; decreased % dams licking pups; none of 2,4-D dams licked anogenital area of pups; increased time spent out of nest); decreased indoleamine levels (dose-related); increased catecholamine levels. The doses used (15, 25, 50 mg/kg/day) are all at or below the expected level of renal clearance saturation. Prolactin levels have been related to the onset and maintenance of maternal behavior in rats. Beyond Pesticides cited the study as showing effects that need to be considered along

with other scientific literature, which they say abounds with evidence that 2,4-D is a dangerous chemical. NRDC cited the study as one that shows that even short-term exposure to 2,4-D at dose levels below toxic levels severely inhibits normal maternal hormones in brain and thereby adversely affects maternal behavior. It is to be noted that pup body weight gain was not affected by 2, 4-D treatment during the test period (PND 1-7), which is not consistent with a previous study by these investigators (Sturtz, 2006 above) where a decrease in pup body weight gain was reported from PND 6 on under similar exposure conditions. Additionally, it is not known whether the diets provided to the lactating dams were adjusted to take into account the fact that lactating dams consume greater amounts of food compared to non-lactating female rats; the doses actually ingested may have been greater than intended. One of the studies NYS DOH requests EPA to consider in any new 2,4-D risk assessment. The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. It is to be noted that a maternal behavioral assessment is one of the many parameters assessed in the extended 1-generation reproductive toxicity study on 2, 4-D. There was no evidence of maternal neglect in the clinical observations performed on the maternal rats in the 2, 4-D extended 1-generation reproductive toxicity study at dose levels approaching metabolic saturation. Additionally, there was no impact on the young with respect to any lack of maternal care. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Ton, *et al.* (2006)

Zebrafish as a Model for Developmental Neurotoxicity Testing.

[Bin: Neurotoxicity]

[Cited: NRDC petition; 2, 4-D Task Force]

[Human Studies Considerations Apply: No]

Study is a screening assay and not directly applicable to human health risk assessment. These data suggest that zebrafish may be an appropriate alternative model to study the mechanism(s) responsible for the developmental neurotoxicity of pesticides and aid in identification of compounds that should be further tested in mammalian systems. Neurotoxicity was already identified as a potential endpoint for 2, 4-D and more appropriate studies have been performed to assess 2, 4-D's developmental neurotoxicity potential. In the extended 1-generation reproductive toxicity study on 2, 4-D (MRID 47972101), young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (*via* the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). An evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation was performed. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. The extended 1-generation reproductive toxicity study on 2, 4-D was performed at three dose levels up to the saturation level for renal clearance. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on myelination. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

Bin: Endocrine

Blair, *et al.* (2000)
The Estrogenic Receptor Relative Binding Affinities of 188 Natural and Xenochemicals: Structural Diversity of Ligands.
[Bin: Endocrine]
[Cited: 2, 4-D Task Force]
[Human Studies Considerations Apply: No]
Study examined the effects of 2, 4-D in a validated (standardized) estrogen receptor (ER) competitive-binding assay, which was used to determine the ER affinity. 2, 4-D was one of many chemicals that were screened at high concentrations to determine whether the chemical competed with [3H]–estradiol for the ER. 2, 4-D did not exhibit any estrogenic activity at the concentrations tested. This is one of several studies referenced by the Task Force that they identified as demonstrating that 2,4-D does not bind to or activate estrogen receptors, contrary to NRDC's assertions that 2, 4-D is a "potent" endocrine disruptor. The Agency used an exposure based approach to select the first list of chemicals to receive Test Orders for the Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery; 2,4-D was included in the list of chemicals. Additionally it should be noted that this study was cited by the Test Order recipient as Other Scientifically Relevant Information (OSRI) in response to the Agency's EDSP Test Order and was evaluated by the Endocrine Disruptor Review Team (TXR No. 0055461). The EDRT concluded that while 2, 4-D was reported as negative in this investigation, no data were supplied to substantiate this conclusion, and this study did not meet Test Order requirements (TXR No. 0055461, page 17). EPA waived the Tier 1 tests most relevant to human endocrine effects for 2,4-D due to the availability of the extended one generation reproduction study with 2,4-D. (Ref. Office of Chemical Safety and Pollution Prevention, EPA, Memorandum from Greg Ackerman to Katie Weyrauch, "**2, 4-Dichlorophenoxyacetic Acid (2,4-D) - Report of the Endocrine Disruptor Review Team - Test Order #: EDSP–031001-120" (December 20, 2010).**) EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D, which include an *in vitro* Estrogen Receptor Binding Assay (890.1250) The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 *in vivo* studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.

Duffard, *et al.* (1995)
Developmental Neurotoxicity of the Herbicide 2,4-Dichlorophenoxyacetic Acid.
[Bin: Endocrine]
[Cited: NRDC petition; 2, 4-D Task Force]
[Human Studies Considerations Apply: No]
The citation only provided an abstract with few details. Study examined the effects of 2, 4-D on neonatal rats exposed during gestation (*i. p.* exposure of pregnant rats from GD 16 to PND 21) and postnatally *via* the diet up to adult life. Vaginal smears were monitored, and prolactin and progesterone levels were measured. Authors stated that these preliminary results demonstrate 2, 4-D adverse effect on the endocrine system based on alterations in the estrous cycle. Study does not provide any dose-response information. The only dose used (70 mg/kg/day) was one expected to exceed rat renal clearance levels. The study does not provide information regarding potential effects of 2, 4-D on estrous cycle or

hormone levels at dose levels below the saturation level for renal clearance. NRDC cited this study as demonstrating endocrine disrupting effects of 2,4-D. The Task Force cited it as one of a series of studies that NRDC contends provides evidence of 2, 4-D endocrine activity. The Task Force does not consider this study as providing compelling evidence of potential environmental or human endocrine health concerns, since it was conducted at a high dose (well above saturation of renal clearance) and, therefore, is of limited value for risk assessment. The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. The citation was considered by the Endocrine Disruptor Review Team (TXR No. 0055461). It is to be noted that the Agency issued a data call-in for a study that assesses thyroid, gonadal, reproductive and other endocrine-sensitive endpoints and while awaiting the study, imposed an additional 10X uncertainty factor to account for the data gap. In response to the data call-in, the Task Force submitted an extended 1-generation reproductive toxicity study on 2,4-D. This study examined potential effects on parental male and female reproductive function, offspring survival and growth, including endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring); sperm parameters; anogenital distance; nipple retention; puberty onset (vaginal opening and balano-preputial separation); adrenal weight, thyroid/parathyroid gland weight, pituitary gland weight, testes and ovarian weight, thyroid hormone effects; and histopathology of a wide range of tissues including the thyroid, adrenal, pituitary, testes, and ovary. The extended one-generation study for 2,4-D showed no treatment-related effects on potential estrogenic effects or androgen-sensitive endpoints (no adverse effects on anogenital distance, nipple retention, age at vaginal opening, estrous cycle length or pattern, mating, fertility, time to mating, gestation length, pre-implantation loss, number of corpora lutea, sperm parameters, ovarian follicle counts, and reproductive organ weights and histopathology; no evidence of hyposadias, ectopic tests, or treatment-related testicular prostate or seminal vesicle histopathology). Anti-androgenic effects in terms of decreased male reproductive organ weights were observed in some animals but they were not statistically significant and were associated with decreased body weight. No treatment-related effects on reproductive organ histopathology were observed. Slight effects were seen in the thyroid (increases or decreases thyroid weight and in T3, T4, and TSH hormones in some animals) but no dose response relationship was shown. These effects were more significant at the highest dose tested but still were considered adaptive and not adverse (i.e., the thyroid responded to insult and corrected itself) due to the fact that this dose exceeded the renal saturation level. Accordingly, the highest dose was considered a No Observed Adverse Effect Level (NOAEL) for thyroid effects. In the extended one generation reproductive toxicity study, there were no treatment-related adverse effects on any of the measured parameters evaluated to assess the estrogen, androgen or thyroid endocrine systems in intact rats following 2, 4-D exposure at dose levels approaching saturation of renal clearance. The changes in estrous cycle noted in the cited study were not replicated in the extended 1-generation reproductive toxicity study.

Haddow, *et al.* (1999)

Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child.

[Bin: Endocrine]

[Cited: NRDC petition]

[Human Studies Considerations Apply: No]

This is a general paper (not 2, 4-D specific) in which the children of women who had thyroid deficiency during pregnancy were assessed for various abilities (15 tests of intelligence, attention, etc.). NRDC cited this study in support of their conclusion that EPA failed to incorporate information on the endocrine disrupting effects of 2, 4-D. NRDC states that thyroid hormone plays a critical role in development of brain; slight thyroid suppression adversely affects neurological development in fetus,

with lasting effects on child learning and behavior. Contrary to the statement in NRDC petition, thyroid effects were identified in the 2, 4-D RED and a data call-in was issued for a 2-generation reproduction study that specifically required examination of the thyroid. HED agrees with NRDC that protection from adverse effects in the thyroid in women of childbearing age is important to protect the developing fetus from adverse outcomes. It is to be noted that 2, 4-D was tested in rats, which are much more sensitive to thyroid perturbations than are humans because the serum half-life of the thyroid hormone, thyroxine, is much shorter in rats (less than 1 day) than in humans (5-9 days). The 10X interspecies uncertainty factor applied to 2, 4-D in the Agency risk assessment to account for the possibility that humans are more sensitive than the test animals is, therefore, more than adequate to protect humans. The 10X intraspecies factor accounts for variability in sensitivity among species and gives protection for women with iodine deficiency. A 10X FQPA database factor was applied to 2, 4-D, which provided added protection pending the outcome of the required studies. The F1-extended one-generation reproductive toxicity study of 2, 4-D provides an assessment of the thyroid; *i.e.,* an evaluation of thyroid hormones (T3, T4 and TSH), thyroid weight and histopathology of the thyroid across life stages, brain morphometry data, and an evaluation of the auditory startle response. In the extended 1-generation toxicity study in rats, the expected hormone response consistent with perturbation in thyroid function was seen in the GD 17 satellite dams. These findings correlated with the thyroid alterations histologically. There was a comparable increase (↑9%) in thyroid weights at the low and high dose levels but no change in thyroid weight at the mid dose. In the F1 PND 4 pups, there were decreases in T3 levels at the low (↓8%) and mid (↓13%) dose groups, but not at the high dose. Decreased T4 levels were seen at the mid (↓15) and high (↓12%) dose groups, but there was no dose response. TSH level was increased (↑19%) at the high dose. In the F1 PND 22 offspring, there was a decrease in T4 (↓20%) only at the high dose. In the F1 PND 62-64 offspring, T3 level was increased (↑11%) only at the high dose. T4 levels were increased (↑19%) both at the mid and high dose levels. There was a dose-dependent increase in TSH levels at the mid (↑11%) and high (↑24%) dose levels. Female offspring (PND 70) displayed decreased absolute thyroid weights at the mid (↓9%) and high (↓5%) dose levels. No treatment-related histopathological lesions were seen in the thyroid glands of P1 rats or F1 offspring. The thyroid effects noted in the extended 1-generation reproductive toxicity study were considered to be adaptive and not adverse. Additionally, no robust effects on auditory startle or statistically significant brain morphometric changes were seen in the DNT component of the extended one-generation reproductive toxicity study; findings otherwise expected as sequelae to any marked thyroid hormone deficiency. The no observed adverse effect level (NOAEL) for thyroid toxicity was established at ≈40 mg/kg/day. This NOAEL is greater than the PODs utilized for the human health risk assessment supporting the 2, 4-D RED. The Agency used an exposure based approach to select the first list of chemicals to receive Test Orders for the Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery; 2, 4-D was included in the list of chemicals. The EDSP is based on a two-tiered approach. Tier 1 consists of a battery of eleven assays to screen chemicals for their potential to interact with estrogen, androgen or thyroid hormone pathways. The fact that a substance may interact with a hormone system does not mean that when the substance is used, it will cause adverse effects in humans or ecological systems. The purpose of Tier 2 testing is to identify and establish a dose response relationship for any adverse effects that might result from interactions with hormone systems identified in Tier 1 screening. The Tier 2 mammalian study is currently the 2-Generation Reproduction and Fertility Effects Study (Note: the OECD Guideline for the Extended F1 One Generation Reproduction Test Guideline is also under consideration). The existing 2, 4-D hazard database includes an Extended F1 One Generation Reproduction Test Guideline. The Agency has determined that the extended one-generation reproductive toxicity study has fulfilled the requirements for the *in vivo* mammalian Tier 1 assays, specifically the Hershberger, Uterotrophic, Male Pubertal and Female Pubertal assays. The

extended 1-generation reproductive toxicity study measured/evaluated endpoints that were not evaluated in the 2-generation reproduction study performed under the old guideline. These include an evaluation of potential effects on parental male and female reproductive function, offspring survival, growth including endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring), evaluation of sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment.

Hurst, *et al.* (2003)

The potential for oestrogenic effects of pesticides in headwater streams in the UK.

[Bin: Endocrine]

[Cited: 2, 4-D Task Force]

[Human Studies Considerations Apply: No]

The referenced study [yeast two-hybrid screen; Yeast Estrogen Screen (YES)] is a screening study in which 2, 4-D was negative. The Task Force referenced this study to dispute NRDC's claim that 2, 4-D is an endocrine disruptor. The Task Force noted that the Agency has on several occasions rejected the position that data gathered under Endocrine Disruptor Screening Program is a prerequisite to a safety determination. Concern for endocrine disruption was identified in the RED. The Agency used an exposure based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2, 4-D was included in this list of chemicals. This study was cited as Other Scientifically Relevant Information (OSRI) in response to the EDSP Test Order and was included in the Endocrine Disruptor Review Team (EDRT) assessment of 2, 4-D (TXR No. 0055461). EDRT did not accept yeast-based assays as OSRI primarily because some test chemicals are unable to penetrate the cell wall of the yeast cells and this may result in a false negative. Tier 1 screening assays are limited to evaluating whether a substance is capable of interacting with the endocrine system and are not sufficient to determine whether a chemical may have an effect in humans that is similar to an effect produced by naturally occurring hormones. The fact that a substance may interact with a hormone system does not mean that when the substance is used, it will cause adverse effects in humans or ecological systems. EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.

Jung, *et al.* (2004)

Anti-estrogenic activity of fifty chemicals evaluated by in vitro assays.

[Bin: Endocrine]

[Cited: 2, 4-D Task Force]

[Human Studies Considerations Apply: No]

The referenced study was conducted using a yeast two-hybrid assay system. 2, 4-D was not reported to inhibit estrogen activity. The Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2,4-D was included in this list of chemicals. This study was cited as Other Scientifically Relevant Information (OSRI) in response to the EDSP Test Order and was included in the Endocrine Disruptor Review Team assessment of 2, 4-D (TXR No. 0055461). As stated above, EDRT did not accept yeast based assays as OSRI primarily because some test chemicals are unable to penetrate the cell wall in yeast-based assays and this failure

may result in a false negative. EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.

Jungbauer, *et al.* (2002)

Yeast reporter system for rapid determination of estrogenic activity.

[Bin: Endocrine]

[Cited: 2,4-D Task Force]

[Human Studies Considerations Apply: No]

When the Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2, 4-D was included in this list of chemicals. Jungbauer and Beck tested 2,4-D in a yeast based system. The concentrations tested were not identified nor were data provided (TXR No. 0055461, page 17). This study was not cited as OSRI by the submitter due to the limitations of yeast-based assays. Some test chemicals are unable to penetrate the cell wall in yeast-based assays and this failure may result in a false negative. As noted above, EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.

Kim, *et al.* (2002)

Mechanism of phenoxy compounds as an endocrine disruptor. (in Korean)

[Bin: Endocrine]

[Cited: NRDC petition; 2,4-D Task Force]

[Human Studies Considerations Apply: No]

This article was written in Korean and was not available to the Agency in English. The Agency identified concern for potential endocrine effects in the RED (as noted above). In response to this and other concerns raised, the registrant conducted the extended one-generation reproductive toxicity study. This study measures several key endpoints. These endpoints include an evaluation of potential effects on parental male and female reproductive function, offspring survival, growth including endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring), evaluation of sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment. In the extended 1-generation reproductive toxicity study on 2,4-D (MRID 47972101) submitted in response to the data call-in, young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (*via* the diet) following weaning. Parameters assessed include sperm parameters, sex gland weights and histopathology; AGD, nipple retention; puberty timing. There were no treatment-related, adverse effects on any of these parameters.

Kim, *et al.* (2005)
Effects of 2,4-D and DCP on the DHT-induced androgenic action in human prostate cancer cells.
[Bin: Endocrine]
[Cited: NRDC petition; 2,4-D Task Force]
[Human Studies Considerations Apply: No]
Kim et al., (2005) studied the ability of 2,4-D and its metabolite to increase the proliferation of 22Rv1 human prostate cancer cells. Negative results were reported. Trancriptional activation studies were conducted with various cell lines. All were reported negative, except 2,4-D was reported to increase the response when coadministered with testosterone. NRDC cited the paper as evidence of endocrine disrupting effects of 2, 4-D, stating that the combination of 2, 4-D and testosterone resulted in synergistic increases in the proliferation of prostate cancer cells. The Task Force commented that this *in vitro* mechanistic study cited by NRDC is based on the earlier report that 2, 4-D increased prostate weight in rats following oral (gavage) exposure at a high dose above the threshold for saturation of renal clearance, which they state renders the usefulness and interpretation of this *in vitro* study of questionable value to potential human risk. As discussed above, the Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2,4-D was included in this list of chemicals. This study was included in the Endocrine Disruptor Review Team assessment of 2, 4-D (TXR No. 0055461); it should be noted that due to the limitations of these studies, the submitter did not claim them as OSRI. Effects of dietary 2, 4-D treatment on prostate weight and pathology were examined in extended 1-generation reproductive toxicity study on 2, 4-D, and there were no treatment-related effects. The NOAEL (45 mg/kg/day) is greater than the PODs utilized for the human health risk assessment supporting the 2, 4-D RED. The objective of the EDSP Tier 1 battery assays is to screen for the potential to interact with the estrogen, androgen and thyroid hormone pathways. EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints. The purpose of Tier 2 testing is to identify and establish a dose response relationship for any adverse effects that might results from interactions identified through Tier 1 screening. The Tier 2 mammalian study is currently the 2-Generation Reproduction and Fertility Effects Study or the Extended F1 One Generation Reproduction Test Guideline. The existing 2, 4-D hazard database includes an Extended F1 One Generation Reproduction Test Guideline.
Kojima, *et al.* (2004)
Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells.
[Bin: Endocrine]
[Cited: 2,4-D Task Force]
[Human Studies Considerations Apply: No]
In an *in vitro* assay (hER α transactivation assay; reporter gene assays for human ERα (hER α) and hERβ and AR (hAR) expression), 2,4-D did not show estrogenic effects or antiestrogenic properties in the hER α transactivation assay; did not show androgenic transcriptional activity; and did not show an inhibitory effect on the androgenic activity induced by DHT. Concern for endocrine disruption potential was identified in the RED. The Agency used an exposure-based approach to select the first list of

chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2,4-D was included in this list of chemicals. This is one of several studies cited as Other Scientifically Relevant Information (OSRI) in response to the EDSP Tier 1 Test Orders and by the Task Force to refute the NRDC evidence of the endocrine disrupting effects of 2,4-D. This citation was evaluated as OSRI in the Endocrine Disruptor Review Team assessment of 2, 4-D (TXR No. 0055461, page 25). The citation does not provide any data to substantiate the reported negative results, and transcriptional assays do not measure receptor binding. The F1-extended one generation reproductive toxicity study of 2,4-D evaluated several key endpoints. These endpoints include an evaluation of potential effects on parental male and female reproductive function, offspring survival, growth including endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring), evaluation of sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment. In addition the test guideline includes options to evaluate development of the immune and nervous systems. The findings for 2,4-D in the extended one-generation study determined there were no adverse effects observed on any of the numerous parameters evaluated, and the NOAEL was established at 45 mg/kg/day in males and 40 mg/kg/day in females. Both NOAELs are greater than the PODs utilized for the human health risk assessment supporting the 2, 4-D RED.

Lin, *et al.* (2000)

In vitro studies of cellular and molecular developmental toxicity of adjuvants, herbicides, fungicides, Minnesota.

[Bin: Endocrine]

[Cited: 2,4-D Task Force]

[Human Studies Considerations Apply: No]

The Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2,4-D was included in this list of chemicals. Lin *et al*., (2000) was cited as Other Scientifically Relevant Information in response to the Test Order. This was also one of several studies cited by the Task Force to refute the NRDC evidence of the endocrine disrupting effects of 2,4-D. Technical grade 2,4-D was positive in the assay, but the reagent grade was negative. It was included in the Endocrine Disruptor Review Team assessment of 2, 4-D (TXR No. 0055461, page 18); that the study may be useful supplementary information but it did not satisfy the requirements of the EDSP Test Order. A cell proliferation assay does not measure receptor binding and may be affected by factors other than binding with the receptor. EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints

Liu, *et al.* (1996)

The Direct Effect of Hepatic Peroxisome Proliferators on Rat Leydig Cell Function in vitro.

[Bin: Endocrine, Cancer]

[Cited: NRDC petition; 2,4-D Task Force]

[Human Studies Considerations Apply: No]

The referenced study is an *in vitro* study designed to address the mode of action for Leydig cell adenomas and peroxisome proliferation. The results suggest that peroxisome proliferators, as a class of

compounds, directly modify the steroidogenic function of Leydig cells *in vitro*. Investigators stated that further investigations were necessary to address the mechanism for the *in vitro* effects on Leydig cells and to clarify the apparent relationship between peroxisome proliferator-induced changes in Leydig cell function and the development of Leydig cell tumors. This study is one of several studies cited by NRDC as the basis for their concern for potential endocrine disrupting effects of 2, 4-D. NRDC believes the findings establish the dangerous endocrine disrupting effects of 2,4-D that requires that 2,4-D be banned in light of the findings that 2,4-D causes slight decrease in testosterone release and significant increase in estrogen release from testicular cells. The Task Force stated that no significant effects in hCG-stimulated release of testosterone were seen in rat Leydig cell cultures. Effects on reproductive hormones were identified in the RED and were addressed in extended 1-generation reproductive toxicity study (MRID 47942101). The F1-extended one generation reproductive toxicity study of 2,4-D evaluated several key endocrine endpoints. These endpoints include an evaluation of potential effects on parental male and female reproductive function, offspring survival, growth including endocrine parameters such as estrous cyclicity (female adult rats and offspring), evaluation of sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment. The F1-extended one generation reproductive toxicity study on 2,4-D demonstrated a lack of interaction with the endocrine system, as evidenced by the lack of effects on male and female reproductive function, anogenital distance (AGD), male and female sexual maturation, reproductive organ weights, or histopathology of reproductive and endocrine organs, or on sperm parameters and estrous cyclicity/periodicity (TXR No. 0055341). The NOAEL was established at 45 mg/kg/day in males and 40 mg/kg/day in females, which are greater than the PODs utilized for the human health risk assessment supporting the 2, 4-D RED.

Malysheva, *et al.* (1997)

Morphological and histochemical changes in the thyroid gland after a single exposure to 2,4-DA herbicide (in Russian)

[Bin: Endocrine]

[Cited: Beyond Pesticides]

[Human Studies Considerations Apply: No]

The study is in Russian and no translation is available. There are no details regarding test methods or dose levels used, and no data are available to substantiate the reported effects. As cited by Beyond Pesticides, decreases in thyroid gland transport and production functions and impairment of hormone iodination in thyroid were observed after acute exposure to 2, 4-D. Thyroid effects were identified in the 2, 4-D RED and a data call-in was issued for a 2-generation reproduction study that specifically required examination of the thyroid. The F1-extended one-generation reproductive toxicity study of 2, 4-D provides an assessment of the thyroid following repeat exposure; *i.e.,* an evaluation of thyroid hormones (T3, T4 and TSH), thyroid weight and histopathology of the thyroid across life stages, and an evaluation of auditory startle and brain morphometry data (reported to be sensitive to thyroid disruption). The thyroid toxicity NOAEL was established at ≈40 mg/kg/day, the highest dose tested. This NOAEL is greater than the POD utilized for the human health risk assessment supporting the 2, 4-D RED. The cited study does not identify a more sensitive endpoint for human risk assessment than already identified by the Agency.

McKinlay, *et al.* (2008)

Endocrine Disrupting Pesticides: Implications for Risk Assessment.

[Bin: Endocrine]

[Cited: Beyond Pesticides]

[Human Studies Considerations Apply: No]

This paper is a review article that cites a paper by Kim, et al., 2005 (discussed above) in which, as cited

by Beyond Pesticides, 2,4-D was reported to promote the proliferation of androgen-sensitive cells by acting synergistically with its main metabolite, DCP. 2, 4-D did not promote the proliferation of androgen-sensitive cells alone. The F1-extended one generation reproductive toxicity study of 2,4-D evaluated several key endpoints. These endpoints included an evaluation of potential effects on parental male and female reproductive function, offspring survival, growth including endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring), evaluation of sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment. There were no adverse effects observed on any of the numerous parameters evaluated in the extended 1-generation reproductive study (TXR No. 0055341), and the NOAEL was established at 45 mg/kg/day in males and 40 mg/kg/day in females. Both NOAELs are greater than the PODs utilized for the human health risk assessment supporting the 2, 4-D RED.

Nishihara, *et al*. (2000)

Estrogenic Activities of 517 Chemicals by Yeast Two-Hybrid Assay.

[Bin: Endocrine]

[Cited:2, 4-D Task Force comments]

[Human Studies Considerations Apply: No]

The referenced study is a yeast two-hybrid assay system, in which 2, 4-D was reported to be negative. When the Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2,4-D was included in the list of chemicals. This study was referenced in a document submitted to the Agency by the 2,4-D Consortium in response to the EDSP Tier 1 Test Order, however it was not cited as Other Scientifically Relevant Information (OSRI). This citation was included in the Endocrine Disruptor Review Team assessment for 2, 4-D (TXR No. 0055461). In yeast-based assays some test chemicals are unable to penetrate the cell wall and this failure may result in a false negative. As noted above, EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.

Oakes, *et al.* (2002)

A Study of the Potential for a Herbicide Formulation Containing 2,4-D and Picloram to Cause Male-Mediated Developmental Toxicity in Rats.

[Bin: Endocrine]

[Cited: 2, 4-D Task Force]

[Human Studies Considerations Apply: Yes; the human data referenced in the citation were not used in the Agency's assessment**]**

Study examines the effects of 2, 4-D on male rats following 9 weeks of exposure with a mixture of 2,4-D and picloram. The treated males were mated with untreated female rats, and the females were sacrificed on GD 20, and fetuses were weighed and examined for either structural malformations or skeletal development. The results did not show any evidence that repeated exposure of male rats was likely to cause male-mediated birth defects in offspring or other adverse reproductive outcomes. Information provided in the report includes 2,4-D serum levels attained at 150 mg/kg/day dose level [daily dose Cmax values of 249-284 µg 2,4-D/mL serum]; serum half-life of ~6 hours; commercial

applicators and forestry workers (no protection) estimated exposures ranging from 0.9 to 160 µg/kg/day, which represents exposure to 0.1-11 mg for 70 kg man (referenced Draper and Street, 1982; Kolmodin-Hedman and Erne, 1980; Lavy, et al, 1987; Nash, et al, 1982); in terms of estimated serum levels, men given an oral dose of 5 mg 2,4-D/kg (=350 mg for 70-kg man) had a Cmax of 40 µg/mL (Kohli, et al., 1974); an 11-mg intake would give an estimated plasma level of 1 µg/mL; negative study at doses up to 150 mg/kg/day 2,4-D, which exceeds renal excretion mechanism. The study was cited by the Task Force as providing evidence that exposure to a herbicide formulation containing 2,4-D and picloram is not likely to cause male-mediated birth defects or other adverse reproductive outcomes, and no effects characteristic of endocrine-mediated response were observed. The findings in the extended 1-generation reproductive toxicity study of 2, 4-D are consistent with this conclusion. There were no adverse effects on any of the numerous parameters monitored in the extended 1-generation reproductive toxicity study of 2, 4-D, which included a detailed assessment of endocrine endpoints. The NOAEL was established at 45 mg/kg/day, which is greater than the POD utilized for the human health risk assessment supporting the 2, 4-D RED.

Soto, *et al* (1995)

The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants.

[Bin: Endocrine]

[Cited: 2, 4-D Task Force]

[Human Studies Considerations Apply: No]

This is one of several studies cited by the Task Force to demonstrate that 2, 4-D does not bind to or activate estrogen receptors. The Task Force referenced this study to refute NRDC's assertions that 2, 4-D has extensive hormone disrupting activity that may occur at low doses. The E-screen is a quantitative assay that compares the cell number achieved by similar inocula of MCF-7 cells in the absence of estrogens (negative control) and in the presence of 17β-estradiol (positive control) and a range of concentrations of chemicals suspected to be estrogenic. The results for 2, 4-D in this assay were reported to be negative, however no data were provided to support this conclusion. A cell proliferation assay could supplement information provided by a binding study, but not substitute for it because it does not directly measure binding. In addition, factors other than binding may stimulate proliferation.

As noted above, EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.

Bin: Dermal Absorption

Brand et al (2007)

Transdermal absorption of the herbicide 2,4-dichlorophenoxyacetic acid is enhanced by both ethanol consumption and sunscreen application

[Bin: Dermal Absorption]

[Cited: NRDC Petition]

[Human Studies Considerations Apply: No]

Both alcohol consumption and topical sunscreen application act as transdermal penetration enhancers for model xenobiotics. The effect of combining these two treatments on transdermal absorption of the herbicide 2, 4-dichlorophenoxyacetic acid (2, 4-D) was examined. Skin from rats ingesting low (1.5 g/kg), medium (4.3 g/kg), or high (6 g/kg) ethanol doses or saline control was treated with a commercially available sunscreen containing titanium dioxide and octyl methoxycinnimate and transdermal absorption of 2,4-D was monitored by excising the skin and placing it into a diffusion chamber. Ethanol increased penetration by a factor of 1.9, 2.0 and 2.5 for animals treated with 1.5, 4.3 and 6 g/kg respectively, demonstrating an ethanol-induced dose response. Sunscreen application to skin from ethanol gavaged rats caused 2, 4-D absorption above that induced by ethanol alone by an additional factor of 1.3, 2.1 and 2.9 for 1.5, 4.3 and 6 g/kg respectively. This *in vitro* study indicates that the use of sunscreens and ethanol can enhance the dermal absorption of 2, 4-D. The use of the study as described in the NRDC petition is reflective of the results of the study. Several human dermal absorption (*in vivo*) studies were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A mean-percent absorbed value of 5.7% of the applied dose was derived from four different human dermal absorption studies. Ultimately, a value of 10% was used by the Agency based on these data and given the variability noted in the results. The studies considered by EPA involved exposure conditions that varied based on application site (forearms, hands), topical dose rates (1.7 to 1100 $\mu g/cm^2$), form (acid or salt), application media (water, ethanol, acetone), and exposure time. The overall average dermal absorption value for all of these data combined (N=34), regardless of design, was 5.7 percent. Examination of these variables, particularly the use of different application vehicles and different anatomical sites, is likely to have captured much of the variability measured in the sunscreen and alcohol *in vitro* studies. EPA used its standard uncertainty factors (UF_A interspecies and UF_H intraspecies) to establish a level of concern for this assessment even though human dermal absorption data were used and a protective value was selected for the risk assessment. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Finally, it should also be noted that the UF_H factor is partially intended to account for uncertainties associated with the relative sensitivity to a pesticide within a population, which includes factors such as differences in dermal absorption that could possibly be attributed to the use of sunscreen, clothing, or occlusion. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk.

Brand et al (2002)
Sunscreens Can Increase Dermal Penetration of 2,4-Dichlorophenoxyacetic Acid
[Bin: Dermal Absorption]
[Cited: NRDC Petition]
[Human Studies Considerations Apply: No]

The purpose of this work was to determine the effect of sunscreen use on the transdermal absorption of a model herbicide, 2, 4-dichlorophenoxyacetic acid. The 2, 4-D was placed on top of the epidermis in an *in vitro* diffusion chamber after pretreatment with one of 9 commercially available sunscreens for 24 hours (dorsal skin from hairless mice CRL:SK1). The total penetrating through the skin in 24 hours ranged from 39.1 ± 1.7% for the no sunscreen control to 81.0 ±2.8% for Neutrogena Oil Free Sunscreen. Six sunscreens led to a significant enhancement of total 2, 4-D penetration as compared to the control. Investigators concluded careful selection of sunscreen during pesticide application could reduce potential exposure. The use of the study in the NRDC petition is reflective of the results. There are methodological issues associated with this study including the use of hairless mice and whether or not

UV radiation was applied to the treated skin during the test as UV radiation can possibly impact dermal absorption. In the 2005 risk assessment, it should be noted that EPA used a protective value for quantifying dermal absorption from a human volunteer study. Further, the Agency still applied its standard uncertainty factors to establish a level of concern, even though these factors typically are intended to establish levels of concern for risks based solely on animal toxicity data. EPA derived an initial dermal absorption factor of 5.7%, based upon the mean-percent absorbed value from four different human dermal absorption studies. However, EPA ultimately used a value of 10%, in order to be protective, based on the upper range of the variability observed in the results.

Brand et al (2008)

The Effect of Chronic Ethanol Ingestion on Skin Penetration of 2,4-D (2008) – title cited (could not find paper with this title; see below Brand, 2004,,). Chronic Ethanol Ingestion Alters Xenobiotic Absorption Through the Skin: Potential Role of Oxidative Stress.

[Bin: Dermal Absorption]

[Cited: NRDC Petition]

[Human Studies Considerations Apply: No]

Study examined the effects of ethanol ingestion on skin penetration of 2, 4-D. Rats pair-fed a diet containing 36% ethanol for 12 weeks; then switched to a non-alcohol diet and monitored for 4 weeks; non-invasive measurements for changes in dermal blood flow using laser Doppler velocimetry (LDV), damage to skin barrier *via* transepidermal water loss (TEWL), and changes in skin moisture content were performed. On 0, 1 day, or 1, 2, 3, 4 weeks after alcohol removal, rats sacrificed and their skin was analyzed for alcohol and aldehyde dehydrogenase, and lipid peroxidation. Transdermal penetration of DEET and 2, 4-D was determined also. Transdermal absorption, LDV, TEWL, skin alcohol and aldehyde dehydrogenase and lipid peroxidation were significantly increased after continuous ethanol exposure; factors remained elevated for up to 4 weeks after alcohol consumption stopped, reflecting fundamental changes in the skin. It was concluded that alcohol consumption triggers increases in levels of enzymes associated with ethanol metabolism, induces physical changes in the skin leading to increased uptake of xenobiotics (e.g., 2, 4-D). One of several studies cited by NRDC (supplement) to support concern for enhanced dermal penetration of 2, 4-D following alcohol consumption. Many factors can alter dermal function (barrier) and impact the magnitude of the dermal absorption factor (e.g. anatomical site of application, presence or absence of penetration enhancers in the pesticide formation, whether liquid formulations are aqueous or organic, species differences, magnitude of dose, and duration of exposure). Several human dermal absorption studies were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A mean-percent absorbed value of 5.7% of the applied dose was derived from four different human dermal absorption studies. Ultimately, a value of 10% was used by the Agency based on these data and given the variability noted in the results. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, in spite of using a dermal absorption value from a human study, the full 10X UF_H was retained. It should also be noted that the UF_H factor is partially intended to account for uncertainties associated with the relative sensitivity to a pesticide within a population, which includes factors such as differences in dermal absorption that could possibly be attributed to the use of sunscreen, clothing, or occlusion. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the

Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective of any potential enhancement to dermal absorption from sunscreen/alcohol use.

Brand et al (2004)

Effects of Chronic Alcohol Consumption on Dermal Penetration of Pesticides in Rats.

[Bin: Dermal Absorption]

[Cited: NRDC Petition]

[Human Studies Considerations Apply: No]

Alcohol consumption acts as a transdermal penetration enhancers for model xenobiotics. The effect of alcohol consumption on transdermal absorption of 2, 4- D was examined. Skin from rats ingesting liquid diets containing 36% ethanol for 6-8 weeks was placed in an *in vitro* flow-through diffusion cell system, and the epidermal side was exposed to 2, 4-D. Transdermal penetration was enhanced by a factor of 2.2. This is a generic issue; alcohol consumption has been shown to adversely affect the skin itself, which can alter dermal function (barrier) and lead to increased dermal absorption. This paper is one of several research papers from the same investigators cited by NRDC to show that the 2007 Brand study confirms prior research showing alcohol consumption enhances 2, 4-D penetration. The existing dermal absorption value of 10% is based on the high end of the range of results in an extensive set of high quality human research results. The Agency considers this the best available data because these data eliminate the interpretative considerations necessary to extrapolate from a different species and an *in vitro* test methodology. Current OPP policy precludes using *in vitro* dermal absorption data as a stand-alone for quantitative use in risk assessment. Corroborating *in vivo* data is also required. This is because OPP does not consider *in vitro* methods to have been sufficiently validated for stand-alone regulatory use[i]. Related to this issue of validation is the issue that the studies cited by NRDC were conducted by a single laboratory, and so have not been independently replicated. Additionally, there are important limitations to *in vitro* models for dermal absorption, one of which is lack of an intact vasculature. This likely limits the use of such a technique for assessing the effects of ethanol on dermal absorption if vasodilation facilitates increased dermal uptake after systemic absorption of ethanol.

The Agency is already aware of the kinds of variables that impact the magnitude of the dermal absorption factor (e.g. anatomical site of application, presence or absence of penetration enhancers in the pesticide formation, whether liquid formulations are aqueous or organic, species differences, magnitude of dose, and duration of exposure). The human volunteer dermal absorption studies that were considered in the 2005 risk assessment addressed several of these variables (skin site, dose level, organic or aqueous dose vehicles) and a conservative, upper-bound estimate of 10% was selected that accounts for these variables. As such, the Agency considers the 10% DAF adequately protective for a wide range of the variables known to impact the magnitude of the dermal absorption factor. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, in spite of using a dermal absorption value from a human study, the full 10X UF_H was retained. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective of any potential enhancement to dermal absorption from alcohol use.

Feldman et al (1974)

Percutaneous Penetration of Some Pesticides and Herbicides in Man

[Bin: Dermal Absorption]

[Cited: NRDC petition; 2,4-D Task Force]
[Human Studies Considerations Apply: Yes]
Twelve different radiolabeled (^{14}C) pesticides and herbicides, including 2,4-D were applied to the forearms of human subjects and their urinary excretion of (^{14}C) was quantified. Data were also obtained via I.V. dosing to correct skin penetration data for incomplete urinary recovery. Approximately 5.8 ± 2.4 % of the 2, 4-D administered dose was excreted. Investigators reported the values for 2, 4-D and other chemicals and acknowledged that many factors can impact dermal absorption (e.g., skin loading rates). NRDC stated that the Agency did not adequately assess the effect of 2, 4-D soaking into clothing or skin exposure covered by clothing or gloves by using this study of six adults to estimate dermal absorption that didn't use any form of occlusion. It is to be noted that long-sleeved shirts, long pants, and/or gloves are protective clothing, the purpose of which is to minimize exposure. One would not expect exposure from soaking through clothing/gloves with proper use of 2, 4-D or any other pesticide. Several human dermal absorption studies, which included this study, were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A mean-percent absorbed value of 5.7% of the applied dose was derived from four different human dermal absorption studies. Ultimately, a value of 10% was used by the Agency based on these data and given the variability noted in the results. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, in spite of using a dermal absorption value from a human study, the full 10X UF_H was retained. It should also be noted that the UF_H factor is partially intended to account for uncertainties associated with the relative sensitivity to a pesticide within a population, which includes factors such as differences in dermal absorption that could possibly be attributed to the use of sunscreen, clothing, or occlusion. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective.

Harris, et al (1992)
Percutaneous penetration of 2, 4-dichlorophenoxyacetic acid and 2, 4-d dimethylamine salt in human volunteers.
[Bin: Dermal Absorption]
[Cited: 2, 4-D Task Force]
[Human Studies Considerations Apply: Yes]
Study examined the effects of 2, 4-D and 2, 4-D dimethylamine salt (DMA) following dermal exposure in five male volunteers (each dosed on separate occasions with both chemicals). Urine samples were collected for 144 hours following dermal application of 10 mg to dorsum of the hand and analyzed for 2,4-D. An average of 4.5% was recovered in urine following 2,4 –D exposure, and 1.8% following DMA; greater amounts of DMA (7.7%) were washed off at 6 hours compared to 2,4-D (5.4%); average half-lives for excretion were ≈40 hours (2,4 –D) and 60 hours (DMA). 2,4-D was applied as a solution in acetone, which can be expected to increase penetration into corneum where it is resistant to removal by washing; thus overestimating dermal penetration under field conditions. These data were evaluated previously by HED (TXR No. 010044, 2/23/1993; several publications including bystander exposure, professional applicators); results were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. This is one of several studies cited by the Task Force that pointed out that EPA's decision regarding glove use is consistent with robust peer-reviewed

literature, which repeatedly demonstrates that use of protective rubber gloves alone dramatically reduces total absorbed dose of 2,4-D.

Moody, et al (1990)

Dermal absorption of the phenoxy herbicides 2,4-D, 2,4-D isooctyl, and 2,4,5-T in rabbits, rats, rhesus monkeys, and humans: a cross-species comparison.

[Bin: Dermal Absorption]

[Cited: 2, 4-D Task Force]

[Human Studies Considerations Apply: Yes]

This 1990 study was conducted to determine whether animal models could be used reliably to predict pesticide dermal absorption in humans and the effect of the pesticide vehicle and anatomic site on pesticide absorption. Dermal absorption was compared among male New Zealand rabbits and male Sprague-Dawley rats (shaved mid-dorsal region), male rhesus monkeys (shaved middorsal forearm or forehead regions), and human volunteers (forehead). A constant dose rate (4 $\mu g/cm^2$) was used and non-occlusive gauze patches were used to protect dosed areas for 24 hours (except forehead). Radiolabel dissolved in water, acetone or Esteron LV96. Urine samples (animals) collected at 4 and 8 hrs on first day, 24-hr intervals for 14 days; human urine collected at 4, 8, and 12 hrs and daily until post-treatment day 7; dose site washed at 24 hrs post dose (all); % dermal absorption and excretion half-lives were calculated. The investigators indicated that the use of volunteers was approved through the institutional review board of the University of California at San Francisco. The investigators concluded that the data demonstrate that none of the animal models consistently approximated dermal absorption in humans. Discussion section of paper states: it is noteworthy that the i.m. correction factor used for the animal data permits interspecies comparison of dermal absorption data and circumvents a potential problem that differential interspecies excretion of the pesticide radiolabel between urinary and fecal routes precludes direct extrapolation. They note that their human data were not corrected for non-urinary excretion since *iv* percent recovery was 100% for 2, 4-D acid in humans (Feldmann, 1974). Assuming a non-urinary excretion of the amine salt and isooctyl ester similar to that obtained in monkeys would tend to increase the human dermal absorption at most by 26-28%, a value consistent with the 20-25% estimated by Grover, et al. (1986) for non-urinary excretion and body storage of 2,4-D acid ingested by humans. Task Force listed this reference but no comments specific to this paper were found.

Moody, et al (1992)

Dermal Absorption of the Phenoxy herbicide 2,4-D Dimethylamine in Humans: Effect of DEET and Anatomic Site.

[Bin: Dermal Absorption]

[Cited: NRDC Petition; 2, 4-D Task Force]

[Human Studies Considerations Apply: Yes]

Percutaneous absorption of ^{14}C-ring labeled 2, 4-D-dimethylamine was examined following topical application to palm and forearm of human volunteers. The effect of two vehicles (water and acetone) and DEET on dermal absorption was also investigated. Urinary data demonstrated that there was no significant difference between palmar and forearm skin permeability to 2, 4-D-amine; DEET had no significant effect on total cumulative palmar permeability, and there was no significant difference between % recoveries obtained with acetone or aqueous vehicles. Results show that 10±11.5% of the ^{14}C -2, 4D-amine was absorbed dermally from the human palm and 7±6.2% was absorbed from the human forearm. The reported forearm data for the monkey was 6±3.4%, which is comparable to that found in this study. The investigators concluded that, from regulatory viewpoint, these data should raise concern since 2, 4-D-amine has been shown to permeate the palm, and hands are the anatomical sites most often contaminated by pesticide applicators. Although this exposure may be somewhat reduced by wearing gloves, NRDC noted that the study shows significant dermal absorption even following

rigorous soap and water washing at 24 hr post treatment. NRDC cited this research as support for their concern that the presence of DEET on skin significantly enhances absorption of 2,4-D. Task Force noted that EPA previously responded to this allegation by pointing out that the conclusion that the data demonstrate a difference between exposure with (14±4.5%) and without (10±11.5%) DEET is not supported given the magnitude of the standard deviation. Task Force also pointed out that in the 2005 RED, the PPE changes implemented mitigated concerns for potential enhancement by DEET, etc. Several human dermal absorption studies were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A value of 10% was used by the Agency based on these data and given the variability noted in the results. Additionally, a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies) were applied. It is to be noted that although a dermal absorption value from human data was used, the full 10X UF_H was retained to account for uncertainties associated with the relative sensitivity to a pesticide within a population. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not underestimate dermal risk and is adequately protective.

Moody, et al (1992)

Effect of the mosquito repellent DEET and long-wave ultraviolet radiation on penetration of the herbicide 2, 4-D and the insecticide DDT in natural rubber gloves.

[Bin: Dermal Absorption]

[Cited: NRDC Petition; 2, 4-D Task Force]

[Human Studies Considerations Apply: No]

The study examined total ^{14}C-ring labeled 2,4-D permeability across a 1.5 cm^2 cut-out of natural rubber latex gloves incubated in laboratory apparatus at 37°C for 48 continuous hours (mean thickness 0.51±0.008 mm; n=4). DEET itself rapidly permeated the glove, but no significant effect of DEET was observed on glove permeability to 2,4-D (2.4±1.8% with DEET vs 3.2±3.46% without DEET); photoenhanced (long-wave ultraviolet (UVA) radiation) permeation of 2,4-D was observed in a natural rubber glove (6.2%±0.73% +UVA vs 0.3%±0.14% -UVA). NRDC cited study as showing that these gloves commonly worn by farmers will not afford adequate dermal protection claiming DEET alone enhanced 2,4-D permeability. NRDC states the gloves were highly permeable to 2,4-D penetration when there was simultaneous exposure to DEET and sunlight. The Task Force noted that the study does not provide definitive conclusions for enhanced risk for following reasons: 1) authors concluded DEET did not significantly impact 2,4-D penetration through rubber; 2) n=2; permeability of 2,4-D was increased to 6.2±0.73% when rubber was simultaneously exposed to UV lamp emitting UVA (not "sunlight"; non-UVA control 0.3±0.14% 2,4-D permeation); 3) neither DEET alone nor UVA alone rendered the rubber "highly permeable to 2,4-D despite the relatively severe experimental conditions; 4) only permeation of total [^{14}C] was measured and thus authors could not exclude that the postulated enhanced permeation with UVA co-treatment was represented by 2,4-D photolysis product(s); and 5) authors stated the field relevance of observations remained to be established. As described elsewhere, Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective.

Pelletier, et al (1989)

Disposition of 2,4-Dichlorophenoxyacetic Acid Dimethylamine Salt by Fischer 344 Rats Dosed Orally and Dermally.

[Bin: Dermal Absorption]

[Cited: 2, 4-D Task Force]

[Human Studies Considerations Apply: No]

This 1989 study was designed to compare the pharmacokinetics of 2, 4-D administered orally and dermally to better understand the relationship between oral toxicity studies and typical dermal field exposure. It was stated that 2, 4-D is rarely commercially available in the acid form due to its poor water solubility and that commercial formulations are usually composed of alkali or amine salts or esters. Dimethylamine salt of 2,4-D was administered to rats orally and dermally. The dermal dose (10 mg/kg) was within upper range that might be expected from typical agricultural use and the oral dose (1 and 0.4 mg/kg), based on preliminary work, which indicated a 4-10% absorption factor for 2,4-D amine through skin, as compared to almost complete absorption from oral route. At 7 hrs, skin wash removed 63% of applied dermal dose. Based on urinary excretion values, the systemic availability was about 10% of the dermal dose, while that of the oral dose was virtually 100 % within 72 hours after exposure. In terms of 2, 4-D concentration, about 1 mg of 2, 4-D was excreted following either a dermal dose of 10 mg/kg or an oral dose of 1 mg/kg. Peak concentrations in blood and kidneys were achieved within 30 minutes (both routes). Oral dose almost completely excreted within 24 hours, whereas about 13% of the dermal dose was systemically available at 72 hours. This paper was referenced by the Task Force, but no specific discussion of it was found.

Pont, et al (2004)

Active ingredients in sunscreens act as topical penetration enhancers for the herbicide, 4-dichlorophenoxyacetic acid.

[Bin: Dermal Absorption]

[Cited: NRDC Petition]

[Human Studies Considerations Apply: No]

The purpose of this work was to determine if the active ingredients in sunscreen also act as dermal penetration enhancers. Full-thickness dorsal skin of female hairless mice was placed into a flow through diffusion chamber with epidermal side facing donor chamber; 2, 4-D amine in ethanol; 9 sunscreen ingredients. Investigators concluded none of the chemicals tested significantly inhibited 2, 4-D diffusion across skin. Based on the data, a sunscreen containing only octocrylene would be optimal to prevent sunscreen-induced penetration enhancement; however, octocrylene only absorbs in the UVB range and, therefore, cannot give broad-spectrum protection alone. The penetration enhancement seen across hairless mouse skin also occurred with human skin (abdominal skin from healthy human volunteers obtained after plastic surgery). EPA agrees that the *in vitro* studies indicate that the use of sunscreens could potentially enhance the dermal absorption of 2,4-D under the tested conditions. As a matter of policy, OPP does not agree that *in vitro* dermal absorption data provide evidence sufficient to be used quantitatively in risk assessment. As a matter of science, the Agency considers the present dermal absorption factor adequately protective, since it is based on human studies that utilized different vehicles and anatomical sites of exposure, the same variables that would be impacted by sunscreen, DEET, and/or ethanol use. The human volunteer dermal absorption studies that were considered in the 2005 risk assessment addressed several of these variables (skin site, dose level, organic or aqueous dose vehicles) and a conservative, upper-bound estimate of 10% was selected that accounts for these variables. As such, the Agency considers the 10% DAF adequately protective for a wide range of the variables known to impact the magnitude of the dermal absorption factor. The use of the study in the NRDC petition is reflective of the results. EPA used its standard uncertainty factors (UF_A interspecies and UF_H intraspecies) to establish a level of concern for this assessment even though human dermal absorption data were used and a protective value was selected for the risk assessment. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Finally, it should

also be noted that the UF_H factor is partially intended to account for uncertainties associated with the relative sensitivity to a pesticide within a population, which includes factors such as differences in dermal absorption that could possibly be attributed to the use of sunscreen. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk.

Riviere, et al (2003)

Percutaneous absorption of topical N, N-diethyl-m-toluamide (DEET): effects of exposure variables and coadministered toxicants.

[Bin: Dermal Absorption]

[Cited: NRDC Petition]

[Human Studies Considerations Apply: No]

The purpose of the study was to determine the effect of co-exposure factors that could modulate transdermal flux of topically applied DEET. Studies were conducted using isolated perfused porcine skin flaps (IPPSFs), *in vitro* porcine skin, and silastic diffusion cells. Results suggest that co-exposure to a number of chemicals that potentially could be encountered in a military environment may modulate the percutaneous absorption of topically applied DEET beyond that seen for normal vehicles at typically applied concentrations. Also observed was that covering DEET dose areas with an occlusive dressing, or even fabric, enhances absorption. NRDC states this study shows occlusion can significantly enhance skin absorption. The Agency is aware that occlusive coverings increase skin hydration status by preventing water loss, and that hydrated skin is generally more permeable to pesticides than un-hydrated skin. Therefore, occlusive coverings would be expected to increase exposure via the dermal route, resulting in increased risk. However, in EPA's evaluation of this study, it found that the Agency's risk assessment and margins of safety established through the application of uncertainty factors, already account for any uncertainty raised by NRDC relative to the findings in this study.

In the 2005 risk assessment, it should be noted that EPA used a protective value for quantifying dermal absorption from a human volunteer study. Further, the Agency still applied its standard uncertainty factors to establish a level of concern, even though these factors typically are intended to establish levels of concern for risks based solely on animal toxicity data. EPA derived an initial dermal absorption factor of 5.7%, based upon the mean-percent absorbed value from four different human dermal absorption studies. However, EPA ultimately used a value of 10%, in order to be protective, based on the upper range of the variability observed in the results. Also, as previously indicated, EPA used its standard uncertainty factors (UF_A interspecies and UF_H intraspecies) to establish a level of concern for this assessment even though human dermal absorption data were used and a protective value was selected for the risk assessment. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Finally, it should also be noted that the UF_H factor is partially intended to account for uncertainties associated with the relative sensitivity to a pesticide within a population which includes factors such as differences in dermal absorption that could possibly be attributed to the use of sunscreen, clothing, or occlusion.

Ross, et al (2005)

Dermal absorption of 2, 4-D: a review of species differences.

[Bin: Dermal Absorption]

[Cited: 2, 4-D Task Force]

[Human Studies Considerations Apply: Yes]

This is a review paper that provides a comparison of dermal absorption studies in animals (rat, mouse, rabbit, and Rhesus monkey) and humans. Several conclusion were presented, including an estimate of

potential human (consumer or professional worker) daily absorbed doses of 2, 4-D following skin exposures to liquid formulations across a range of applied dose densities (1-1000 µg of 2, 4-D per cm^2 of skin), and for contact durations of 24 hours or less. A mean percent absorbed value of 5.7% of the applied dose (derived from four different human dermal absorption studies) was considered appropriate. The use of the results of this study in the Task Force Comments on the NRDC petition appears reasonable. Several human dermal absorption studies, which are included in this review, were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A mean-percent absorbed value of 5.7% of the applied dose was derived from four different human dermal absorption studies. Ultimately, a value of 10% was used by the Agency based on these data and given the variability noted in the results. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, in spite of using a dermal absorption value from a human study, the full 10X UF_H was retained. It should also be noted that the UF_H factor is partially intended to account for uncertainties associated with the relative sensitivity to a pesticide within a population, which includes factors such as differences in dermal absorption that could possibly be attributed to the use of sunscreen, clothing, or occlusion. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective.

Wester, et al (1998)

Percutaneous Absorption of Salicylic Acid, Theophylline, 2,4-D Dimethylamine, Diethyl Hexyl Phthalic Acid, and p-Aminobenzoic Acid in the Isolated Perfused Procine Skin Flap Compared to Man In Vivo.

[Bin: Dermal Absorption]

[Cited:2, 4-D Task Force]

[Human Studies Considerations Apply: Yes]

Study compares percutaneous absorption in the isolated perfused porcine skin flap (IPPSF) system with that in man *in vivo*. Six human subjects (males and postmenopausal women) were topically dosed 2, 4-D (dimethylamine salt) in ethanol on the ventral forearm (site not occluded). Urine collected for day of dosing and subsequent 6 days; at 24 hr, site washed with soap and water. At 7 days after application, skin-dosing site was cellophane tape-stripped 10 times. Percutaneous absorption was determined from ^{14}C–urinary excretion. Five IPPSF were surgically prepared, harvested and perfused in a non-re-circulating chamber and then dosed with [^{14}C] 2, 4-D –dimethylamine one hour after equilibrium; a 10-cm^2 area on the IPPSF was dosed in a manner identical to the *in vivo* human study. Total penetration after 8 hours was estimated as the radiolabel detected in perfusate plus treated skin. Human *in vivo* percutaneous absorption was 1.1±0.3 % dose absorbed compared to penetrated dose in IPPSF (3.8±2.4 % dose absorbed). It was concluded that the IPPSF system appears to be a good model for predicting percutaneous absorption relative to man. This paper was referenced by the Task Force, but no specific comments were found.

Bin: Metabolism

Saghir, et al (2006)

Strategies to Assess Systemic Exposure of Chemicals in Subchronic/Chronic Diet and Drinking Water Studies.

[Bin: Metabolism]

[Cited:2, 4-D Task Force]

[Human Studies Considerations Apply: No]

Study objective was to develop a practical method to determine the steady-state daily systemic dose of a chemical to animals in repeat dose toxicity studies by determining the area under the blood/plasma concentration time curve (AUC-24 h) for compounds of different blood/plasma half-life. The approach involved collection of a number of blood samples within a 24 hours period and then examining various combinations of 1-3 time points predictive of the actual AUC values. This information was considered to provide a scientific basis (e.g., sampling times in reference to light/dark cycle) for choosing the minimum number and best combination of time points in order to predict the daily systemic bioavailability of a chemical (AUC-24 h) as well as daily highest (C_{max}) and lowest (C_{min}) body burden and the time of their occurrence. The study also investigated the use of terminal blood concentrations from a rat subchronic study collected after 18 h of fasting/removal of diet to roughly estimate elimination half-life of chemicals, as well as the use of time-course urinary elimination as an indicator of the circulating blood levels/AUC-24 h at steady-state exposure levels. Rats were dosed with 2, 4-D at dietary levels of 5 mg/kg/day and 100 mg/kg/day. 2,4-D concentrations in plasma and AUC values increased in a distinctly nonlinear fashion between 5 and 100 mg/kg/day (4-week exposure); nonlinear increase was apparent from the 89-fold increase in Cmax, and a 69-fold higher plasmaAUC-24 hr, far exceeding the expected 20-fold difference between doses; higher than expected plasma AUC-24 hr was also supported by the saturation of urinary excretion at high dose; only 28-fold higher 2,4-D was recovered during 18 hr of collection instead of 69-fold as expected from difference in circulating levels. The study demonstrates that a dose of 2,4-D of 100 mg/kg/day can be considered an excessive dose. This information (saturation of the renal excretion mechanism) was available previously and consider in the RED. The Task Force cited this paper with respect to Sturtz (2000) citation by NRDC regarding lactational exposure at 50, 70, 100 mg/kg/day. Task Force states that toxicity observed in the resulting non-linear pharmacokinetics range does not inform human risk assessment because nonlinear doses are unlikely to occur in humans. Similar data by these authors were submitted (MRID 47414901 and 47414902) in conjunction with the extended 1-generation reproduction study on 2, 4-D and are incorporated into the overall assessment for the study.

Saghir, et al (2009)

A Dietary Dose Range-Finding and Toxicokinetic (TK) Study of 2,4-Dichlorophenoxyacetic Acid (2,4-D) in Adult Crl:CD(SD) Rats and Their Offspring. 1. Toxicokinetics.

[Bin: Metabolism]

[Cited:2, 4-D Task Force]

[Human Studies Considerations Apply: No]

The citation is an abstract. Study examined the effects of 2, 4-D dietary exposure to rats from pre-breeding through weaning and F1 offspring exposure through PND 35. 2, 4-D AUC 24 h was calculated from 3 plasma samples collected at 6 am, 9 am, and 5 pm on designated sample day. 2,4-D in milk determined on lactation days 4 and 14 and in plasma of nursing pups by collection of single 9 am sample on PND 4, 14, 21, and 28. Due to saturation of renal clearance, nonlinear TK was observed in parental males at 1600 ppm (test day 28) and 1200 ppm (test day 71). Onset of nonlinear TK in parental females was observed at <400 ppm (test day 29 and 95; AUC 100 ppm = 25; AUC 400 ppm = 201 µg h mL-1);

during gestation and lactation, female 2,4-D levels were higher due to higher diet consumption; nonlinear TK occurred at <400 ppm (GD 17 and LD 14) and > 400 ppm and <800 ppm (LD 4); a shift in nonlinear TK on LD 4 may have been due to excretion into milk (levels ≈50% of female plasma); plasma 2,4-D in pups 30-70% lower than dam on LD 4 (M/F TK nonlinear <800 ppm) but similar to or higher than dam on LD 14 (M/M TK nonlinear <400 ppm). Maximal 2,4-D in pups was reached on PND 21 when pups were consuming 2,4-D *via* the diet in addition to nursing, but dropped to levels equal to dams on test day 29 and 95 levels on PND 35. Study results showed a kinetically-derived maximum dose of 400 ppm (≈25 mg/kg/day) for females and 1200 ppm (≈65 mg/kg/day) for males. Increased dam and pup AUCs during lactation supported dietary adjustment during lactation. It was concluded by the investigators that the TK data support a definitive study high dose for females and pups lower than that derived by traditional toxicity testing. These dose selection data (MRID 47414901) were discussed with the Agency prior to the extended 1-generation reproduction study initiation. The Task Force cited this study with respect to Sturtz (2000) citation by NRDC regarding lactational exposure at 50, 70, 100 mg/kg/day. Task Force states that toxicity observed in the resulting non-linear pharmacokinetics range does not inform human risk assessment because nonlinear doses are unlikely to occur in humans. The results are incorporated into the overall assessment of the extended 1-generation reproduction study.

van Ravenzwaay, et al (2003)

Comparative Metabolism of 2,4-Dichlorophenoxyacetic Acid in Rat and Dog.

[Bin: Metabolism]

[Cited:2, 4-D Task Force]

[Human Studies Considerations Apply: No]

Study examined the effects of 2, 4-D on rats and dogs of both sexes dosed orally with either 5 mg/kg or 50 mg/kg ^{14}C-2, 4-D. The rates and routes of excretion and plasma toxicokinetics and biotransformation were assessed. Elimination of 2, 4-D from rat plasma was significantly faster than in the dog ($T_{1/2}$ were 1.3-3.4 hrs for rat and 99-134 hr for dog) following 5 mg/kg or 50 mg/kg, respectively; AUC for rat was 21-57 µ eq.hg-1 and 4889-5298 µ eq.hg-1 for dog at 5 mg/kg; 122-2358 µ eq.hg-1 for rat and 34100-44296 µ eq.hg-1 for dog at 50 mg/kg; major route of excretion for rat was in urine; essentially complete after 24 hrs (low)/48 hrs (high) dose; elimination incomplete in dog over sampling period (50% of dose recovered); urine principal route at low dose, but equal amounts excreted in urine and feces at high dose over 120 hrs; body burden of 2, 4-D in dog significantly higher than in rat for an equivalent dose, which is consistent with the increased sensitivity of dog to 2,4-D toxicity. It was concluded that in the case of certain organic acids (2, 4-D), the rat is a better model for assessing the relevance to man of the toxicity of these compounds than the dog. These data were considered previously by the Agency. The toxicological endpoints for use in human risk assessment for 2, 4-D were selected from the rat database, based on the determination that the rat is a better predictor than the dog of the potential toxicity of 2,4-D to man. The data at 50 mg/kg in the rat suggested the clearance mechanism was saturated. The Task Force used this reference in its discussion of lactational exposure (Sturtz 2006 study) where ↓pup BW observed at 15 mg/kg/day. Task Force points out that this effect level was not replicated in 2009 Marty dietary life-stage study, where BW decreases were not observed at 400 ppm (25, 28, 37-57 mg/kg/day). Effects were observed at 800 ppm, which occurred under conditions in which 2,4-D pharmacokinetic behavior in lactating dams was distinctly non-linear, thus rendering these responses of limited relevance to human risk assessment; also, cited with respect to Sturtz (2000) citation by NRDC regarding lactational exposure at 50, 70, 100 mg/kg/day; toxicity observed in the resulting non-linear pharmacokinetics range does not inform human risk assessment because nonlinear doses are unlikely to occur in humans. The extended 1-generation reproduction study on 2, 4-D will provide data on potential effects following lactational exposure. *No further action required.*

Bin: Mutagenicity

Arias 2003, 2007
Sister chromatid exchange induction by the herbicide 2, 4-dichlorophenoxyacetic acid in chick embryos Cytogenetic Effects of Short- and Long-term Exposure of Chick Embryos to the Phenoxyherbicide 2, 4-D.
[Bin: Mutagenicity]
[Cited: NRDC Petition; NYS DOH]
[Human Studies Considerations Apply: No]
In the first study, concentrations of 0, 0.5, 1, 2, and 4 mg/embryo of either the pure grade of 2,4-D (99%) or the commercial formulation, Agroxone containing 37% 2,4-D were injected into the air cell of White Leghorn chicken eggs (9-12/dose). Treatment lasted for 3 days; eggs were automatically rotated at 37.8ºC and BrdU (150 µg/embryo) was injected into the air cell. On Day 4, embryos were removed following a 2-hour exposure to colchicine (0.05%). Chromosome preparations were made from harvested embryos and slides were stained using the fluorescent-plus-Giemsa technique; the report did not indicate whether slides were coded. A total of 30-40 second-division metaphases were scored for SCE in the first five pairs of macrochromosomes. The frequency of first (M1), second (M2) and third (M3) division metaphases from 200 cells were scored for mitotic delay and MIs were determined from the count of 1000 cells. No tabular presentation of the data was available. Results, shown in graphs, indicated a significant ($p<0.05$) increase in SCEs for the high concentration of the commercial formulation (~1.43 SCEs/ cell vs. ~1.22 SCEs/cell for the solvent control, acetone) and a nonsignificant increase (~1.41 SCEs/ cell vs. ~1.22 SCEs/cell for acetone) for pure 2,4-D. Although the dose response was concentration related for both test materials, the increase at the high dose was only 1.2-fold higher, compared to the solvent control and 1.2-fold compared to the average spontaneous background of 1.15 SCEs/cell. Additionally, data were presented as the mean and standard error of the mean, which suggests wide variation in the data. The investigator also stated that cell cycle delay was observed at the high concentration (2 and 4 mg/embryo) and that the MI was significantly ($p<0.01$) reduced by both test materials at the high dose (4 and 8% reductions for the commercial and pure 2, 4-D, respectively). Based on these considerations, the values for SCE induction, cell cycling and MIs provide questionable evidence of a positive response. The study looks only at DNA damage (SCE), not mutagenic effects.

For the second study, Arias (2007) used the same purity of the test materials at similar concentrations and the same protocol with the exception of including a 7- and 10-day extended exposure time and increasing the sample size to 35 embryos/concentration. Results were similar to the previous study showing "significant ($p<0.05$)" increases in SCEs/cell at 4.0 mg/embryo (1.1-fold increase) at 4, 7 and 10 days for the commercial product and at 4.0 mg/embryo (1.1 fold-increase) at 7 and 10 days for pure 2,4-D. Similarly, slight but significant ($p<0.01$) effects were reported on cell cycle delay and the MI (≈23.3 for the commercial product and ≈23.1 for the pure substance at the high dose vs. 24 for acetone), which was not associated with prolonged exposure. Although these effects were statistically significant, the biological relevance is unclear.

Charles et al (1999a)
Ames Assays and Unscheduled DNA Synthesis Assays on 2,4-Dichlorophenoxyacetic Acid and its Derivatives.
[Bin: Mutagenicity]
[Cited: 2, 4-D Task Force; NCASI]
[Human Studies Considerations Apply: No]

Charles et al (1999a) reported the results of the independently conducted Ames test plate incorporation and the preincubation modification to the plate incorporation assay. Additionally, independently performed the *in vitro* primary rat hepatocyte unscheduled DNA synthesis (UDS) assay and the *in vivo* test procedure with hepatocyte treated with 2,4-D acid were investigated. For the Ames assay, *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, and TA100 were exposed to 5 to 7 dilutions of the test articles in the presence or absence of Aroclor 1254-induce rat liver homogenates. Note: TA 1538, which is considered a duplicate of TA98, was not used for all test articles. All bacterial assays were performed for each test agent with a high concentration in excess of the limit dose for this test system (5000 µg/plate) or up to a cytotoxic concentration; negative (solvent) and the appropriate positive controls were included. A minimum of triplicate plates per dose per strain per condition was also included in each assay. Representative results are presented in Table 1. As shown none of the eight test articles induced a mutagenic response in any of the tester strains at any test article concentration. All negative and positive controls responded in the expected manner. Accordingly, all bacterial assays were considered negative and fulfill the regulatory requirements for acceptable bacterial gene mutation assays. *In vitro* UDS assays were conducted with isolate primary rat hepatocytes exposed to the five to six test article dilutions using solubility or cytotoxicity as the starting concentration. Hepatocytes were doses for 18-20 hours and scored for net nuclear grain (NNG) counts and the percentage of cells with ≥5 NNG. Data from these assays can be seen in Table 1 and clearly show that neither 2, 4-D acid nor its salts or esters induced a genotoxic effect. By contrast, the positive control induced marked increases in NNG and the percentage of cells with ≥5 NNG. Accordingly, these assays are negative and fulfill the regulatory requirements for acceptable *in vitro* UDS assays.

Only 2, 4-D acid was tested in the *in vivo* UDS assay conducted with male Wistar Han rats administered single oral gavage doses of 400 and 1000 mg/kg. Five animals per dose received the selected test material doses, vehicle (1% methylcellulose) or positive controls and were sacrificed at 2-4 and 12-14 hours after dosing. Hepatocytes were recovered from three animals per group, cultured with [^{3}H] thymidine and scored for NNG. Dose selection was based on the findings of a preliminary range finding study with 0, 600, 800 or 1000 mg/kg. Clinical signs of toxicity in the range finding study were loss of balance and red stains on the nose and mouth "shortly after dosing" with 600 mg/kg. At 800 mg/kg, lethargy, abnormal breathing, unsteady gait and rigid body tone were seen. Similar signs were observed at 1000 mg/kg within 5 hours and two rats were killed in extremis after 3 days (Bus, 2009). Accordingly, 1000 mg/kg was selected as the maximum tolerated dose (MTD). Findings from the main test indicated that 2, 4-D did not increase the frequency of NNG at any dose or treatment time.

Charles et al (1999b)
In Vivo Micronucleus Assays on 2,4-Dichlorophenoxyacetic Acid and its Derivatives.
[Bin: Mutagenicity]
[Cited: 2, 4-D Task Force]
[Human Studies Considerations Apply: No]
The final publication in this series of Industry Task Force II Genetic Toxicology investigations on 2, 4-D details the *in vivo* mouse bone marrow micronucleus assays conducted with 2, 4-D (96.1%), 2, 4-D DEA (73.8%) 2, 4-D DMA (66.2%), 2, 4-D IPA (50.15%), 2, 4-D TIPA (70.9%), 2, 4-D BEE (95.6%), 2, 4-D EHE (98%), and 2, 4-D IPE (97.1%). Doses selected for the assays were based on the findings of mortality in groups of three male and three female administered 3 to 5 oral gavage doses of each agent. In the main study, groups of 5 male and 5 female mice/dose/sacrifice intervals received a single administration of three selected doses of each test agent via oral gavage. Animals were sacrificed at 24, 48 and 72 hours postdosing and bone marrow cells were harvested, processed, spread on to slides, stained and scored; there was no indication whether slides were coded prior to analysis. Appropriate vehicle and positive controls were included in each assay. The incidence of micronucleated polychromatic erythrocytes (MPCE) and the PCE to normochromatic (NCE) ratio were determined by analyzing 1000 PCEs/animal.

All test agents were examined up to the maximum tolerated dose (MTD), based on mortality; the generally comparable MTDs estimate for each test agent were:

- 400 mg/kg 2, 4-D
- 443 mg/kg 2, 4-D DEA
- 397 mg/kg 2, 4-D DMA
- 500 mg/kg 2, 4-D EHE

400 mg/kg 2, 4-D IPE
376 mg/kg 2, 4-D IPA
542 mg/kg 2, 4-D TIPA
375 mg/kg 2, 4-D BEE

Results of the individual micronuclei analysis indicated that neither 2, 4-D nor its seven derivatives were cytotoxic to the bone marrow since all PCE: NCE ratios were generally comparable to the vehicle control values. Similarly, none of the test treatments with 2, 4-D or its seven derivatives induced an aneugenic or clastogenic effect at any dose or sacrifice time in mouse bone marrow. Accordingly, the test agents were adequately tested and found to be negative in these acceptable assays that fulfill the regulatory requirements for *in vivo* mammalian erythrocyte micronucleus assays.
Figg et al., 2000
Increased lymphocyte replicative index following 2,4-dichlorophenoxyacetic acid herbicide exposure.
[Bin: Mutagenicity]
[Cited: NRDC Petition; 2, 4-D Task Force]
[Human Studies Considerations Apply: No]
Figg et al. (2000) found no evidence of micronuclei (MN) induction in the peripheral lymphocytes of 12 applicators spraying only 2, 4-D during a 6-month period. In this study, 12 applicators were compared to a control group consisting of 9 controls (matched for gender, age, alcohol and tobacco uses, general health and geographical location). Blood and urine samples were collected prior to spraying and at every other week after a typical day of 2,4-D application. The concentration of 2, 4-D in the collected urinary samples was determined. Lymphocyte cultures were grown for 44 hours, and Cytochalasin B was added to prevent complete cytokinesis. At 72 hours, lymphocytes were separated; slides were prepared, stained with antikinetochore antibody and coated with fluorescent anti-goat human IgG, counterstained with the DNA dye, 4, 6-diamidino-2-phenylindole, refrigerated for 1 week, coded and

scored. At least 1000 binucleated lymphocytes were scored for micronuclei; cell division kinetics was calculated for the count of 400 cells per sample (duplicate cultures). The replicative index (RI) was determined by calculating the percentage of cells with one, two, three or more nuclei per individual. Hematological end points such as hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, red cell count, white blood cell count and leukocyte differential examinations were also conducted. The 2, 4-D concentration 'grand mean among all applicators after spraying 2, 4-D was 240 ± 100 (±SE) ppb. The authors stated, "Urinary concentrations of 2, 4-D increased as hours spent spraying the herbicide increased." Consequently, the 12 individuals were grouped according to hours spent spraying 2, 4-D according to the following urinary concentrations: <20 ppb (4 subjects), 20-40 ppb (3 subjects) or > 40 ppb (5 subjects). Results of the lymphocyte analysis revealed that there was no significant increase in the incidence of micronuclei in the exposed workers. However, the replicative index (RI) for lymphocytes was significantly ($p<0.05$) higher in applicators compared to non-applicants and in applicators after spraying compared to before spraying ($p<0.016$). It was stated that the increase in RI suggests "stimulated cell growth that could contribute to carcinogenesis." Nevertheless, the authors concluded that their findings did not support a "genotoxic pathway" for 2, 4-D at mean urinary levels of 12 to 1285 ppb.

Filkowski et al. (2003)

Genotoxicity of 2,4-D and dicamba revealed by transgenic Arabidopsis thailiana plants harboring recombination and point mutation markers.

[Bin: Mutagenicity]

[Cited: Beyond Pesticides; NYS DOH]

[Human Studies Considerations Apply: No]

Filkowski et al. (2003) exposed transgenic seedlings of *Arabidopsis thaliana* to 0, 3, 10, 30, 100, 200 and 300 µg/L 2, 4- D (>98%) for 3 weeks under standard growth conditions; the number treated was not reported. Plants were collected, stained with 5-bromo-4-chloro-3-indolyl glucuronide, incubated for 48 hours and bleached with ethanol. Intrachromosomal homologous recombinations and point mutations were scored as blue sectors on white plants. It was indicated that "1000 'mutation'- and 200 'recombination'-monitoring plants were scored". The experiment was repeated three times. Data were only presented in graphs; thus variability in the data could not be ascertained. The mid-to low-doses of 2, 4-D (3, 10 and 30 µg/L) but not the high doses (100 and 300 µg/L) induced significant ($p<0.05$) increases in A → G point mutations; no explanation was provided for the lack of an effect at the high doses. Results also show that significant ($p<0.05$) increases in the induction of homologous recombinations (suggestive of DNA double strand breaks) occurred at all tested concentrations of 2, 4-D; a clear dose response was, however, not evident. The authors stated, "…the increase in the recombination frequency at low concentrations of 2, 4-D correlated with the increase in the levels of A → G point mutations".

Gollapudi et al., 1999
Evaluation of the genotoxicity of 2,4-dichlorophenoxyacetic acid and its derivatives in mammalian cell cultures.
[Bin: Mutagenicity]
[Cited: 2, 4-D Task Force]
[Human Studies Considerations Apply: No]
Gollapudi et al.(1999) continued the Industry Task Force II efforts on 2, 4-D and investigated several 2, 4-D derivatives [2, 4-D BEE (95.6%), 2, 4-D IPA (50.15%) and 2, 4-D TIPA (70.9%)] in mammalian cells performing the Chinese hamster ovary (CHO) cell forward gene mutation assay at the HGPRT locus and the rat lymphocyte *in vitro* cytogenetic assay. For the CHO/HGPRT assay, concentrations ranges of 150 to 1400µg/mL +/-S9 (2, 4-D BEE), 500 to 3000 µg/mL +/-S9 (2, 4-D IPA), or 800 to 5000 µg/mL +/-S9 (2, 4-D TIPA) were tested. Duplicate cultures per concentration were exposed to the selected test material doses and the appropriate solvent and positive controls for 4-5 hours, allowed a 7-9 day expression time, selected and plated for mutants and survivors. Each experiment was independently repeated at least once. The highest concentration of each test agents was selected to represent either to the limit dose of 5000 µg/mL for the test system (2, 4-D TIPA), the limit of solubility (2, 4-D BEE) or severe cytotoxicity (2, 4-D IPA +/-S9). None of the 2, 4-D derivatives induced a mutagenic effect in CHO cells in either the absence or presence of S9 activation at any concentration tested.
Gonzalez et al., 2005
Genotoxicity of the herbicide 2,4-dichlorophenoxyacetic and a commercial formulation, 2, 4-dichlorophenoxyacetic acid dimethylamine salt.
[Bin: Mutagenicity]
[Cited: NRDC Petition]
[Human Studies Considerations Apply: No]
Gonzalez et al. (2005) evaluated 2,4-D (purity not reported) and commercial grade 2,4-D DMA (containing 60.2% 2,4-D as the active ingredient) for the ability to cause DNA damage in CHO cells using the SCE and Comet tests. Concentration ranges of 2- 10 µg/mL were used in both assays in the absence of S9 activation; treatment times for the SCE assay were 24 and 36 hours and 90 minutes and 36 hours for the comet test. For both the SCE assay and the Comet test, duplicate cultures were prepared for each dose; three independent experiments were conducted; and cultures treated with the solvent acetone were included. The percentage of cells in M1, M2 and M3 division in 200 metaphases/sample, the proliferative rate and the MIs/2000 cells were determined. There were no significant differences for cell-cycle progression or alterations in the rate of cell proliferation. By contrast, 2, 4-D at 24 hours caused 25 and 35% reductions in the MI at 6 and 10 µg/mL; respectively, and 42 and 46% reductions at 6 and 10 µg/mL, respectively (36 hours). For 2, 4-D DMA, reduction of ~38 % (24 hours) and ~28% (36 hours) were recorded for both 6 and 10 µg/mL. SCE data were presented in graphs and showed significant ($p<0.01$) and dose-related increases in SCE/cell at all levels (2, 6 and10 µg/mL) for both test materials, regardless of time. The fold increase at the high dose was 1.5 and 1.8 X for 2,4-D at 24 and 36 hours, respectively and 1.6 and 2.1 X for 2,4-D DMA, at 24 and 36 hours, respectively. Similar evidence of DNA damage was seen in the comet test with significantly ($p<0.01$) increased and dose-related percentages of cells with tail comets, indicative of DNA damage, at 2.0, 6.0 and 10.0 µg/mL in the absence of severe cytotoxicity (≥85% viability) at 90 minutes. At 36 hours, regardless of the test material, all cells had tail comets. The Agency concludes in agreement with the investigators that these data provide clear and reproducible evidence of the DNA-damaging activity of 2, 4-D and the DMA derivative. However, DNA damage alone, whether in vitro or in vivo cannot be

interpreted as causal event leading to mutagenicity or other adverse outcomes in rodents or humans because of the ability of cells or whole animals to repair damage to DNA.

Holland et al., 2002

Micronucleus frequency and proliferation in human lymphocytes after exposure to herbicide 2,4-dichlorophenoxyacetic acid in vitro and in vivo.

[Bin: Mutagenicity]

[Cited: NRDC Petition]

[Human Studies Considerations Apply: Yes]

In the *in vitro* micronucleus assays of Holland et al. (2002), two experiments were conducted with either 2 or 5 volunteers (total of 3 females and 2 males, nonsmokers, 26- 45 years). Note: one of the subjects from the first test participated in the second test. Whole blood cultures as well as isolated lymphocyte assays were examined in the first test; the second test only used isolated lymphocytes. Collection of blood samples, culture preparation, treatment conditions and scoring of binucleated lymphocytes was conduced as described above in Figg et al. (2000). The only procedural exceptions were that lymphocytes smears were not stained with antikinetochore antibody or coated with anti-goat human IgG and MIs were determined from the count of 1000 cells/culture. In the first test, samples of whole blood and isolated lymphocytes were exposed *in vitro* to pure (98%) 2, 4-D at concentrations of 0.001-1.0 mM and analyzed for micronucleus induction. In the second test, only isolated lymphocytes were exposed to both pure 2, 4-D or commercial 2, 4-D (9.4%2, 4-D but adjusted to 98% pure 2, 4-D) at concentrations of 0.005 and 0.3 mM.

Cultures exposed to concentrations ranging from 0.001- 1.0 mM of pure 2, 4-D (98%) for 4 hours showed only "a minimal increase in MN…at doses approaching cytotoxic levels" in either whole blood or isolated lymphocytes. Similar findings were obtained for the commercial product at 0.3 mM 2, 4-D in isolated lymphocytes. However, the study authors conceded that these increases were within the "lower range of normal MN for healthy adults". RIs were significantly ($p<0.001$) decreased (~19%) at 0.3 mM of either pure or commercial 2, 4-D, regardless of the solvent (ethanol or culture medium) used. These data suggest a cytotoxic effect. Although Holland et al. (2002) claimed that 0.005 mM of commercial grade 2, 4-D induced an increase (nonsignificant) in the RI of isolated lymphocytes, an examination of the data in Table 1 of this paper revealed that the pooled average RI for both commercial 2,-4 –D groups (10 samples) was 1.65 vs. 1.62 for the pooled solvent control groups. This "increase" cannot be considered evidence of a proliferative response. Similarly, MIs were unaffected by treatment with 0.005 or 0.3 mM 2, 4-D (commercial or pure). Thus, the lack of a meaningful increased RI at this low dose is supported by data showing that MIs were unchanged. The overall conclusions are that 2, 4 –D, regardless of the formulation, did not induce micronuclei in human lymphocytes up to levels approaching cytotoxicity.

Madrigal-Bujaidar et al., (2001)

Induction of sister chromatid exchanges by 2,4dichlorophenoxyacetic acid in somatic and germ cells of mice exposed in vivo.

[Bin: Mutagenicity]

[Cited: NRDC Petition; 2, 4-D Task Force]

[Human Studies Considerations Apply: No]

In the study of Madrigal-Bujaidar et al. (2001), SCE induction in mouse bone marrow and spermatogonia were investigated. Groups of five male mice received oral doses of 0, 50, 100 or 200 mg/kg 2,4-D (98.95%) in alkalinized H_2O (pH 10.5) or the vehicle, followed at 2 hours by an intraperitoneal (ip) injection of BrdU/charcoal and at 21 hours by an ip injection of colchicine. Bone marrow cells were harvested, stained by the fluorescent-plus-Giemsa technique, and scored; it was not

reported whether slides were coded prior to scoring. Thirty second-division metaphases/animal were scored for SCE induction; the MIs, cell cycle delay and average generation time were also determined. For the examination of spermatogonial cells, groups of five male mice received the test material and BrdU/charcoal as described; cell division was halted by the subcutaneous injection of colchicine at 51 hours after BrdU administration and testes were harvested 4 hours later. Prepared slides were stained as described and 25 mitosis /animal were scored for SCEs. The presented data show that there were no adverse effects on the MI, cell cycle progression on the average generation time in the bone marrow cells. By contrast, significant ($p<0.05$) and dose-related increases in SCE induction (2-fold or approaching 2-fold increases) at 100 and 200 mg/kg were recorded for both cell types. It is also of note that the authors speculate that the fate of the genotoxic damage on the spermatogonial cells is unclear since "teratogenic and embryotoxic studies on rodents treated with 2,4-D have given conflicting results and the dominant lethal mutation assay on mouse and rats has produced negative data. Thus cell repair, cell lethality, and zygote damage remain as three possible fates of the observed genotoxic alterations in spermatogonia." However, these results should be viewed with caution since the extended 1-generation reproductive toxicity study in rats, designed to detect defects in sperm or poor reproductive performance in males, was negative. The Agency notes that similar speculations can be raised for the fate of DNA damage in bone marrow cells since chronic and oncogenic studies in rats and mice were negative. It is noted that this *in vivo* study only looked at a genotoxic, as opposed to a mutagenic, endpoint (sister chromatid exchange).

Maire et al. (2007)

2, 4-Dichlorophenoxyacetic acid: Effects on Syrian hamster embryo (SHE) cell transformation, c-Myc expression, DNA damage and apoptosis.

[Bin: Mutagenicity]

[Cited: NYS DOH]

[Human Studies Considerations Apply: No]

In a series of *in vitro* investigations, Maire et al. (2007) exposed Syrian hamster embryo (SHE) cells to 2, 4-D (98%) at concentrations of 0, 4.5, 11.5, 23 or 34 µM (equivalent to 0, 1, 2.5, 5 or 7.5 µg/mL, respectively) and evaluated the cultures for malignant cell transformation, apoptosis, expression of the *c-myc* gene and gene product, and DNA damage (Comet test). For the cell transformation assay, X-irradiated SHE feeder cells were seeded and grown for 25 hours. Target cells were seeded on top of the feeder cells, allowed a 24-hours period of quiescence and 10 dishes /concentration were treated with the selected test article levels of 2, 4-D for 7 days. At the end of exposure, dishes were washed, fixed and stained. Appropriate solvent (H_2O) and positive controls were included in each assay. One thousand colonies/concentration were counted and examined for morphology; cloning efficiency (CE) and the transformation frequency (TF) were determined.

For apoptosis, cells were also sampled 48 hours after seeding, trypinized, reseeded and treated with of 0, 4.5, 11.5 and 23 µM 2, 4-D for 5 and 24 hours. After treatment, cells were suspended in culture medium deprived of growth factors for 3 hours, harvested and centrifuged. Cell pellets were stained with fluorescent acridine orange and ethidium bromide, mixed, placed on slides, and scored for the number of apoptotic cells/100 cells/slide. Three slides/concentration were examined.

For the RT-PCR determination of gene expression, cells were again sampled 48 hours after seeding, trypinized, reseeded and treated with of 0, 4.5, 11.5 and 23 µM 2, 4-D for 5 hours. After treatment, total RNA was extracted and complementary DNA (c-DNA) was synthesized from 1µg of total RNA using oligo d (T) as a primer and reverse transcriptase. PCR amplification of the c-DNA was performed with *c-myc, bcl-2* and *bax* primers specific to SHE cells. Preparations were denatured and PCR products

were sequenced, cloned and electrophoresed. Signals were analyzed by densitometry and data were expressed as density ratios of the transcript corresponding to the *c-myc* gene. Three different experiments were conducted with different batches of SHE cells. Western blot analysis was conducted in parallel with RT-PCR to assess effects on *c-myc* protein.

For the comet assay, SHE cells were trypsinized 48 hours after seeding, reseeded and treated with 0, 4.5, 11.5, or 23µM 2, 4-D for 5 and 24 hours. At least three experiments were performed and included in each assay were the appropriate solvent and positive control. Cells were recovered and electrophoresed; nuclear DNA was stained with ethidium bromide and scored blind. Cells (75/slide/concentration) were scored randomly and the percentage of DNA in the tail was determined as a measure of DNA damage.

Results indicate that 2, 4-D induced a dose-dependent and significant ($p<0.01$) increases in morphological cell transformation of SHE cells at 11.5 (2.4%) and 23µM (3.8%); relative cloning efficiency at 11.5 and 23µM was 14%. No significant increase in transformed cells was observed at the high dose of 34 µM 2, 4-D. There were also no significant increases in apoptotic cells 5 or 24 hours after exposure to the selected doses of 2,4-D. These data are supported by the lack of an effect on expression of *bcl-2* and *bax* genes, which are associated with regulation of the apoptotic pathways. By contrast, a significant ($p<0.05$) increase in the up-regulation of *c-myc* RNA at 11.5 and 23 µM was reported; the increases represented 2.3-fold at these levels and immunoblotting confirmed the increase in the *c-myc* protein. Thus, 2, 4-D induced over expression of *c-myc* at both the RNA and the protein level; this finding is consistent with the transforming activity of 2, 4-D at comparable doses. Data from the comet test revealed that treatment with 11.5 and 23 µM for 5 or 24 hours also induced a dose-dependent and significant ($p<0.01$) increase in the number of DNA-damaged cells. No dead cells were found at any dose or harvest time.

Overall, the investigators concluded that 2, 4-D induces morphologic cell transformation in SHE cells at doses that extend into the cytotoxic range and caused overexpression of *c-myc* (at both the mRNA and the protein levels) and DNA damage. The Agency notes that the SHE morphologic transformation assay is an *in vitro* test for carcinogenesis, not for mutagenicity. Further, all of the findings reported by Maire *et al.,* (2007) were generated from *in vitro* studies. It is, therefore, not likely that these effects will be expressed *in vivo* because of the negative findings in acceptable/guideline chronic/carcinogenic bioassays in rats and mice for cancer detection.

Soloneski et al., 2007
Herbicide 2,4-dichlorophenoxyacetic acid (2, 4-D induced cytogenetic damage in human lymphocytes in vitro in presence of erythrocytes
[Bin: Mutagenicity]
[Cited: NRDC Petition]
[Human Studies Considerations Apply: No]

In another study of SCE induction, Soloneski et al. (2007) assayed 2, 4-D (purity not reported) and commercial grade 2, 4-D DMA (purity not listed) at levels of 10 to 100 µg/mL in human whole blood (WBC) and plasma leukocyte cultures (PLC). Treatment time was 72 hours. Duplicate cultures were prepared for each dose or the solvent control, acetone and three independent experiments were conducted. Smears were prepared and stained as previously described and metaphases were scored blind for cell cycle progression, MIs and SCE induction. Results show that both test materials significantly (generally $p<0.05$) affected cell cycle progression in WBCs at doses ≥ 25 µg/mL (note: the 100-µg/mL group was not scored for 2, 4-D) and only at 100 µg/mL (2, 4-D) for PLCs. Mean SCE frequencies were also significantly ($p\leq0.05$) increased at 10, 25 and 50 µg/mL (2, 4-D) and at 25, 50 and 100 µg/mL

(2, 4-D DMA) when WBCs were tested. No activity was seen at any dose when PLCs were examined. Significant reductions in the proliferative rates and the MIs were found in WBCs and PLCs after treatment with 50 or 100 µg/mL 2, 4-D, respectively. For 2, 4-D DMA reduced proliferative rates and MIs were noted at the high dose but only in WBC. The discrepancies in the data between WBCs and PLCs were considered by the authors to be related to the detoxificating properties of erythrocytes, which are present in the PLC cultures but not the purified leukocyte cultures. The authors, citing several investigators, further speculated that the cellular response caused by both test agents in this study could be related to oxidative damage since lipid peroxidation has been reported for 2,4-D.

Tripathy et al. (1993)

Genotoxicity of 2,4-dichlorophenoxyacetic acid tested in somatic and germ-line cells of Drosophila

[Bin: Mutagenicity]

[Cited: NRDC Petition]

[Human Studies Considerations Apply: No]

In a *Drosophila melanogaster* wing spot test and sex-linked recessive lethal assay, Tripathy et al. (1993), exposed second- and third- instar *mwh* + / + fl^3 transheterozygous larvae to 2.5, 5.0, 10.0 mM 2, 4-D (98%) prepared in 3% ethanol + 1% Tween 80 and incorporated into the feeding medium for 48 and 72 hours (i.e., "the rest of their larval life"); the high dose represents 50% of the $LD_{50.}$ Following exposure, 40 wings/treatment or control group of "eclosed" flies (i.e., flies that have wings that are highly folded and compact; within an hour, each wing expands) were examined for the type and number of spots. Duplicate experiments were performed; the data were pooled because there were no statistically significant differences among experiments.

In the sex-linked recessive lethal assay, Oregon-R larvae were fed diets containing comparable doses of 2, 4-D as described for 3 days; male flies were mated to 3 *Basc* virgin females for 3 days to obtain the F_1 *Basc/*Y siblings (Brood 1). Brood 1-males and females were mated (1:1) to produce Brood 2 (Brood 2 represents the sampling of germ cells that were primarily late or middle spermatids). X chromosomes from 60-65 males/test group (~1230-1330 X chromosomes/test group) and 150 pooled vehicle controls (3130 X chromosomes) were scored; the X-linked recessive lethals were calculated. Duplicate experiments were performed; the data were pooled because there were no statistically significant differences among experiments.

Results indicated that after 48 hours of exposure to 5.0 and 10.0 mM 2, 4-D, a significant ($p<0.05$) and dose-related increase in the number of spots/per wing (small single and large single wing spots) as well as the total spots was induced. Following 72 hours of exposure, significant increases were obtained only at the high dose for small single and large single and twin wing spots and total spots. The low dose (2.5 mM) was not active. Based on these data, it was concluded that 2, 4-D induced somatic cell genotoxic damage in the primordial wing cells of *Drosophila*.

Significant ($p<0.05$) and dose-related increases in the lethal frequencies were calculated at 5.0 and 10.0 mM 2, 4-D (48-hour exposure) and at all doses (72-hour exposure). Accordingly, the authors concluded that in addition to somatic cell damage, 2, 4-D is also genotoxic in the male germinal cells of *Drosophila*. The Agency notes that positive findings with D. melanogaster studies have not received the same degree of confidence as assays that are part of the regulatory test battery for mutagenicity. It is further doubtful that these data would contribute much to a weight-of-the-evidence approach for the mutagenicity of 2-4-D because their relevance to human health is not clear.

Tuschl and Schwab (2003)
Cytotoxic effects of the herbicide 2, 4-dichlorophenoxyacetic acid in HepG2 cells.
[Bin: Mutagenicity]
[Cited: NRDC Petition]
[Human Studies Considerations Apply: No]
Tuschl and Schwab (2003) found that exposure of the human hepatoma cell line, HepG2 to 2, 4-D (purity no stated) in 1% ethanol induces apoptosis but only at high doses (4, 8 or 16 mM; equivalent to 0.9, 1.8 or 3.6 mg/mL) that induced severe effects on cell cycling, increased necrotic cells and increased breakdown of mitochondrial membranes. The data in this article were only presented in graphic form. It is of note, however, that these doses are ≈ 1000X higher than the doses reported by Maire et al. (2007). The relevance of the findings is unclear because of severe cytotoxicity at all levels. Studies on cytotoxicity alone do not provide evidence of genotoxicity.
Venkov et al. (1993)
Genotoxicity of 2,4-dichlorophenoxyacetic acid tested in somatic and germ-line cells of Drosophila.
[Bin: Mutagenicity]
[Cited: NRDC petition]
[Human Studies Considerations Apply: No]
In this assay, Venkov et al. (2000) exposed *Saccharomyces cerevisiae* D7*ts1* to 2, 4-D (100%) test concentrations at 4, 8, or 16 mM (~900, 1800 or 3600 µg/mL, respectively) for 1 to 8 hours; cells were recovered and plated for survival and the reversion and mitotic gene conversion rates were calculated. Additionally, 100% pure phenoxyacetic acid, 4-chlorophenoxyacetic acid and 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T) were tested in this yeast assay following a 4-hour treatment. Results show that 2, 4-D at 8 mM induced gene mutations and gene conversions at levels causing ~40% reduction in cell survival. The effect was time and dose dependent with the peak response occurring after 8-hours of treatment with 8 mM. No evidence of genetic activity was seen for phenoxyacetic acid or 2,4, 5-T; 4-chlorophenoxyacetic acid at 16 mM induced mitotic gene conversion but did not increase reversion to isoleucine protrotrophy.

These investigators also examined the cytotoxic effects of 2, 4-D (16, 160 and 1600 µM) and 2, 4, 5-T (16, 160 and 1600 µM) in human transformed hematopoietic cells lines (LAMA-84 cells, derived from chronic myelogenous leukemia cells and U-937 cells, derived from histocytic lymphoma cells). Both test agents were 100% lethal at the high concentration. At 160 µM 2, 4-D, cell survival was 13.4 and 52.5 % for the LAMA-84 and U-937 cell lines, respectively. For the lowest dose of 2, 4-D, survival for both cell lines was ≥ 89%. The data indicate that 2, 4-D was slightly more cytotoxic than 2, 4, 5-T since cell survival at 160 µM 2, 4, 5-T was 41 and 53% for the LAMA-84 and U-937 cell lines, respectively. Based on these findings, the investigators concluded that 2, 4-D is cytotoxic, mutagenic and recombinogenic in yeast and cytotoxic in transformed human cells. Data on the cytogenetic analysis of C57b1 male and female mice is discussed under the section on chromosome aberrations and micronucleus induction. The Agency notes that this is an *in vitro* test for carcinogenicity, not a test for mutagenicity. The results are not consistent with the tumor profile for 2, 4-D.

Venkov et al. (2000)
Genotoxicity of 2,4-dichlorophenoxyacetic acid tested in somatic and germ-line cells of Drosophila.
[Bin: Mutagenicity]
[Cited: 2, 4-D Task Force]
[Human Studies Considerations Apply: No]
As part of the analysis of 2, 4-D and other substituted phenoxyacetic acids in yeast and cytotoxicity assays in human transformed hematopoietic cells (see Section, Assays in Yeast, Plants and Insects), Venkov et al. (2000) also investigate 100% pure 2, 4-D and 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T) for the induction of structural chromosome aberrations following single ip administrations of both agents at 3.5 and 4.0 mg/kg, respectively. Accordingly, bone marrow cells were harvested from groups of four male and four female mice at 12 and 24 hours postdosing. The appropriate vehicle (0.9% NaCl) and positive controls were included. At least 100 metaphases per animal were scored for aberrant structural morphology and the MIs were determined from the count of 1000 cells per animal. It was not stated whether slides were coded prior to analysis. 2, 4-D at 3.5 mg/kg induced significant ($p<0.0.5$) increases in the frequency of cells with aberrations at both sacrifice times (3.4 and 5.5% vs. 1.5 % for the vehicle control at 12 and 24 hours, respectively); the predominant type of aberration induced by 2,4-D was chromatid breaks, suggesting cytotoxicity, and exchanges suggesting clastogenicity. 2, 4-D also reduced the MIs by 69% at 12 hours and 35% at 24 hours. By contrast, 2, 4, 5-T was neither cytotoxic nor clastogenic in bone marrow cells harvested 12 postexposure to 4.0 mg/kg. Based on the findings from this investigation, the authors concluded that 2, 4-D was clastogenic in mouse bone marrow cells when administered via ip injection.

Zeljezic and Garaj-Vrhovac, (2004)
Chromosomal aberrations, micronuclei and nuclear buds induced in human lymphocytes by 2, 4-dichlorophenoxyacetic acid pesticide formulation.
[Bin: Mutagenicity]
[Cited: NRDC Petition; 2, 4-D Task Force]
[Human Studies Considerations Apply: No]
Zeljezic and Garaj-Vrhovac (2004) exposed human lymphocytes, harvested from two young, healthy non-smoking volunteers, to Deherban A®, a commercial formulation of 2, 4-D adjusted to contain 0.4 or 4 µg/mL 2, 4-D in the absence and the presence of S9. The S9 fraction was derived from the livers of male Sprague-Dawley rats treated with intraperitoneal (ip) injections of Deherban A® adjusted to contain 500 mg/mL 2, 4-D for 3 consecutive days. Lymphocytes were treated for 24 hours, recovered, processed, stained with Giemsa and scored (1000 metaphases/concentration) for chromosome aberrations. Similar cultures were treated for 24 hours with 0.4 or 4 µg/mL 2, 4-D +/-S9; cytochalasin B was added and cells were harvested, processed, and stained as previously described for the *in vitro* micronucleus assay. Cells were scored (1000 binucleated lymphocytes/concentration) for micronuclei and nuclear buds. Appropriate solvent and positive control cultures were included. The article did not indicate whether slides were coded to eliminate bias prior to scoring.

Both concentrations of 2, 4-D induced statistically significant ($p<0.05$) increases in the percentage of cells with aberrant chromosome aberrations (primarily chromatid and chromosome breaks) in lymphocytes from both donors. The number of micronuclei and nuclear buds at both nonactivated and S9-activated test material concentrations was also significantly increased ($p<0.05$) at 0.4 and 4 µg/mL +/-S9 in cells also harvested from two different donors. The cytogenetic, micronuclei and nuclear bud responses were uniformly and significantly ($p<0.05$) higher in cells treated in the presence of S9 at both test levels. Hence, it can be concluded that S9 activation was not required to demonstrate the

clastogenic effect induced by 2, 4-D in this study.

Hazard Data Summary: Studies of several types and designs were cited in the NRDC petition on 2, 4-D and various related documents (see attached for detailed review of each citation). These included subcutaneous and intraperitoneal exposures to maternal animals with resulting gestational and/or lactational exposure of the offspring; subcutaneous exposure of offspring, and oral exposures to the maternal rat and/or offspring. Potential effects identified in these studies included effects on the endocrine system, including the thyroid, estrogenic and androgenic effects, developmental neurotoxicity and effects on neurotransmitters (dopamine and serotonin), myelination, and maternal behavior. Overall, these studies do not identify any hazard not already identified by the Agency in its assessment. Additionally, the studies cited by NRDC were performed at dose levels above the saturation level for renal clearance (i.e., doses that overwhelm the body's ability to excrete 2, 4-D). Further, in many cases, the studies were performed at only one dose level, which does not provide dose-response data; important in determining cause and effect. A series of doses provides information showing that with an increase in dose, there's an increase in response (magnitude of effect and/or severity of effect). Other citations were *in vitro* endocrine screening assays, many of which have been included in the Endocrine Disruptor Review Team assessment of 2, 4-D (see TXR No. 0055461). These citations were submitted in support of concern for the potential of 2, 4-D to interact with the estrogen, androgen and thyroid hormone systems. All totaled, the results of the cited studies do not yield information that would cause the Agency to modify its conclusions from the 2005 Reregistration Eligibility Document (RED) for 2,4-D (EPA 738-R-05-002) that pertain to hazard.

As required in the RED, and in response to the data call-in, the 2,4-D Task Force has submitted an F1-extended 1-generation reproductive toxicity study on 2,4-D (MRID 47417901), which provides a comprehensive evaluation of the adult rat reproductive systems, including an evaluation of gonadal function, the estrous cycle, sperm parameters, mating performance, conception, gestation, parturition and lactation, and selected systemic toxicity parameters, as well as survival, growth and development of the offspring. A separate satellite group of dams was included for assessments of gross pathology, clinical pathology, thyroid hormones, toxicokinetics, and selected reproductive and systemic parameters during gestation. F1 offspring were evaluated for potential effects on the developing nervous system, immune system, reproductive and endocrine systems, thyroid function, and other systemic toxicity parameters. This study has been evaluated (TXR Nos. 0055341 and 0050697) and is classified acceptable/non-guideline.

All of the effects identified in the cited studies have been considered in conjunction with the results from the rat extended 1-generation reproductive toxicity study on 2, 4-D (MRID 47972101), which evaluated the potential of 2, 4-D to affect the various endpoints identified by NRDC as potential adverse effects following 2, 4-D exposure. Regarding concerns expressed in the petition with respect to endocrine disrupting potential, the F1 extended 1-generation reproductive toxicity study on 2, 4-D evaluated thyroid hormones (T3, T4 and TSH), thyroid weight and histopathology across life stages, and also evaluated auditory startle and brain morphometry data, which have been reported to be sensitive to thyroid disruption, in the DNT component of the study. Parameters sensitive to anti-androgenicity or androgenicity (balano-preputial separation, anogenital distance, quantitative F1 nipple retention, spermiology, organ weights and detailed histopathological evaluations of male reproductive organs) and estrogen sensitive parameters (estrous cyclicity assessment in parental females and F1 offspring, time to vaginal opening including assessment of vaginal thread retention, anogenital distance, quantitative ovarian follicle count in F1 females, reproductive organ weight evaluations and detailed histopathology

of female reproductive organs) were evaluated. There were no adverse effects observed on any of the numerous thyroid parameters, estrogen-sensitive parameters, and anti-androgenicity or androgenicity parameters evaluated in the extended 1-generation reproductive toxicity study, and the NOAEL was established at 45 mg/kg/day in males and 40 mg/kg/day in females. Both NOAELs are greater than the PODs utilized for the human health risk assessment supporting the 2, 4-D RED.

Regarding concerns expressed in the petition with respect to developmental neurotoxicity, in the extended 1-generation reproductive toxicity study on 2, 4-D, offspring were exposed during gestation, lactation (*via* the milk), and directly (via the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity, and acoustic startle response (ASR). An evaluation of myelin was performed on the PND 22 and PND 60 offspring, and the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. At the highest dose tested (82 mg/kg bw/day in males/59 mg/kg bw/day in females), there was no evidence of developmental neurotoxicity and no adverse effect on myelination at any dose level. Both NOAELs are greater than the PODs utilized for the human health risk assessment supporting the 2, 4-D RED.

Regarding concerns expressed in the petition with respect to mutagenicity/genotoxicity, based on a review of the cited studies on mutagenicity and the rather extensive Genetic Toxicology database on 2, 4-D, the only conclusion that can be reached with assurance is that 2, 4-D induces DNA damage both *in vitro* and *in vivo* and gene mutations and/or chromosome mutations in yeast, plants and insects. However, DNA damage alone, whether *in vitro* or *in vivo,* cannot be interpreted as a causal event leading to mutagenicity or other adverse outcomes in rodents or humans because the ability of cells or whole animals to repair damage to DNA cannot be overlooked. Between the *in vitro* and *in vivo* tests, the latter carry the greater weight in assessing mutagenic potential because *in vitro* tests do not capture how a living body responds to a toxic insult, including its ability to detoxify putative mutagens and genotoxicants.

The F1-extended one-generation reproductive toxicity study evaluated thyroid hormones (T3, T4 and TSH), thyroid weight, and histopathology across life stages, and also evaluated auditory startle and brain morphometry data. Exposure related effects in females in the F1-extended one-generation study were limited to non-statistically significant decreases in T3 and T4 and increased TSH, associated with adaptive changes (depleted colloid) in the thyroid follicles of GD 17 females at a dose that clearly exceeded the threshold for saturation of renal clearance. No robust effects on auditory startle or statistically significant brain morphometric changes were seen in the DNT component of the extended one-generation reproductive toxicity study. Myelin deposition was also characterized at PND 22 and 60, and no exposure-related changes were observed. Overall, there are no findings suggesting an effect on the thyroid at doses below the saturation threshold for renal clearance. There were no adverse effects observed on any of the numerous thyroid parameters evaluated in the extended 1-generation reproductive study, and the NOAEL was established at 45 mg/kg/day in males and 40 mg/kg/day in females. Both NOAELs are greater than the PODs utilized for the human health risk assessment supporting the 2, 4-D RED. In summary, several studies were cited in the NRDC petition and the associated documents. Most were cited correctly as reported by the investigators, but the overall results have little or no impact on the hazard assessment conclusions that have been developed by the Agency for 2,4-D. None of the issues of concern identified in the NRDC petition identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. Additionally, the

results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. The results of the extended 1-generation reproductive toxicity study support the previously selected PODs utilized for the human health risk assessment supporting the 2, 4-D RED.

An exposure pathway identified in the NRDC petition is lactational exposure to 2, 4-D. The Agency currently is addressing the issue of a lack of information regarding the levels of 2, 4-D that can occur in human breast milk. The studies cited by NRDC established effects that occurred at very high dose levels that exceed renal clearance capabilities. This allowed investigators to examine effects associated with 2,4-D exposure in order to postulate mechanisms of action and to guide future research. In fact, the information contained in these studies informed the design of the recent extended 1-generation reproduction study in rats. The extended 1-generation reproductive toxicity study more definitively evaluated the impacts of lactational exposure of treated offspring and, as such, is considered a more definitive source for evaluating these phenomena. Along with being accounted for in the hazard characterization of 2, 4-D, the potential for dietary consumption of 2, 4-D from milk has been adequately considered. Residues in all manner of milk containing food forms (e.g., milk fat, nonfat milk solids, etc.) have been accounted for in the dietary exposure assessment based on monitoring data from the USDA Pesticide Data Program (PDP). There were no detections of 2,4-D in any samples so one half of the detection limit was used as the basis for all analyses and the most highly exposed group (children 1-2 years old) in the analysis accounted for only 33 and 8.5 percent of the acute and chronic levels of concern, respectively, when all dietary consumption was considered. This finding indicates that residues in the actual food supply would have to be much greater to be of concern. Further, exposures from human breast milk are expected to be lower than from cow's milk because residues in cow's milk are expected to greatly exceed the values that would be present in human breast milk because exposure of dairy cattle to pesticides are generally significantly higher than humans (i.e., tolerances for grass (hay) and grass (forage) for 2,4-D are 300 and 360 ppm, respectively, while the 2,4-D tolerances for various human foods are all much lower in the single digits or less than 1 ppm as described in 40 CFR 180.142). There are uncertainties associated with attempting to characterize exposures from breastmilk. In order to address these EPA is seeking monitoring data for 2, 4-D in human breastmilk (e.g., EPA's National Children's Study).

NRDC also discussed factors that can impact dermal absorption and cited several studies, which EPA reviewed as well as those provided by other commenters. These studies examined the effects of alcohol consumption in conjunction with topical sunscreen and/or DEET application on dermal absorption of 2,4-D. They also discussed the impact of occlusion on absorption. Several human dermal absorption studies were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A value of 10% was used by the Agency based on these data and given the variability noted in the results. Additionally, a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies) were applied. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective. It is to be noted that although a dermal absorption value from human data was used, the full 10X UF_H was retained to account for uncertainties associated with the relative sensitivity to a pesticide within a population. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not underestimate dermal risk and is adequately protective.

4.0 Overview of Exposure/Epidemiological Data

A summary of the available exposure data is included below. In some cases specific papers were utilized in the NRDC petition itself and in one or more subsequent, related comments. In other cases citations were utilized only in single comments. For clarity, this section summarizes the actions associated with each citation and the results are presented in alphabetical order based on the name of the primary author. Additionally, it is noted where each citation has been used.

Alexander et al (2007)
Biomonitoring of 2,4-dichlorophenoxyacetic acid exposure and dose in farm families
[Bin: Population Based Monitoring]
[Cited: 2,4-D Task Force]
[Human Studies Considerations Apply: Yes]
This study is part of the industry FFES (Farm Family Exposure Study) conducted by industry to compare with the Agricultural Health Study conducted by the NIH. Families were evaluated in South Carolina and Minnesota using similar methods to the Ag Health Study. The use of the results of this study in the 2,4-D Task Force Comments on the NRDC Petition appears reasonable and consistent with other studies of this type. The investigators reported urine samples were collected for 3 days after 2,4-D application in both applicators and their family members. Median urine 2,4-D concentrations at baseline and day after application were 2.1 and 73.1 µg/L for applicators, below the limit of detection, and 1.2 µg/L for spouses, and 1.5 and 2.9 µg/L for children. The geometric mean systemic doses (micrograms per kilogram body weight) were 2.46 (applicators), 0.8 (spouses), 0.22 (all children), 0.32 (children 4–11 years of age), and 0.12 (children ≥ 12 years of age). The 2,4-D task force indicated EPA estimates of exposure were reasonable and cited the systemic dose values reported by the investigators.

Arbuckle et al (2002)
Predictors of herbicide exposure in farm applicators.
[Bin: Population Based Monitoring]
[Cited: 2,4-D Task Force]
[Human Studies Considerations Apply: Yes]
This study monitored a subset of participants in a retrospective cohort study of Ontario farm families. Immediately prior to and after handling phenoxy herbicides (including 2,4-D) for the first time during the season, 126 applicators provided pre-exposure spot urine samples and a subsequent 24 hour urine sample. They also completed a questionnaire on pesticide use and handling practices for the 1st day of application. The validity of the questionnaire as a predictor of exposure was examined. The arithmetic means for 2,4-D in urine were 27.6 and 2.6 µg/L for applicators and those not involved in application, respectively. The maximum reported 2,4-D concentrations were 410.0 and 66.0 µg/L for applicators and those not involved in application, respectively. Assuming that the presence of 2,4-D in the urine was a measure of true exposure and that questionnaire indications of 2,4-D use were the exposure classification subject to error, then the questionnaire's prediction of exposure had a sensitivity of 56.7% and specificity of 86.4%. Investigators also indicated the only protective factor identified in the 2,4-D multivariate model was the wearing of rubber gloves during mixing or loading of the herbicide. The 2,4-D task force indicated the use of gloves dramatically reduces exposure which is consistent with the conclusions of the investigators even when no precautions were taken regarding glove condition.

Figgs et al (2000)
Increased lymphocyte replicative index following 2,4-dichlorophenoxyacetic acid herbicide exposure
[Bin: Epidemiology & Population Based Monitoring]

[Cited: 2,4-D Task Force]
[Human Studies Considerations Apply: Yes]
The objective of this study was to evaluate peripheral blood lymphocyte proliferation (replicative index: RI) and micronuclei frequency (MF) among 2,4-D herbicide applicators. Twelve applicators (one 17 years old) spraying only 2,4-D provided a blood and urine specimen upon enrollment, several urine samples during the spraying season, and a blood specimen at the end of the study. Nine controls also provided blood and urine specimens. Standard methods were used to quantify impacts and 2,4-D residues. Applicator RI and MF were compared before and after spraying and with controls. Applicators contributed 45 urine specimens and concentrations ranged from 1.0 to 1700 µg 2,4-D/g creatinine/L urine. Applicator RI increased after spraying (p=0.016), independent of tobacco and alcohol use, and demonstrated a weak dose-response with increasing urinary 2,4-D levels (p=0.15). Among 2,4-D applicators pre-exposure complete blood counts and lymphocyte immunophenotypes were not significantly different from post-exposure measurements. Investigators concluded urinary 2,4-D concentration may be associated with lymphocyte replicative index, a cell proliferation biomarker. Investigators also indicated the results of this study do not support a genotoxic pathway (see additional review under Mutagenicity above, page 43) . In the NRDC petition, this study is cited in a manner that reflects the conclusions drawn by the investigators except it was not explicitly stated by the investigators that there is an increased risk of chronic toxicity due to the apparent exceedance of clearance mechanisms. The 2,4-D task force also cited this study correctly but included characterization elements which were not raised by NRDC including indicating the authors described the study as preliminary, pilot investigation, and the first to show a relationship between RI and urine levels. The task force also cited the investigator indication that the link between 2,4-D function and lymphocyte replicative index is unclear and that the mechanism, our reports and other recent investigations do not support a genotoxic pathway.

Garabrant et al (2002)
Review of 2,4-dichlorophenoxyacetic acid (2,4-D) Epidemiology and Toxicology
[Bin: Epidemiology]
[Cited: 2,4-D Task Force Comments]
[Human Studies Considerations Apply: Yes]
A literature review was completed using a variety of animal data and epidemiological evidence to determine if 2,4-D or its salts or esters damage DNA under physiologic conditions or induce oncogenic or carcinogenic effects. Investigators concluded overall the available evidence from epidemiologic studies is not adequate to conclude that any form of cancer is causally associated with 2,4-D exposure. There is no human evidence of adverse reproductive outcomes related to 2,4-D. The available data from animal studies of acute, subchronic, and chronic exposure to 2,4-D, its salts, and esters show an unequivocal lack of systemic toxicity at doses that do not exceed renal clearance mechanisms. There is no evidence that 2,4-D in any of its forms activates or transforms the immune system in animals at any dose. At high doses, 2,4-D damages the liver and kidney and irritates mucous membranes. Although myotonia and alterations in gait and behavioral indices are observed after overwhelming doses of 2,4-D in the microgram/kg/day range, it is unlikely that 2,4-D has any neurotoxic potential at doses below those required to induce systemic toxicity. This study was conducted for the 2,4-D Industry Task Force II on Research Data to provide an update of the evidence on human toxicity and cancer risks related to 2,4-D on research completed between 1995 and 2001. The use of the results of this study in the Task Force Comments on the Petition appears reasonable. No environmental media sampling or direct exposure monitoring from the considered studies were reported.

Garry et al (2002)
Birth Defects, Season of Conception, and Sex of Children Born to Pesticide Applicators Living in the

Red River Valley of Minnesota, USA
[Bin: Epidemiology]
[Cited: 2,4-D Task Force]
[Human Studies Considerations Apply: Yes]
This study was a cross-sectional study of 695 families and 1,532 children conducted during 1997 to 1998 in the Red River Valley of Minnesota. This study was a follow up to a previous study conducted in 1989-1991. Birth defects, season of conception and the sex of children born to pesticide applicators were evaluated. The investigators concluded that the present and previous studies point out that *a*) herbicides applied in the spring may be a factor in the birth defects observed and *b*) fungicides can be a significant factor in the determination of sex of the children of the families of the RRV. They also indicated use of the phosphonamino herbicides (glyphosate, Roundup) was overrepresented in the adverse birth and developmental effect group. Forty-three percent of the children (6 of 14) who had parent-reported ADD/ADHD used phosphonamino herbicides (OR = 3.6; CI, 1.35–9.65). No other commonly used pesticide compared by major organ and/or functional system was uniquely associated with specific adverse birth or developmental effects including the phenoxy acids. The 2,4-D task force indicated the results of this study showed no statistically significant correlations between 2,4-D use and excess adverse birth or neurodevelopmental effects which is consistent with the conclusions made by the investigators.
Garry et al (1996)
Pesticide Appliers, Biocides, and Birth Defects in Rural Minnesota
[Bin: Epidemiology]
[Cited: NRDC Petition; Beyond Pesticides; 2,4-D Task Force]
[Human Studies Considerations Apply: Yes]
This study evaluated the hypothesis that offspring of pesticide appliers might have increased risks of birth anomalies. This was investigated by linking 4,935 births to 34,772 state-licensed, private pesticide appliers in Minnesota occurring between 1989 and 1992 to the Minnesota state birth registry containing 210,723 live births in the same timeframe. Western Minnesota, a major wheat, sugar beet, and potato growing region, showed the highest rate of birth anomalies per 1000 live births: 30.0 for private appliers versus 26.9 for the general population of the same region. The highest frequency of use of chlorophenoxy herbicides and fungicides also occurred in western Minnesota. Births in the general population of western Minnesota showed a significant increase in birth anomalies in the same three birth anomaly categories as appliers and for central nervous system anomalies. This increase was most pronounced for infants conceived in the spring. The seasonal effect did not occur in other regions. The male/female sex ratio for the four birth anomaly categories of interest in high phenoxy herbicide/fungicide use is 2.8 for appliers versus 1.5 for the general population of the same region (p=0.05). The use of the results of this study in the NRDC Petition and in the comments from Beyond Pesticides reflect the specific conclusions described in the study but NRDC and Beyond Pesticides ignored the comments provided in later publications by the same investigator which were cited by the 2,4-D task force comments on the petition which generally indicate lower incidence rates. The 2,4-D task force commented the study of Garry and co-workers (1996) assessing potential associations of herbicide use and birth defect outcomes in an agricultural region of Minnesota was later described by this same research team as "a first effort" that suffered from a "limited reporting timeframe" (Garry et.al., 2002). In the latter report, the 2,4-D task force indicated the authors concluded that a more detailed cross-sectional analysis of this area showed no statistically significant correlations between 2,4-D use and excess adverse birth or neurodevelopmental effects.
Garry et al (2001)
Biomarker Correlations of Urinary 2,4-D Levels in Foresters: Genomic Instability and Endocrine

Disruption
[Bin: Population Based Monitoring & Epidemiology]
[Cited: Beyond Pesticides]
[Human Studies Considerations Apply: Yes]
Forest pesticide applicators were selected for consideration in this study of chlorophenoxy herbicides. Aberrations in G-banded chromosomes, reproductive hormone levels, and polymerase chain reaction-based V(D)J rearrangement frequencies were examined in these applicators whose exposures were mostly limited to chlorophenoxy herbicides. Data from appliers where chlorophenoxy use was less frequent were also examined. The biomarker outcome data were compared to urinary levels of 2,4-dichlorophenoxyacetic acid (2,4-D) obtained at the time of maximum 2,4-D use. Further comparisons of outcome data were made to the total volume of herbicides applied during the entire pesticide-use season. Twenty-four applicators and 15 minimally exposed foresters (control) subjects were studied. Categorized by applicator method, men who used a hand-held, backpack sprayer in their applications showed the highest average level (453.6 ppb) of 2,4-D in urine. Investigators concluded that herbicide applicators with high urinary levels of 2,4-D (backpack and boom spray applications) exhibited elevated LH levels. They also indicated workers exhibited altered genomic stability as measured by V(D)J rearrangement frequency, which appears reversible months after peak exposure. Finally investigators commented though highly detailed, the limited sample size warrants cautious interpretation of the data. The use of the results of this study in the comments from Beyond Pesticides appears to be reasonable and it reflects the specific conclusions described by the investigators (i.e., Garry "found a direct correlation of urinary levels of 2,4-D with serum levels of luteinizing hormone (LH) and high testosterone levels at the time of highest exposure suggest a direct effect on hormonal levels by the chlorophenoxy herbicide).
Grover et al (1986)
Factors Affecting the Exposure of Ground-rig Applicators to 2,4-D Dimethylamine Salt
[Bin: Planned Exposure Monitoring]
[Cited: 2,4-D Task Force]
[Human Studies Considerations Apply: Yes]
A total of 30 individual spray operations using 2,4-D dimethylamine salt were monitored. These operations involved farmers carrying out spray operations with tractor-drawn ground rigs, involving handling, transferring, mixing, and applying to wheat. Air sampling using a typical personal sampling pump method was used. Dermal exposure was measured using patches and a handwash method. Patches were worn under 2 work clothing and on the exterior. Some applicators wore protective gloves. Most applicators in the study used tractors with no cabs but 13 of 30 had some sort of enclosure. Urine was also collected and analyzed for 2,4-D residues. A total of nine subjects participated in the research and all but one were monitored multiple times with one worker being monitored during 7 spray operations. Hand exposures accounted for 80 to 90 percent of the potential cumulative exposure. The 2,4-D on the rest of the body ranged from 10 to 20 percent of the potential cumulative exposure. Urinary 2,4-D excretion accounted for 1 to 2 percent of the potential cumulative exposure. The median air concentration was 2.1 $\mu g/m^3$ with a range of <0.01 to 101 $\mu g/m^3$. Dermal (non-hand) dose values had a median of 27.9 µg/kg BW with a minimum of 7.5 µg/kg BW and a maximum of 278 µg/kg BW. Hand dose values had a median of 120 µg/kg BW with a minimum of 0.1 µg/kg BW and a maximum of 552 µg/kg BW. There is no mention in the study of consideration by an institutional review board. This study was conducted prior to the initiation of the Pesticide Handlers Exposure Database (PHED). The use of the results of this study in the comments from the 2,4-D task force appears to be reasonable and it reflects the specific conclusions described by the investigators. Direct exposure monitoring was reported and could be considered for quantitative use. However, it should be noted that PHED is currently used

for routine assessments of this type rather than individual studies.

Hardell, et al (1999)

A Case–Control Study of Non-Hodgkin Lymphoma and Exposure to Pesticides

[Bin: Epidemiology]

[Cited: Beyond Pesticides]

[Human Studies Considerations Apply: Yes]

A population-based case–control study in northern and middle Sweden encompassing 442 cases and twice as many controls was performed. Exposure data were ascertained by comprehensive questionnaires, and the questionnaires were supplemented by telephone interviews. In total, 404 cases and 741 controls answered the questionnaire. Increased risk for NHL was found for subjects exposed to herbicides (odds ratio [OR], 1.6; 95% confidence interval [CI], 1.0 –2.5) and fungicides (OR, 3.7; 95% CI, 1.1–13.0). Among herbicides, the phenoxyacetic acids dominated (OR, 1.5; 95% CI, 0.9 –2.4); and, when subclassified, one of these, 4-chloro-2-methyl phenoxyacetic acid (MCPA), turned out to be significantly associated with NHL (OR, 2.7; 95% CI, 1.0–6.9). For several categories of herbicides, it was noted that only exposure during the most recent decades before diagnosis of NHL was associated with an increased risk of NHL. Investigators concluded exposure to herbicides in total, including phenoxyacetic acids, during the decades before NHL diagnosis resulted in increased risk for NHL. Thus, the risk following exposure was related to the latency period. The combination of 2,4-D and 2,4,5-T, which constituted Agent Orange in U.S. warfare in Vietnam, was the most predominantly used herbicides in Swedish forestry. In all cases the results presented by the investigators included both 2,4-D and 2,4,5-T concurrently which may confound results based on current cultural practices which have eliminated 2,4,5-T concurrent use. Also, TCDD contamination has been eliminated by improved manufacturing processes for 2,4-D. Finally, improved requirements for personal protective equipment are used in current practices which would also impact the OR (Odds Ratio) values. The citation of this study by Beyond Pesticides does not account for the confounding factors of 2,4,5-T and TCDD exposures in their attribution of enhanced rates of non-Hodgkins Lymphoma associated with 2,4-D use.

Hines et al (2001)

Distributions and Determinants of Pre-emergent Herbicide Exposures Among Custom Applicators

[Bin: Planned Exposure Monitoring]

[Cited: 2,4-D Task Force]

[Human Studies Considerations Apply: Yes]

The primary objective of this study was to characterize the exposure distributions of the herbicides alachlor, atrazine, 2,4-D 2-ethylhexyl ester (2,4-D EH), and metolachlor among a group of applicators during the spring pre-emergent spray season. A secondary objective was to evaluate determinants of exposure and to estimate within- and between-worker variance components. Fifteen applicators were sampled using a systematic design that included spray and non-spray days and multiple measurements (five to seven) on each applicator. Air, patch, and handwash samples were collected on 89 applicator-days. Applicator-days were classified into three categories: target herbicide sprayed, non-target herbicide sprayed, and no herbicide sprayed. Mixed-model regression analysis was used. For all exposure metrics, adjusted mean herbicide exposures were significantly higher on days when target herbicides were sprayed as compared to non-spray days. For 2,4-D EH only, adjusted mean exposures on non-target herbicide spray days were significantly higher than on non-spray days. For all exposure metrics, the within-worker variability (GSDW 2.1–5.6) was greater than the between-worker variability (GSDB 1.2–2.7). Investigators concluded wearing gloves significantly reduced adjusted mean hand exposure for all herbicides (4–20 fold). This study evaluated several determinants of worker exposure and also produced distributions of exposure under varied field use circumstances. The use of the results of this study in the comments from the 2,4-D task force appears to be reasonable as they reflect the

specific conclusions described by the investigators in that the study illustrated that protective gloves significantly reduce exposures. It should be noted that PHED is currently used for routine assessments of this type rather than individual studies but the values generated in this study could be used to calculate risks if desired.

Lavy et al (1987)

Exposure of Forestry Ground Workers To 2,4-D, Picloram, and Dichlorprop

[Bin: Planned Exposure Monitoring]

[Cited: 2,4-D Task Force]

[Human Studies Considerations Apply: Yes]

Urine samples from 80 forest workers applying 2,4-D plus dichlorprop or picloram were included. Four crews of 20 workers each were studied during application by backpack, injection bar, hypohatchet or a hack and squirt methods. Each worker was evaluated under two operational modes. In the first (T1), the workers wore their usual clothing and followed the work habits they ordinarily used on their job. In the second (T2), conducted 6 days after the first, workers were given new leather gloves and boots and asked to take all feasible precautions (e.g., wash hands, bathe, wash clothes, avoid sprayed areas) to prevent herbicide exposure and to wash immediately if they come in contact with the chemical. For all application methods except backpacks the T-2 treatment decreased the absorbed dose. During both T-1 and T-2 the clothing of backpack sprayers often became saturated with spray, dew and perspiration, and these workers received a higher absorbed dose of 2,4-D (0.04 – 0.24 mg/kg/bw) than did workers in other crews. Hypohatchet workers received a greater dose than did injection bar or hack-and-squirt workers. Investigators evaluated the utility of using improved hygiene practices with fresh gloves and boots as a means of reducing exposures. In cases where exposures impinge mainly on the feet and hands the T-2 conditions described by the authors coupled with enhanced hygiene appear to reduce exposures. As noted by the authors, T-2 conditions and enhanced hygiene were not as effective with backpack applicators where much of the exposure occurs around the neck and upper chest/back area and they were noted to have been working in dense vegetation areas which had been previously treated (i.e., 2 to 7 meters high). Investigators also calculated various margins of safety with the lowest one being 272 for the highest exposed backpack applicators in T-2 conditions (based on geometric mean and NOEL of 24 mg/kg/d). It was concluded that having a calculated MOS less than the level of concern was attributable to very low dermal absorption since the backpack applicators had very high levels of contact with the 2,4-D spray solution as evidenced by their wet clothing and observed activities This study evaluated several determinants of worker exposure and also produced distributions of exposure under varied field use circumstances. The use of the results of this study in the comments from the 2,4-D task force appears to be reasonable in that they reflect the specific conclusions described by the investigators (e.g., T2 conditions tend to reduce exposures and 2,4-D has a low dermal absorption level). It should be noted that PHED is currently used for routine assessments of this type rather than individual studies but the values generated in this study could be used to calculate risks if desired.

Lerda, et al (1991)

Study of Reproductive Function In Persons Occupationally Exposed to 2,4-D

[Bin: Population Based Monitoring & Epidemiology]

[Cited: NRDC Petition & Beyond Pesticides]

[Human Studies Considerations Apply: Yes]

The reproductive function of 32 male farm sprayers who were exposed to 2,4-D was evaluated. These evaluations were completed in March through July 1989. The exposure periods were August to September 1988 with effects being determined in March 1989 and possible recovery being evaluated in July 1989. Controls were evaluated in April 1989. Sperm morphology, numbers, volume and other factors were evaluated. The levels of 2,4-D in urine were also determined. It is not clear when the urine

samples were collected relative to exposure events. The results indicate a mean urinary level of 2,4-D of 9.02 mg/liter in the 32 farmers selected for this study. The sperm of these 32 farmers show a significant increase in the incidence of asthenospermia, necrospermia, and teratospermia compared to the measured control group. Over time, asthenospermia and necrospermia diminished but the abnormal spermatozoa (teratospermia) continued. Investigators concluded that exposure to 2,4-D at the measured concentrations produces harmful effects on germinal epithelium, causing alterations of spermatogenesis. In the petition NRDC cited the results of this study in a manner reflective of the conclusions of the investigators. Beyond Pesticides also cited the results of this study in a manner reflective of the conclusions of the investigators. No environmental media sampling was completed. The direct exposure monitoring could be considered for quantitative use. However, PHED is currently used for routine assessments of this type rather than individual studies. No explanation of the circumstances of how exposures occurred was included in the discussions in the paper so those aspects of the study could not be considered. Additionally, the locale of this study was not described so it is not clear that the practices evaluated in this study are reflective of current cultural practices in the United States.

McDuffie, et al (2005)

Insect Repellents, Phenoxyherbicide Exposure, and Non-Hodgkin's Lymphoma

[Bin: Epidemiology]

[Cited: NRDC Petition]

[Human Studies Considerations Apply: Yes]

A hypothetical explanation was tested of contradictory results in studies of phenoxyherbicides and non-Hodgkins lymphoma (NHL), that the exposure of rubber gloves recommended for use by farmers when mixing or applying pesticides simultaneously to 2,4-D (2,4-dichlorophenoxyacetic acid), DEET (N,N-diethyl-m-toluamide), and ultraviolet rays increased their permeability to 2,4-D. A case (NHL n _513)/control (n _ 1506) study among men was conducted using age; province of residence; exposure to insect repellents containing DEET, phenoxyherbicides, or dicamba; and gloves when handling pesticides. The stratum with reported exposure to mecoprop, to DEET and the use of rubber gloves had higher odds ratios (3.86; 95% confidence interval _ 1.57–9.49) compared with strata with other combinations (e.g., 2,4-D with OR = 1.77 and range from 0.90 –3.45). The investigators acknowledged that they did not determine the exact type of rubber gloves used, which may vary in their permeability or whether gloves were specifically used while mixing or applying 2,4-D or other phenoxyherbicides of interest. The temporal relationship between the mixing or application of pesticides and the use of DEET is unknown. Lack of information in each of these instances would tend to bias the results toward the null. They also acknowledged the case–control design which they used has inherent limitations, including recall bias and selection bias. Investigators concluded the etiologic complexity of NHL was demonstrated. The investigators indicated in future research sample sizes must be large enough to statistically address the issues of exposure to multiple pesticides, either simultaneously or concurrently and other effect modifiers or confounders, which include personal hygiene habits during and after handling pesticides and other agricultural chemicals, personal and family medical history, and cigarette-smoking practices. NRDC indicates in the petition that there is a relationship between NHL and phenoxyacid herbicide use, DEET use and the use rubber gloves. NRDC also indicated that this study suggests that herbicides could be penetrating the skin even when gloves are worn and this could be leading to development of NHL in humans. While increased OR values were calculated by the investigators there are several critical uncertainties which have not been addressed (e.g., glove type and timing of DEET use). The investigators also did not indicate if enhanced dermal absorption was the mechanism or glove permeability failure contributed to higher exposures and subsequent higher OR values.

Moody, et al (1992)

Effect of the Mosquito Repellent DEET and Longwave Ultraviolet Radiation On Permeation of the Herbicide 2,4-D and the Insecticide DDT in Natural Rubber Gloves

[Bin: Planned Exposure Monitoring]

[Cited: NRDC Petition & 2,4-D Task Force Comments]

[Human Studies Considerations Apply: No]

A laboratory based permeation cell test was conducted to determine the effect of the commonly used insecticide DEET on the permeability of 100 percent latex rubber gloves (Becton Dickson N36-124). These gloves could be worn by applicators as part of a chemical protective clothing ensemble. Glove permeation analysis was conducted with an automated in vitro diffusion analysis (AIDA) method employing an in-house, flow through permeation cell design. Permeation of 14C ring labeled 2,4-D in natural rubber glove material was 2.4±1.81 percent at 48 hours after treatment of the glove with 2,4-D applied with DEET; this was not significantly different (Student's t-test; $p<0.05$) from 3.2±3.46 percent permeation of 2,4-D observed without DEET. Investigators concluded that because chemical protective clothing is commonly exposed to solar UVA this study raises concerns about the efficacy and safety of protective equipment in general. This study describes the permeability changes in a single glove material after exposure to UVA and also DEET coupled with 2, 4-D. The manner in which this study is cited by NRDC is not appropriate because they indicate that the gloves are highly permeable when the glove is exposed to DEET and UVA. The investigators reported that with DEET the permeation rate of the glove material was similar with 2,4-D whether or not DEET was present. The latex material tested did indicate increased permeability to UVA. NRDC indicates it then follows that enhanced exposures would occur. This conclusion is not warranted because it is not clear the application of UVA in this study mimics real world conditions (e.g., intensity of light beam). Also, only one type of latex rubber glove material was evaluated and its composition was not clear (e.g., UV stabilization or not?). Current production methods likely use UVA and UVB stabilizers in the manufacturing process. It is also easy to recommend other types of glove materials, which are UVA/UVB impervious. Finally, it should be noted that in PHED, exposures under real world conditions with actual glove use are measured and used to develop risk estimates. While chamber permeability tests of glove materials are useful they do not necessarily accurately predict protection factors for gloves, which can also be impacted by design and seam sealing methods. The Agency concurs with the comments of the 2,4-D task force on the NRDC use of this study.

Morgan, et al (2008)

Adult and children's exposure to 2,4-D from multiple sources and pathways

[Bin: Population Based Monitoring & Planned Exposure Monitoring]

[Cited: NRDC Petition Supplement, 2,4-D Task Force Comment & New York State Comment]

[Human Studies Considerations Apply: Yes]

This study investigated the 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide exposures of 135 preschool-aged children and their adult caregivers at 135 homes in North Carolina (NC) and Ohio (OH). Participants were randomly recruited from six NC and six OH counties. Monitoring was performed over a 48-h period at the participants' homes. Environmental samples included soil, outdoor air, indoor air, and carpet dust. Personal samples collected by the adult caregivers concerning themselves and their children consisted of solid food, liquid food, handwipe, and spot urine samples. 2,4-D was detected in all types of environmental samples but most often in carpet dust samples, with detection frequencies of 83% and 98% in NC and OH, respectively. The median level of 2,4-D in the carpet dust samples was about three times higher in OH homes compared to NC homes (156 vs. 47.5 ng/g, $P<0.0002$). For personal samples, 2,4-D was more frequently detected in the handwipe samples from OH participants (>48%) than from NC participants (<9%). Handwipe levels at the 95th percentile were about five times higher for OH children (0.1 ng/cm^2) and adults (0.03 ng/cm^2) than for the NC children (0.02 ng/cm^2) and

adults (<0.005 ng/cm^2). 2,4-D was detected in more than 85% of the child and adult urine samples in both states. The median urinary 2,4-D concentration was more than twice as high for OH children compared to NC children (1.2 vs. 0.5 ng/ml, P<0.0001); however, the median concentration was identical at 0.7 ng/ml for both NC and OH adults. The intraclass correlation coefficient of reliability for an individual's urinary 2,4-D measurements, estimated from the unadjusted (0.31–0.62) and specific gravity-adjusted (0.37–0.73) values, were somewhat low for each group in this study. The variability in urinary 2,4-D measurements over the 48-h period for both children and adults in NC and OH suggests that several spot samples were needed to adequately assess these participants' exposures to 2,4-D in residential settings. Investigators concluded results from this study showed that children and their adult caregivers in NC and OH were likely exposed to 2,4-D through several pathways at their homes. In addition, our findings suggest that the OH children might have been exposed to higher levels of 2,4-D through the dermal and nondietary routes of exposure than the NC children and the NC and OH adults. This study was designed using the CTEPP (Children's Total Exposure To Persistent Pesticides and Other Persistent Organic Pollutants) multi-media approach. The key difference is was that in this case potential exposures were quantified for children who did not attend daycare and their adult caregiver's exposure was also evaluated. The investigators concluded 2,4-D urinary levels were low compared to established reference doses established by IRIS. This study was cited appropriately by the 2,4-D task force including in its description of possible body burden values that could be experienced in households (i.e., NC and OH families were sampled). Additionally, the reported house dust levels were compared by the 2,4-D task force to previous values defined by Nishioka (1996) cited by NRDC. Additionally, investigators indicated levels did not indicate a health concern compared to IRIS and existing BE values. The New York Departments of Environmental Conservation and Health both indicated that EPA should consider this study in its 2,4-D risk assessment. The values could be considered quantitatively in an assessment but the results would always be compared to those calculated using applicable existing methods (e.g., SOPs For Residential Exposure Assessment). Finally, this study was cited in the NRDC supplement to the petition. It was cited properly and NRDC indicated residues can be tracked into indoor environments. NRDC also indicated the Agency did not account for this exposure pathway. NRDC indicates the data from this study could be used quantitatively for risk assessment purposes. In fact, in its updated SOPs for Residential Exposure Assessment the Agency has proposed a method for completing such assessments. It could be incorporated as appropriate.

Nishioka, et al (1996)

Transport of Lawn-Applied Herbicide Acids from Turf to Home: Correlation of Dislodgeable 2,4-D Turf Residues with Carpet Dust and Carpet Surface Residues

[Bin: Population Based Monitoring & Planned Exposure Monitoring]

[Cited: NRDC Petition, 2,4-D Task Force & Beyond Pesticides Comments]

[Human Studies Considerations Apply: Yes]

Transport of lawn-applied herbicides into the home via walking over treated turf, defined here as track-in, was measured at five different times out to 1 week after application. At five discrete times after herbicide application, study participants walked through a treated turf plot and then over a section of carpet to simulate the transfer that might occur with a homeowner walking across turf (e.g., to a mailbox). Dislodgeable turf residues were collected prior to walking; dislodgeable carpet surface residues and carpet dust were collected after walking. Residues of turf-applied 2,4-D and dicamba were measured in carpet dust and on the carpet surface after track-in. Both carpet dust levels and carpet surface dislodgeable residue levels were highly correlated with turf dislodgeable residue levels. Turf dislodgeable residues were 0.1−0.2% of turf application levels. Transfer of herbicides from turf to carpet dust was 3% of the turf dislodgeable residues. Transfer from turf to carpet surface was 0.3% of the turf dislodgeable residues. Herbicide 2,4-D was measured in the carpet dust of suburban homes at the 0.1−5

μg/g levels that are predicted by this efficiency of mass transfer. New tools used to collect these samples included the polyurethane foam roller dislodgeable residue sampler and the high-volume solid surface sampler. Investigators concluded they expect to find 2,4-D in residential carpet dust up to 1 year after turf application at concentrations of approximately 0.5 μg/g and at carpet loadings of approximately 0.3 $\mu g/m^2$. These estimates are in agreement with the range of 2,4-D found in nine residential dust samples: 0.1-5 μg/g and 0.02−1.1 $\mu g/m^2$. These 2,4-D levels suggest track-in as a contributing transport mechanism, with the number of children correlated with both carpet loading ($r^2 = 0.49$; $p = 0.04$) and with dust concentration ($r^2 = 0.35$; $p = 0.09$) as measured in regression analyses. The investigators also indicated in a recently reported study, the urine from matched populations of children in two Arkansas towns was analyzed for 2,4-D and 2,4-D was detected in urine of 18% of the children from the town containing a 2,4-D manufacturing plant and was present in the urine of 23% of the children in the town with no manufacturing plant. The similarity of exposure rates and detection levels indicated an exposure source other than the manufacturing plant, though the source was unknown. Both NRDC and Beyond Pesticides cited the conclusions by the investigators that 2,4-D persists indoors for up to one year. This value was not empirically derived through measurements but instead it was concluded by the investigators based on a series of assumptions and sampling which only occurred out to 1 week after an application of 2,4-D. The Agency does not concur with these conclusions by the investigators. The 2,4-D task force correctly indicates that the levels were calculated by the investigators and then compared them to empirically derived dust concentrations by Morgan (2008) which were lower than those calculated by the investigators. The 2,4-D task force did not discuss the temporal considerations associated with the interpretation in this comparison. Also, the Agency does not concur with the conclusions by the investigators because the assumptions do not account for any degradation processes nor is the short sampling timeframe of 1 week discussed as an uncertainty in the conclusions.

Yeary, et al (1986)

Urinary Excretion of 2,4-D In Commercial Lawn Specialists

[Bin: Planned Exposure Monitoring]

[Cited: 2,4-D Task Force Comments]

[Human Studies Considerations Apply: Yes]

Occupational exposure to the herbicide 2,4-D was measured in 45 commercial lawn care specialists who had been spraying the herbicide on lawns on a daily basis, excluding Sundays, for a period of at least 3 weeks. Exposure was determined by collecting all urine voided during a 24 hour period, and analysis of 2,4-D was made by gas chromatography. The 24 hour urinary excretion of 2,4-D was considered to be a reasonable estimate of the daily absorbed dose. Workers wore latex gloves, an apron or coveralls, goggles or faceshield, and rubber boots when handling concentrated product. When spraying dilute mixtures workers wore rubber boots, long pants, and short-sleeved shirts. Glove use was optional. The urinary concentrations of 2,4-D ranged from non-detectable at a sensitivity of 0.01 ppm to 2.29 ppm with a median of 0.18 ppm. The quantity excreted on a body weight basis (mg/kg/day) was 0.0032 ± 0.0010, 0.0063 ± 0.0018, 0.00035 ± 0.0005, and 0.00138 ± 0.0005 mg/kg respectively at 4 work locations. Investigators concluded these values are considerably less than the WHO/FAO acceptable daily dietary intake. The use of the results of this study in the comments from the 2,4-D task force appears to be reasonable in that they reflect the specific conclusions described by the investigators. However, it should be noted that, as written, the 2,4-D task force summary of the study could be misleading because they indicate exposures did not exceed a level of 6 μg/kg/day which was really the maximum mean value from particular site with the highest average exposures. Exposures on a individual basis actually ranged about 10 times higher that this value as noted in the highest urinary concentration reported of 2.29 ppm. The direct exposure monitoring could be considered for quantitative use. However, PHED is currently used for routine assessments of this type rather than

individual studies.

Exposure and Epidemiological Data Summary: Several types of studies were cited in the NRDC petition on 2,4-D and various related documents. These included exposure monitoring in several forms, epidemiological analyses, environmental measurements, and methodological analyses. All totaled, the results of these efforts do not yield information which would cause the Agency to modify its conclusions from the 2005 Reregistration Eligibility Document (RED) for 2,4-D (EPA 738-R-05-002) that pertain to occupational and residential risks. In summary, some risks were identified and the Agency implemented appropriate risk management measures to address them.

Exposure Studies: A number of exposure monitoring studies cited in the NRDC petition and associated comments included biological monitoring results, a measurement of exposure using passive dosimetry methods, or some measure of environmental contamination of 2,4-D (e.g., house dust levels cited by Nishioka 1996). None of these values as reported seem to indicate exposures at levels which are of concern using the current endpoints for short- and intermediate term exposures as referenced in the 2005 Agency RED human health risk assessment. In fact, many of these values represent risk levels that are far below the Agency level of concern and they are mostly below or in more limited cases similar to the levels used to characterize the potential risks associated with 2,4-D use in the Agency RED. The process used in Agency risk assessments is to evaluate current cultural practices but with the stipulation that the upper range of anticipated exposures (i.e., maximum application rate and extensive acres treated coupled with central tendency exposure rates compared to subchronic endpoints) be considered in order to determine if all proposed label uses should be allowable. It could be argued that some of the cited studies could provide additional insight into the types of job tasks that could be associated with 2,4-D use but this research does not represent current agricultural practices which would add uncertainty to the analysis (e.g., Lavy *et al* describes different tasks in forestry management but was completed in 1986 before current requirements for worker protection were established). Also, some studies investigated the factors which impact exposure (e.g., Hines et al 2001). Many of the referenced studies provide similar information on exposure factors but this issue is much broader than the studies considered herein and for consistency the Agency uses the Pesticide Handlers Exposure Database (PHED) to predict occupational handler exposures to alleviate such concerns. In addition to the studies mentioned by NRDC and petition comments, similar trends are noted in more current research. Aylward *et al* (2009)[4] concluded that "current use patterns and risk management efforts" are keeping levels "well below current noncancer reference values" established by the Agency. This paper used biomonitoring equivalents as the basis for the analysis defined by Aylward and Hays (2008).[5] It should be noted that when compared to the studies cited by NRDC and petition comments that the values also do not indicate a concern. Finally, Thomas *et al* (2009)[6] reported biomarker urine concentrations from the Agricultural Health Study cohort (www.Aghealth.org) lower than current Agency levels of concern which is consistent with the results of the cited studies as well. In summary, the exposure monitoring studies cited by NRDC do not indicate risks of concern and the values presented are similar to or lower than those used for risk

4 Aylward LL, Morgan MK, Arbuckle TE, Barr DB, Burns CJ, Alexander BH, et al. 2010. *Biomonitoring Data for 2,4-Dichlorophenoxyacetic Acid in the United States and Canada: Interpretation in a Public Health Risk Assessment Context Using Biomonitoring Equivalents*. Environ Health Perspect 118:177-181. doi:10.1289/ehp.0900970

5 Aylward LL, Hays, SM (2008) *Biomonitoring Equivalents (BE) dossier for 2,4-dichlorophenoxyacetic acid (2,4-D) (CAS No. 94-75-7)*, Regulatory Toxicology and Pharmacology 51 (2008) S37–S48

6 Thomas, K.W., Dosemeci, M., Hoppin, J., Sheldon, L.S., Croghan L.S., Gordon, S.M., Jones, M.L., Reynolds, S.J., Raymer, J.H., Akland, G.G., Lynch, C.F., Knott, C.E., Sandler, D.P., Blair, A.E., and Alavanj, M.C. (2009) *Urinary biomarker, dermal, and air measurement results for 2,4-D and chlorpyrifos farm applicators in the Agricultural Health Study*, Journal of Exposure Science and Environmental Epidemiology (2009), 1–16

assessment purposes by the Agency.

Consideration of Dust as Exposure Source: To examine whether 2,4-D contamination of indoor dust might significantly alter the findings of the risk assessment, EPA considered how the indoor residue values in the Nishioka studies would affect the risk assessment. EPA assumed for screening purposes that toddlers consume 100 mg/day of dust containing the highest dust concentration found in Nishioka studies (67 µg/g). The 2,4-D levels in dust in the Nishioka studies were generally much lower than 67 µg/g (e.g., 1996 maximum is 4.85 µg/g, and 2001 median is 10 µg/g). The value of 100 mg/day for dust consumption is drawn from the EPA's Child Specific Exposure Factors Handbook and is the same value assumed for soil consumption. This value was also used in the Nishioka studies. Additional conservatisms in this screening assessment are the assumption that (1) that exposures from dust occur assumed despite the uncertainties noted in the 2009 FIFRA Scientific Advisory Panel Report where the recently revised *SOPs For Residential Risk Assessment* were reviewed; and (2) 2,4-D residues do not to decline over time even though 2,4-D is known to dissipate quickly**.** Based on these assumptions, margins of exposure range from approximately 32,000 to 150,000 depending upon whether the duration of exposure considered is acute, short- or intermediate term in duration.[7]

As such, use of this highest dust concentration value would not impact the findings of the current risk assessment. If it is further assumed that dusts persist in impacted residences in such a way that ingestion of the highest concentration would occur in a chronic exposure pattern and that the highest noted concentration in dust would never dissipate, which is counter-intuitive given how 2,4-D is used and its known rapid dissipation characteristics, risks are still not of concern. In such situations, dust would be the predominant source for chronic exposures but margins of exposure still would exceed 11,000 based on the chronic dietary point of departure (5 mg/kg/d).[7]

Epidemiological Research: The appropriate consideration of epidemiological research into regulatory decision making is difficult due to the inherent uncertainty associated with interpreting such data.[8] In fact the Agency has been undertaking a process to develop a more rigorous approach for incorporating epidemiological research into regulatory decision making.[9] With regards to specific pesticides such as 2,4-D the Agency has been actively considering epidemiology in an extensive way because of the nature of the available research and the potential concerns which can be identified based on this research. In fact, the FIFRA Scientific Advisory Panel evaluated this type information applicable to 2,4-D in 1994 and the panel concluded that "data are not sufficient to conclude that there is a cause and effect relationship between exposure to 2,4-D and non-Hodgkin's lymphoma." In 1996 and then in 2004 further reviews were conducted of additional epidemiological studies by Blondell (D311464, 12/8/04) both essentially with similar conclusions. In some cases studies completed by the same investigators who were cited in Blondell (2004) were also cited in the NRDC petition and the other pertinent documents (e.g., Garabrant *et al* (2002)). All the additional data identified in the NRDC petition do not indicate major differences from the Agency conclusions based on epidemiological evidence outlined by Blondell (2004).

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7 Calculated using: POD (mg/kg/d)/[(67µg 2,4-D/1000 mg soil*100 mg soil ingestion)/15 kg child body weight]
where Acute POD = 67 mg/kg/d, Short-term POD = 25 mg/kg/d, Intermediate-term POD = 15 mg/kg/d, and chronic POD = 5 mg/kg/d

8 Similar conclusions are drawn in CA OEHHA (2009) *Public Health Goals For Chemicals in Drinking Water: 2,4-D* available at http://oehha.ca.gov/water/phg/pdf/24dphg010209.pdf

9 http://www.epa.gov/scipoly/sap/meetings/2010/020210meeting.html

The cited epidemiological studies were of different designs, focused on a variety of exposed groups, and also contained other types of information such as biological monitoring results or environmental measurements. In some cases the NRDC analysis ignored additional research completed by investigators which impacted the overall conclusions drawn by investigators regarding the public health implications of 2,4-D. One such example is the use of Garry *et al* (1996) by the NRDC which ignored the conclusions drawn in Garry *et al* (2002) which indicated less concerns than in the original paper.

In some cases investigators were concerned about possible causal links to certain effects. The studies where results are related to 2,4-D and that appear to present more likely results of concern are discussed further below. Selected issues are described below along with a rationale regarding how the findings of these specific studies should be considered.

Case-control studies: In these studies investigators tried to determine if 2,4-D exposure can affect genomic stability, reproductive hormone levels (luteinizing hormone), non-Hodgkin's lymphoma (NHL) and increased birth defects. Case-control studies identify factors that may contribute to a medical condition by comparing patients who have that condition (the cases) with subjects (the controls) who do not have the condition, but shared the common characters and experience within a defined time period (including the exposure situation). The studies discussed below do not demonstrate causation.

In 1998, **Hardell and Eriksson** conducted a case-control study to determine the importance of phenoxyacetic acids and other pesticides in the etiology of NHL. Although the study reported exposure to phenoxyacetic acids including 2,4-D (significantly with MCPA) and glyphosate resulted in increased risk NHL, authors admitted that the in-depth knowledge of active ingredient concentration was lacking, and recall bias in providing information for pesticide might have occurred since the exposures occurred a few decades ago (i.e., the exposure assessment aspects of this study were uncertain).

Garry et.al. (2001)mentioned that cases with high urinary 2,4-D levels had elevated LH levels, and exhibited altered genomic stability; however, to what extent the cumulative chromosome effect observed were related to adjuvant use was unsure, also the sample size was small (only 24 exposed and 15 controls) making the results uncertain. It should also be noted that the mean 2,4-D residue level in urine (454 ppb) is also not a level where saturation of the LH mechanism would be anticipated and thus would not be of concern.

Literature Review: A review of the epidemiological and toxicological studies from 1995 to 2001 by Garabrant and Philbert, concluded that 2,4-D did not have any genotoxic potential; also it did not have carcinogenic and reproductive toxic effects. However, they suggested an association between exposure to 2,4-D and neurological effects such as myotonia, persistent peripheral polyneuropathy, demyelination and ganglion degeneration in CNS. They also indicate 2,4-D exposure may have an association with suicide, depression, anxiety, and post-traumatic stress syndrome. However, these results are uncertain because the studies did not control for the effects of age, exposure to dioxin, and other confounding factors. These studies also did not adequately quantify the exposure aspects of these suggested relationships. The reviewed articles were from 1995-2001, and this review was done at the request of the, "Industry task force II". It should also be noted that the use patterns and possible use of adjuvants or dioxin contamination

differs in current practice from the timeframe when these studies were conducted.

Ecological Study: Garry et.al. (1996) conducted ecological study. They found that rate of birth defects were increased in offspring born to pesticide applicators, infants conceived in spring, and offspring born to the general population residing in pesticide (chlorophenoxy herbicide) high use region. However, analysis was done at the group level, rather than individual level. Thus individuals were assigned an exposure classification that might not represent their true exposure making this aspect of the study highly uncertain. Although this study considered the pregnancy risk factors, it did not mention in detailed if important maternal factors (such as, infections, vaccines, drug use) that cause birth defects were also considered.

A broad array of epidemiological research was cited in the NRDC petition and associated comments. This review indicates that described by Blondell (2004) are still pertinent (i.e., 2,4-D does not appear to be carcinogenic). With regard to other possible effects investigators have yet to prove that there is a causal relationship between 2,4-D and these effects at exposure levels which would be encountered in user populations, especially given current use practices and worker protection requirements (e.g., neurotoxicity). However, more recent research may necessitate further investigation into the possible effects of 2,4-D as illustrated by findings in the Agricultural Health Study (e.g., rhinitis)[10] and possible links to cardiovascular disease.[11] Any issues pertaining to the validity of these studies will also be addressed (e.g., Schreinemachers use of data which do not represent current use practices).

Exposure Methods: Some studies cited in the NRDC petition and associated documents provide methodological information pertinent to the risk assessment and risk management process (e.g., cell permeation test for breakthrough of latex rubber glove material conducted by Moody et al 1992). Such studies, while well conducted, may provide little impact on the health outcomes associated with 2, 4-D use because different glove materials could be recommended for use by applicators which could be less permeable to 2,4-D end use products or less susceptible to environmental conditions (e.g., because they are UV stabilized). Differences in glove materials are considered in the development of end-use product pesticide labels according to the nature of the challenge agent which should account for issues noted in these studies.[12] Addtionally, NRDC cited the Riviere et al. study to claim that occlusion can significantly enhance skin absorption of 2, 4-D. In normal pesticide exposure situations, fabric occlusion would be anticipated under normal work clothing. Guidelines for evaluating dermal toxicity of pesticides require that a gauze dressing be placed over the dosage site which closely simulates fabric occlusion. This method, coupled with the consideration of appropriate uncertainty factors accounts for any uncertainty raised by NRDC. Finally, a study NRDC was cited by Moody et al. regarding the permeability of worker gloves in conjunction with simultaneous exposure to DEET and sunlight. However, EPA does not concur with the conclusions made by NRDC because the conditions of this research do not represent likely exposure conditions which would be expected currently in agriculture. This is because DEET would have to be present in all circumstances where workers use gloves at similar concentrations and new glove materials account for sunlight exposure through UV stabilization. It should also be noted that exposures used to calculate risks have been measured under real world

10 Slager RE, Poole JA, LeVan TD, Sandler DP, Alavanja MC, Hoppin JA. Rhinitis associated with pesticide exposure among commercial pesticide applicators in the Agricultural Health Study. Occup Environ Med. 2009 Nov;66(11):718-24. Epub 2009 Mar 15. PubMed PMID: 19289390; PubMed Central PMCID: PMC2936571.

11 Schreinemachers DM (2010) Perturbation of lipids and glucose metabolism associated with previous 2,4-D exposure: a cross-sectional study of NHANES III data, 1988-1994. Environ Health. 2010 Feb 26;9:11.

12 http://www.epa.gov/oppfead1/labeling/lrm/chap-10.pdf

conditions with actual glove use, which also accounts for possible effects of field conditions on glove efficacy in risk assessment.

Summary: The Agency generally concurs with the analysis of this evidence by the 2,4-D task force. Several studies were cited in the NRDC petition and associated comments. Most were cited correctly as reported by the investigators but, overall, the results do not impact the risk conclusions which have been developed by the Agency for 2, 4-D. This is because of several critical considerations including some information is outdated. For example, 2, 4-D use patterns have changed in agriculture since the timeframes where the cited data were generated. Additionally, possible confounding aspects such as potential dioxin contamination is no longer a factor in the modern manufacturing processes for 2, 4-D. In many studies there are significant uncertainties that were identified, including preliminary conclusions were cited by NRDC not accounting for later research by the same investigators. Also, in many cases the exposure related aspects on the analyses were inadequately addressed.

The Agency is currently involved in processes to refine many of its exposure assessment inputs and to establish better methods for the considering epidemiological research into the regulatory process (i.e., 2010 SAP review). The Agency is also re-evaluating pesticide risks on a cyclical basis under its registration review process. Given these two efforts, the Agency will further evaluate research related to 2,4-D under registration review. It has also been actively participating in epidemiological research efforts such as the Agricultural Health Study and, as part of this process, will pursue additional information related to 2,4-D and the potential for health effects in potentially exposed populations.

5.0 Overview of Pet Incidents

A summary of the available pet incident data is included below. In some cases specific papers were utilized in the NRDC petition itself or in one or more subsequent, related comments. In other cases citations were utilized only in single comments. For clarity, this section summarizes the actions associated with each citation and the results are presented in alphabetical order based on the name of the primary author. Additionally, it is noted where each citation has been used.

Hayes, et al (1991)
Case-Control Study of Canine Malignant Lymphoma: Positive Association With Dog Owner's Use of 2,4-Dichlorophenoxyacetic acid herbicides
[Bin: Pet Exposure Monitoring]
[Cited: Beyond Pesticides]
[Human Studies Considerations Apply: No]
A hospital-based case control study of companion dogs which examined developing canine malignant lymphoma associated with the use of chemicals in and around the home. A self administered owner questionnaire and/or phone interviews were completed for 491 cases, 466 nontumor controls, and 479 tumor controls. Dogs diagnosed between January 1984 and February 1988 were identified from 3 veterinary teaching hospitals (MI, IN, CO). Dogs living in households where 2,4-D was applied more frequently had significantly higher odds ratios that control situations (Odds Ratio=1.3). Risk of canine malignant lymphoma rose to twofold excess with four or more yearly owner applications of 2,4-D. A positive trend ($P<0.02$) was observed for the yearly number of applications. Other possible factors (e.g., flea product use, cigarette use, carpet presence) were evaluated but found not to be a factor. Known susceptible breeds (e.g., boxers, mastiffs) were also addressed. The investigators commented on how

recall biases were addressed in the study but they acknowledged a major weakness is a lack of precise exposure data. No environmental or personal sampling was completed and the author s acknowledge significant uncertainty pertaining to a lack of exposure data. It is also not clear how germane the results of this study are given the timeframe over which this study was conducted compared to modern use patterns associated with 2,4-D. The citation of this study by Beyond Pesticides reflects the conclusions of the investigators but it does not account for the uncertainty associated with the exposure predictors acknowledged by the investigators in this study. It should also be noted that in the follow-up 1995 paper to this investigators note significant levels of criticisms that are addressed in the subsequent document described below. Please refer to that summary of Hayes *et al* 1995 for additional information. It should be noted that the NRDC petition does not make any mention of exposure to pets or via pets.

Hayes, et al (1994)

On the Association Between Canine Malignant Lymphoma and Opportunity for Exposure to 2,4-Dichlorophenoxyacetic Acid

[Bin: Pet Exposure Monitoring]

[Cited: Beyond Pesticides]

[Human Studies Considerations Apply: No]

This article was written in response to specific criticisms leveled at the Hayes et al 1991 article described above. Several specific considerations were addressed including how control groups were treated, whether responses were via mail or phone, potential hospital selection bias, difficulties associated with exposure classification including frequency of use, and knowledge related to the specific use of 2,4-D products. The authors addressed each of the applicable criticisms. The results of Hayes et al 1991 were supported in their analysis. The authors clarified the conclusions to better address uncertainty issues. The investigators also acknowledged that the reported values could be explained by bias or confounding factors. It was also indicated that the results should be interpreted with caution because of low exposures. The investigators also concur with several commenters that the study does not prove that 2,4-D exposure in a home environment is a cause of malignant lymphoma in companion dogs. They again indicate it does warrant further research on the issue. The citation of this study in the Beyond Pesticides comments to the NRDC petition is correct. However, it is somewhat misleading because they do not reiterate the level of uncertainty voiced by the investigators in their comments about the applicability of the results and the call in both Hayes 1991 and 1995 for additional research to investigate this relatively weak relationship between 2,4-D exposure and canine malignant lymphoma.

Reynolds, et al (1994)

Canine Exposure To Herbicide Treated Lawns and Urinary Excretion of 2,4-Dichlorophenoxyacetic Acid

[Bin: Pet Exposure Monitoring]

[Cited: Beyond Pesticides]

[Human Studies Considerations Apply: No]

A recent study by Hayes *et al* (J NatI. Cancer. Inst., *83:*1 226-i 231 , 1991) found an increased risk of malignant Iymphoma associated with exposure to 2,4-dichlorophenoxyacetic acid (2,4-D) in pet dogs. A study was conducted to determine the extent to which dogs absorb and excrete 2,4-D in urine after contact with treated lawns under natural conditions. Among 44 dogs potentially exposed to 2,4-D-treated lawns an average of 10.9 days after application, 2,4-D concentrations greater than or equal to 10.0 µg/l were found in 33 dogs (75%) and concentrations of ≥50 µg/l were found in 17 (39%). Among 15 dogs with no known exposure to a 2,4-D-treated lawn in the previous 42 days, 4 (27%) had evidence of 2,4-D in urine, 1 at a concentration of ≥50 µg/l . The odds ratio for the association between exposure to a 2,4-D-treated lawn and the detection of ≥50 µg/l 2,4-D in urine was 8.8 (95% confidence interval, 1 .4-56.2). Dogs exposed to lawns treated within 7 days before urine collection were more than 50 times

as likely to have 2,4-D at concentrations ≥50 µg/l than dogs with exposure to a lawn treated more than 1 week previously (odds ratio = 56.0; 95% confidence interval, 10.0-312.2). The highest mean concentration of 2,4-D in urine (21 3 mg/l) was found in dogs sampled within 2 days after application of the herbicide. Investigators indicated these findings demonstrate that dogs living in and around residences with recent 2,4-D lawn treatment absorb measurable amounts of the herbicide for several days after application and thus may constitute a useful animal model for evaluation of the effects of herbicides on the induction of lymphoid cancer. This study was considered in the comments of Beyond Pesticides and was cited appropriately except that the comparison made to previous data could not be verified. It should be noted that the NRDC petition does not make any mention of exposure to pets or *via* pets.

Pet Exposure Summary:

EPA evaluated the studies cited by Beyond Pesticides and the studies do not change the Agency's current conclusions. The two studies conducted by Hayes et al. in 1991 and 1995 focused on an epidemiological evaluation (i.e., case control study) of the relationship between 2,4-D use on lawns and the incidence of malignant lymphoma in companion dogs. This evaluation used a hospital-based recall questionnaire approach. There were several criticisms of this paper which were addressed in 1995 by Hayes et al., including issues associated with the exposure assessment process and the uncertainties associated with low odds ratios. The authors acknowledged a major weakness with the studies were a lack of environmental or personal sampling and possible recall bias. Further, significant uncertainty exists due to the lack of exposure data. In addition, the 2005 RED required that, in order for a product to be eligible for reregistration, label language needed to be added restricting pet access to treatment areas during and immediately after application. All labels now have this language, which changes the use patterns that existed during the time of the *Hayes* studies in the early 1990s because now pets should not be in the treatment area during or immediately after application. Further, the authors concurred with several commenters that the studies do not prove that 2,4-D exposure in a home environment is a cause of malignant lymphoma in companion dogs. Therefore, Hayes *et al* does not impact the Agency's previous assessment.

Reynolds et al. 1994 attempted to supplement Hayes et al. by attempting to refine the ability to predict exposures from lawn herbicides like 2,4-D by collecting exposure characteristics using an interview/questionnaire approach and by monitoring of 2,4-D in pet urine. They concluded that gathering data via owner questionnaires could be a good surrogate predictor of exposure. Among 44 dogs potentially exposed to 2,4-D-treated lawns an average of 10.9 days after application, 2,4-D concentrations greater than or equal to 10.0 µg/l were found in 33 dogs (75%) and concentrations of ≥50 µg/l were found in 17 (39%). Among 15 dogs with no known exposure to a 2,4-D-treated lawn in the previous 42 days, 4 (27%) had evidence of 2,4-D in urine, one at a concentration of ≥50 µg/l . The odds ratio for the association between exposure to a 2,4-D-treated lawn and the detection of ≥50 µg/l 2,4-D in urine was 8.8 (95% confidence interval, 1 .4-56.2). Dogs exposed to lawns treated within 7 days before urine collection were more than 50 times as likely to have 2,4-D at concentrations ≥50 µg/l than dogs with exposure to a lawn treated more than one week previously (odds ratio = 56.0; 95% confidence interval, 10.0-312.2). The highest mean concentration of 2,4-D in urine (21 3 mg/l) was found in dogs sampled within two days after application of the herbicide. The authors indicated that these findings demonstrate that dogs living in and around residences with recent 2,4-D lawn treatment, absorb measurable amounts of the herbicide for several days after application and, thus, may constitute a useful animal model for evaluating the effects of herbicides on the induction of lymphoid cancer. EPA believes

the use of interviews/questionnaires may have some utility as a surrogate means of predicting exposures in epidemiological research. However, with regard to the specific issue of establishing a model specific to 2,4-D exposure and cancer induction the results are incomplete and have considerable uncertainty, which is consistent with the findings of the investigators who concluded that additional research on this matter is needed.

In summary, the studies cited by Beyond Pesticides, in its comments to support its assertion that 2,4-D has been shown to induce lymphoma, did not address key uncertainties (lack of sampling, timing of the studies, and recall bias) in the study results. In particular, Beyond Pesticides failed to note the relatively weak association between exposure and lymphoma onset described by Hayes et al. Further, because these studies were conducted prior to the risk mitigation measure identified in the RED, and now reflected in product label language, limiting pet access during and immediately following application, the resulting new use patterns should result in reduced exposure. As such, while the Agency concurs with Hayes et al that perhaps additional research is warranted, it does not believe that there is evidence of critical animal health issues which warrant changes to its current conclusions.

6.0 Summary

Several types of studies were cited in the NRDC petition on 2,4-D and comments from other sources. These included a variety of toxicity, exposure, epidemiological, and pet incident data. The position set forth in the petition is that there is sufficient scientific evidence to justify the cancellation of 2,4-D containing products because it is not possible to make a safety finding. Comments from other sources both support (i.e., Beyond Pesticides) and countermand the conclusions made by NRDC (e.g., 2, 4-D Task Force). After considering the analyses and conclusions included in these documents the Agency does not believe the petition or other related comments provide any information that would cause the Agency to modify its conclusions on endpoints used in risk assessment from the 2005 Reregistration Eligibility Document (RED) for 2, 4-D (EPA 738-R-05-002).

The toxicity data that were cited included subcutaneous and intraperitoneal exposures to maternal animals with resulting gestational and/or lactational exposure of the offspring; subcutaneous exposure of offspring, and oral exposures to the maternal rat and/or offspring. These studies included evaluations of the endocrine system, including the thyroid, estrogenic and androgenic effects, developmental neurotoxicity and effects on neurotransmitters (dopamine and serotonin), myelination, and maternal behavior. Some investigations were conducted *in vivo* and others *in vitro*. Overall, these studies do not alter the POD used in the 2005 RED and human health risk assessment. Additionally, the studies cited by NRDC and others were performed at dose levels above the saturation level for renal clearance (*i.e.,* at levels that overwhelm the body's ability to excrete 2, 4-D) and in many cases were performed at only one dose, which does not provide dose-response data; i.e., a no-effect dose and another dose where the effects are observed but at a different magnitude, were not identified. Other citations were *in vitro* endocrine screening assays, many of which have been included in the Endocrine Disruptor Review Team assessment of 2, 4-D. The Tier 1 battery assays were designed as screens. Their intended purpose is to evaluate whether a substance is capable of interacting with the endocrine system The EDSP Tier 1 data is due to the Agency in the fall of 2011. On completion of the review of Tier 1 data, the Agency will conduct a Weight-of-Evidence (WoE) to consider the potential of the chemical to disrupt either the estrogen, androgen or thyroid (EAT) hormone systems. The WoE approach that is been drafted provides general guidance in which basic principles and criteria are described for an interpretive process for evaluating the EDSP Tier 1 screening results together with other considerations and supportive data

sources to identify candidate chemicals for Tier 2 testing. Chemicals with demonstrated evidence of a potential to interact with EAT hormone systems will be considered as candidates for Tier 2 testing. Currently, the Agency is developing Tier 2 EDSP tests for ecological species, which may include the following: Amphibian 2-Generation Study, Avian 2-Generation Study, Fish Lifecycle Study and the Invertebrate Lifecycle Study. The Tier 2 mammalian study is currently the 2-Generation Reproduction and Fertility Effects Study (Note: the OECD Guideline for the Extended F1 One Generation Reproduction Test Guideline is also under consideration). It should be noted that the fact that a substance may interact with a hormone system does not mean that when the substance is used, it will cause adverse effects in humans or ecological systems.

In addition to the studies considered in the NRDC and associated analyses, the 2, 4-D Task Force submitted an F1-Extended 1-Generation Reproductive toxicity study in response to the Data Call-In for a 2-generation reproduction study and a developmental neurotoxicity study. The Extended 1-Generation Reproduction study included an assessment of several key endpoints: an evaluation of potential effects on parental male and female reproductive function, offspring survival and growth. Additional endpoints included endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring), sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment. The test guideline and the 2, 4-D study also included options that evaluated development of the immune and nervous systems.

The results of the 2, 4-D extended 1-generation reproductive toxicity study support the current conclusions of the Agency outlined in the RED. In summary, several studies were cited in the NRDC petition and the associated documents. Most were cited correctly as reported by the investigators, but the overall results have little or no impact on the endpoints selected for human health risk assessment by the Agency for 2,4-D. This is because none of the studies identified an effect of concern at a lower dose than previously identified by the Agency in the 2005 RED, and the majority of the cited studies were performed at dose levels that exceed the renal clearance mechanism for 2, 4-D.

With regard to the exposure and epidemiological information presented by NRDC and in the related comments, conclusions are similar in that most studies were cited correctly as reported by the investigators but, overall, the results do not impact the risk conclusions that have been developed by the Agency for 2,4-D. This is because of several critical considerations. For example, 2, 4-D use patterns have changed in agriculture since the timeframes where the cited data were generated. Additionally, possible confounding aspects such as potential dioxin contamination are no longer a factor in the modern manufacturing processes for 2, 4-D. In many studies there are significant uncertainties, which were identified including preliminary conclusions that were cited by NRDC not accounting for later research by the same investigators. Also, in many cases the exposure related aspects on the analyses were inadequately addressed.

No studies were cited related to the issue of pet exposure except in the Beyond Pesticides comments. The results of these three studies were cited correctly in the Beyond Pesticides comments (i.e., 2, 4-D can be shown to induce lymphoma) but the key uncertainties in the results were not addressed. In particular, a discussion of the relatively weak association between exposure and lymphoma onset was not included and the impact of changing use practices as well as differences in formulations was not addressed.

In summary, the Agency did not conclude that the information provided by NRDC or in the associated comments warrants alteration of the conclusions described in the RED for 2,4-D. It should be noted that the Agency is re-evaluating pesticide risks on a cyclical basis under its registration review process. Concurrent to this effort, the Agency is also involved in many efforts to refine its risk assessment policies including the development of updated exposure assessment inputs and methods; establishing better methods for the considering epidemiological research in the regulatory process; and consideration of the issues identified in recent National Academy of Science reviews related to risk assessment (e.g., Toxicity Testing in the 21st Century); and more active participation with several epidemiological cohorts focused on agriculture and the use of pesticides (e.g., Agricultural Health Study). More current use information and knowledge of current cultural practices will also be available for the registration review process. Given this construct, the Agency will further evaluate research related to 2, 4-D under registration review and will modify the conclusions from the RED as appropriate.

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Study Identification				Summary of Citation Use							
*	Study Authors	Study Name	Study Year	Original NRDC Petition, Nov 6, 2008	Supplement to NRDC Petition, Feb 23, 2009	Task Force Comments on Petition, Feb 23, 2009	Beyond Pesticides Comment, Feb 23, 2009	Newton Comment, Feb 15, 2009	NY State Comment, Feb 23, 2009	NCASI Comment, Feb 20, 2009	Bin
1	Alexander, B.H., Mandel, J.S., Baker, B.A., Burns, C.J., Bartels, M.J., Acquavella,J.F., Gustin, C.	Biomonitoring of 2,4-dichlorophenoxyacetic acid exposure and dose in farm families.	2007			X					PB
2	Arbuckle, T.E., Burnett, R., Cole, D., Teschke, K., Dosemeci, M., Bancej, C., Zhang, J.	Predictors of herbicide exposure in farm applicators.	2002			X					PL
3	Arias E.	Cytogenetic Effects of Short- and Long-term Exposure of Chick Embryos to the Phenoxyherbicide 2,4-D.	2007						X		MUT
4	Arias E.	Sister chromatid exchange induction by the hervicide 2, 4-dichlorophenoxyacetic acid in chick embryos.	2003	X							MUT
5	Blair, R.M., Fang, H., Branham, W.S., Hass,, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R., Sheehan, D.M.	The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands.	2000			X					EN
6	Bon, E., Barbe, U., Nunez Rodriguez, J., Cuisset, B., Pellissero, C, Sumpter, J.P., LeMenn, F.	Plasma vitellogenin levels during the annua reprroductive cycle of the female rainbow trout (Onchorhynchus mykiss): Establishment and validation of an ELISA.	1997			X					ECO
7	Bortolozzi A et al.	Asymmetrical Development of the Monoamine Systems in 2,4-dicholorophenoxyacetic Acid Treated Rats	2003						X		MET
8	Bortolozzi A, Evangelista de Duffard AM, Dajas F, Duffard R, Silveira R.	Intercerebral administration of 2, 4-dichlorophenoxyacetic acid induces behavioral and neurochemical alterations in the rat brain.	2001	X		X					IP/SUBQ
9	Bortolozzi AA et al.	Effects of 2,4-dichlorophenoxyacetic acid exposure on dopamine D2-like receptors in rat brain.	2004						X		IP/SUBQ

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10	Bortolozzi AA et al.	Behavioral Alterations Induced in Rats by a Pre- and Postnatal Exposure to 2,4-Dichlorophenoxyacetic Acid	1999						X		IP/SUBQ
11	Brand and Jendrzejewski	The Effect of Chronic Ethanol Ingestion on skin Penetration of 2,4-D.	2008		X						DA
12	Brand RM, Charron AR, dutton L, Gavlic TL, et al.	Effects of chronic alcohol consumption on dermal penetration of pesticides in rats.	2004	X							DA
13	Brand RM, McMahon L, et al.	Transdermal absorption of the herbicide 2,4-dichlorophenoxyacetic acid is enhanced by both ethanol consuption and sunscreen application.	2007	X							DA
14	Brand RM, Spalding M, Mueller C.	Sunscreens can increase dermal penetration fof 2, 4-dichlorophenoxyacetic acid	2002	X							DA
15	Brusco A et al.	2,4-Dichlorophenoxyacetic Acid Through Lactation Induces Astrogliosis in Rat Brain.	1997						X		IP/SUBQ
16	Brusick DL. et al.	Genetic Toxicology. In: Principles and methods of Toxicology, ed. AW HaX.	2008							X	NR
17	Bukowska B.	Effects of 2,4-D and its metabolite 2,4-dichlorophenol on antioxidant enzymes and level of glutathione in human erythrocytes.	2003	X							MET
18	CalamariD, and Vighi M.	A proposal to define quality objectives for aquatic life for mixtures of chemical substances.	1992				X				ECO
19	Charles JM, Cunny HC, Wilson RD, Bus JS	Comparative subchronic studies on 2,4-dichlorophenoxyacetic acid, amine, and ester in rats.	1996	X		X	X			X	NC

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20	Charles JM, Cunny HC, Wilson RD, Bus JS, Ivett Jl, Murli H, and Gollapudi B.	In Vivo Micronucleus Assays on 2,4-Dichlorophenoxyacetic Acid and its Derivatives.	1999			X					MUT
21	Charles JM, Cunny HC, Wilson RD, Bus JS, Lawlor TE, Cifone MA, Fellows M, and Gollapudi B.	Ames Assays and Unscheduled DNA Synthesis Assays on 2,4-Dichlorophenoxyacetic Acid and its Derivatives	1999			X					MUT
22	Charles JM, Hanley TR Jr, Wilson RD, van Ravenzwaay B., Bus JS.	Developmental toxicity studies in rats and rabbits in 2,4-dichlorophenoxyacetic acid and its forms.	2001			X					NR
23	Cox, C.	2,4-D Herbicide Factsheet	2005				X				NR
24	Duffard R, Bortolozzi A, Ferri A, Garcia G, Evangelista de Duffard AM.	Developmental neurotoxicity of the herbicide 2,4-dichlorophenoxyacetic acid	1995	X		X					NEU
25	Duffard R, Garcia G, rosso S, Bortolozzi A, Madariaga M, di Paolo O, Evangelista de Duffard AM.	Central nervous system myelin deficit in rats exposed to 2,4-dichlorophenoxyacetic acid throughout lactation.	1996	X		X				X	NEU
26	Evangelista de Duffard AM, Bortolozzi A, Duffard RO.	Altered behavioral responses in 2, 4-dichlorophenoxyacetic acid treated and amphetamine challenged rats.	1995	X		X					NEU
27	Feldmann R.G., Mailbach, H.I..	Percutaneous penetration of some pesticides and herbicides in man.	1974			X					DA
28	Ferri A, Duffard R, Evangelista de Duffard Am.	Selective oxidative stress in brain areas of neonate rats exposed to 2,4-dichlorophenoxyacetic acid throught mother's milk.	2007	X		X					NEU
29	Figgs LW, Holland NT, Rothmann N, Zahm SH, et al.	Increased lymphocyte replicative index following 2,4-dichlorophenoxyacetic acid herbicide exposure.	2000	X		X					EPI

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30	Filkowski J, Besplug J, Burke P, Kovalchuk I, and Kovalchuk O.	Genotoxicity of 2,4-D and dicamba revealed by transgenic Arabidopsis thailiana plants harboring recombination and point mutation markers.	2003				X		X		ECO
31	Garabrant D.H., Phibert M.A.	Review of 2,4-dichlorophenoxyacetic acid (2,4-D) Epidemiology and Toxicology.	2002			X				X	EPI
32	Garcia G, Taliaferro P, et al.	Study of tyrosine hydroxylase immunoreative neurons in neonate rats lactationally exposed to 2,4-dichlorophenoxyacetic acid	2004	X		X					NEU
33	Garcia GB, Konjuh C, Duffard RO, Evangelista de Duffard AM.	Dopamine-beta-hydroxylase immunohistochemical study in the locus coeruleus of neonate rats exposed to 2,4-dichlorophenoxyacetic acid through mother's milk.	2006	X		X					NEU
34	Garry VF, Hawkins ME, Erickson LL, Long-Simpson LK, Holland SE, Burroughs BL.	Birth defects, season of conception, and sex of children born to pesticide applicators living in the Red River Valley of Minnesota, USA.	2002			X					EPI PB
35	Garry VF, Schreinemachers D, Harkins ME, et al.	Pesticide appliers, biocides, and birth defects in rural Minnesota.	1996	X		X	X				EPI PB
36	Garry VF, Tarone RE, Kirsch IR, Abdallah JM, Lombardi DP, Long LK, Burroughs BL, Barr DB, and Kesner JS.	Biomarker correlations of urinary 2,4-D levels in foresters: genomic instability and endocrine disruption.	2001				X				EPI PB
37	Giasson BI, Lee VM.	A new link between pesticides and Parkinson's disease.	2000			X					MUT NEU

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38	Glickman LT, Raghavan M, Knapp DW, Bonney PL, and Dawson MH.	Hervicide exposure and the risk of transitional cell carcinoma of the urinary bladder in Scottish Terriers.	2004				X				PI
39	Gollapudi BB, Charles JM, Linscombe VA, Day SJ, and Bus JS.	Evaluation of the genotoxicity of 2,4-dichlorophenoxyacetic acid and its derivatives in mammalian cell cultures.	1999			X					MUT
40	Gonzalez M, Soloneski S, Reigosa MA, Larramendy ML.	Genotoxicity of the herbicide 2,4-dichlorophenoxyacetic and a commercial formulation, 2, 4-dichlorophenoxyacetic acid dimethylamine salt.	2005	X							MUT
41	Grover Cessna AJ, Muir NI, Reidel S, Franklin C, Yoshida K.	Factors affecting the exposure of ground-rig applicators to 2,4-dimethylamine salt.	1986			X					PL
42	Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J, O'Heir CD, Mitchell ML, Hermos RJ, Waisbren SE, Faix JD, Klein RZ.	Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child.	1999	X							NEU
43	Hardell, L. and Ericsson, M.	A case-control study of non-Hodgkin lymphoma and exposure to pesticides.	1999				X				EPI PB
44	Harris SA, Solomon KR.	Percutaneous penetration of 2, 4-dichlorophenoxyacetic acid and 2, 4-d dimethylamine salt in human volunteers.	1992			X					DA (H)
45	Hayes, HM, Tarone RE, and Cantor KP.	On the Association between Canine Malignant Lymphoma and Opportunity for Exposure to 2,4-Dichlorophenoxyacetic Acid.	1995				X				PI
46	Hayes, HM, Tarone RE, Cantor KP, Jessen CR, MrCurnin DM, and Richardson RC.	Case- Control Study of Canine Malignant Lymphoma: Positive Associatiion With Dog Owner's Use of 2,4-Dichlorophenoxyacetic Acid	1991				X				PI PL

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		Herbicides.									
47	Hines CJ, Deddens JA, Tucker SP, Hornung RW.	Distributions and Determinants of Pre-Emergent Herbicide Exposures among Custom Applicators.	2001			X					PL
48	Holland NT, Duramad P, Rothman N, Figgs LW, et al.	Micronucleus frequency and proliferation in human lymphocytes after exposure to herbicide 2,4-dichlorophenoxyacetic acid in vitro and in vivo.	2002	X							???
	Howard, Phillip H.	Handbook of Environmental Fate and Exposure Data for Organic Chemicals.	1991			X					NR
49	Hurst MR, Sheahan DA.	The potential for oestrogenic effects of pesticides in headwater streams in the UK.	2003			X					ECO
50	Hutchinson TH. Et al.	Screening and Testing for Endocrine Disruption in Fish Biomarkders as Signposts, Not Traffic Lights in Risk Assessment.	2006							X	ECO
51	Hwang, U-G.	Effect of 2, 4-dichlorophenoxyacetic acid on vitellogenin synthesis and E2-ER binding affinity or hepatocytes in rainbow trout.	2002			X					ECO
52	Ibrahim MA, Bond GG, Burke TA, Cole P, Dost FN, Enterline PE, Gough M, Greenberg RS, Halperin WE, McConnell E, and et al.	Weight of the evidence on the human carcinogenicity of 2,4-D.	1991				X				CAN
53	INCHEM.	Environmental Health Criteria For 2,4-Dichlorophenoxyacetic Acid.					X				CAN

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54	Jung J, Ishida K, Nishihara T.	Anti-estrogenic activity of fifty chemicals evaluated by in vitro assays	2004			X					EN
55	Jungbauer A, Beck V.	Yeast reporter system for rabid determination of estrogenic activity.	2002			X					EN
56	Kim HJ, Kim WD, et al.	Mechanism of phyenoxy compounds as an endocrine disrupter.	2002	X		X					EN
57	Kim H-J, Park YI, Dong M-S.	Effects of 2,4-D and DCP on the DHT-induced androgenic action in human prostate cancer cells.	2005	X			X				EN
58	Kojima H, Katsura E, Takeuchi S, Niiyama k, Kobayashi K.	Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells.	2004			X					EN
59	Konjuh C, Garcia G, Lopez L, de Duffard AM, Brusco A, Duffard R.	Neonatal hypomyelination by the herbicide 2, 4-dichlorophenoxyacetic acid. Chemical and ultrastructural studies in rats.	2008	X	X	X				X	NEU
60	Kramer V. Blewitt C, Gersich M.	Comments on "Evaluation of Estrogenic Activities of Aquatic Herbicides and Surfactants Using a Rainbow Trout Vitellogenin Assay	2008			X				X	ECO
61	La Salle T, and Hepperly P.	Regenerative 21st Century Farming: a Solution to Global Warming.	2008				X				NR
62	LaChapelle, AM et al.	The hormonal herbicide, 2,4-dichlorophenoxyacetic acid, inhibits Xenopus oocyte maturation by targeting translational and post-translational mehanisms.	2007						X		ECO
63	Lavy TL, LA Norris, JD Mattice and DB Marx.	Exosure of forest ground workers to 2,4-D, picloram and dichlorprop.	1987			X					PL

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64	Lerda D, Rizzi R.	Study of reproductive function in persons occupationally exposed to 2,4-D.	1991	X			X				EPI PB
66	Lin N., Garry VF.	In vitro studies of cellular and molecular developmental toxicity of adjuvants, herbicides, and fungicides commonly used in Red River Vally, Minnesota.	2000			X					EN
67	Liu RC, Hahn C, Hurtt ME.	The direct effect of hepatic peroxisome proliferators on rat leydig cell function in vitro.	1996	X							EN
68	Madrigal-Bujaidar E, Hernandez-Ceruelos A, Chamorro G.	Induction of sister chromatid exchanges by 2,4dichlorophenoxyacetic acid in somatic and germ cells of mice exposed in vivo.	2001	X		X					MUT
69	Maire MA et al.	2, 4-Dichlorophenoxyacetic acid: Effects on Syrian hamster embryo (SHE) cell transformation, c-Myc expression, DNA damage and apoptosis.	2007						X		MUT
70	Malysheva LN, and Zhavoronkov AA.	Morphological and histochemical changes in the thyroid gland after a single exposure to 2,4-DA herbicide.	1997				X				EN
71	Marty MS, SA Saghir, AJ Clark, CL Zablotny, JS Bus, AW Perala, BL Yano and BH Neal.	A dietary dose range-finding and toxicokinetic (tk) study of 2, 4-dichlorophenoxyacetic acid (2,4-D) in adult crl:cd(sd) rats and their offspring: II. Toxicity.	2009			X					NR
72	McDuffie HH, Pahwa P, Robson D, Dosman JA, Fincham S, Spinelli JJ, McLaughlin JR.	Insect repellents, phenoxyherbicide exposure, and non-Hodgkin's lymphoma.	2005	X							EPI PB
73	McKinlay R, Plant JA, Bell JNB, and Voulvoulis N.	Endocrine disrupting pesticides: Imlications for risk assessment.	2008				X				EN

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74	Moody RP, Franklin CA, Ritter L, Maibach HI.	Dermal absorption of the phenoxy herbicides 2,4-D, 2,4-D isooctyl, and 2,4,5-T in rabbits, rats, rhesus monkeys, and humans: a cross-species comparison.	1990			X					DA
75	Moody RP, Nadeau B.	Effect of the mosquito repellent DEET and long-wave ultraviolet radiation on permeation of the herbicide 2, 4-D and the insecticide DDT in natural rubber gloves.	1992	X		X					PL
76	Moody RP, Wester RC, melendres JL, Mailbach HI.	Dermal absorption of the phenoxy herbicide 2,4D dimethylamine in humans: effect of DEET and anotomic site.	1992	X		X					DA (H)
77	Morgan MK, LS Sheldon, KW Thomas, PO Egegly, CW Croghan, PA Jones, JC Chuang, and NK Wilson.	Adult and children's exposure to 2, 4-D from multiple sources and pathways.	2008			X			X		PL
79	Nishihara T, J Nishikawa, T. Kanayama, F. Dakeyama, K. Saito, M. Imagawa, S. Takatori, Y. Kitagawa, S. Hori, h. Utsumi.	Estrogenic activites of 517 chemicals by yeast two-hybrid assay.	2000			X					EN
80	Nishioka, MG Burkholder HM, Brinkman MC, Gordon SM.	Measuring lawn transport of lawn-applied herbicide acids from turf to home: Correlation of dislodgeable 2, 4-D turf residues with carpet dust and carpet surface residues.	1996	X		X	X				PB
81	NRDC	Natural Resources Defense Council's petition to revoke all tolerances and cancel all registrations for the pesticide 2, 4-D.	2008			X					NR

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82	Oakes DJ, and Pollak JK.	The in vitro evaluation of the toxicities of three related herbicide formulations containing ester derivative of 2,4,5-T and 2,4-D using sub-mitochondrrial particles.	2000				X				NC
83	Oakes DJ, Webster WS, Brown-Woodman PDC, Ritchie HE.	A study of the potential for a herbicide formulation containing 2,4-D and picloram to cause male-mediated develpmental toxicity in rats.	2002			X					EN
84	Pelletier O, Ritter L, Caron J, Somers D.	Disposition of 2,4-dichlorophenoxyacetic acid dimethylamine salt by Fischer 344 rats dosed orally and dermally.	1989			X					MET
85	Petit F, Le Goff P, Cravedi J-P, Volataire Y, Pakdel F.	Two complementary bioassays for screening the estrogenic potency of xenobiotics; recombinant yeast for trout estrogen receptor and trout hepatocyte cultures.	1997			X					ECO
86	Pont AR, Charron AR, Brand RM.	Active ingredients in sunscreens act as topical penetration enhancers for the herbicide ,4-dichlorophenoxyacetic acid.	2004	X							DA
87	Rawlings NC, Cook SJ, Waldbilling D.	Effects of the pesticides carbofuran, chlorpyrifos, dimethoate, lindane, triallate, trifluralin, 2,4-D and pentachlorophenol on the methbolic endocrine and reproductive endocrine system in ewes.	1998			X				X	ECO
88	Reynolds PM, Reif JS, Ramsdell HS, and Tessari JD.	Canine exposure to herbicide-treated lawns and urinary excretion of 2,4-dichlorophenoxycetic acid.	1994				X				PI

Appendix A: Data Summary

Study Identification				Summary of Citation Use							
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89	Riviere JE, Baynes RE, Brooks JD, Yeatts JL, Monteiro-Riviere NA.	Percutaneous absorption of topical N, N-diethyl-m-toluamide (DEET): effects of exposure variables and coadministered toxicants.	2003	X							DA
90	Rodwell DE, Brown WR.	A dietary two-Generation Reproduction Study in Fischer 344 Rats with 2,4-dichlorphenoxyacetic acid.	1986			X					NR
91	Ross RH, Driver JH, Harris SA, Maibach HI.	Dermal absorption of 2,4-D: a review of species differences.	2005			X					DA
92	Rosso B et al.	2,4-Dichlorophenoxyacetic Acid Disrupts the Cytoskeleton and Disorganizes the Golgi Apparatus of Cultured Neurons.	2000						X		NEU
93	Rosso SB et al.	Effects of 2,4-dichlorophenoxyacetic acid on central nervous system of developmental rats.	1997						X		NEU
94	Rosso SB, Garcia GB, Madariaga MJ, Evangelista de Duffard AM, Duffard RO.	2, 4-Dichlorophenoxyacetic acid in developing rats alters behaviour, myelination and the regions brain gangliosides pattern	2000	X		X				X	NEU
95	Sachs PD.	Handbook of Successful Ecological Lawn Care.	1996				X				NR
96	Saghir SA, Marty MS, Clark AJ, Zablotny CL, Bus JS, Peral AW, Yano BL, neal BH.	A dietary dose range-finding and toxicokinetic (tk) study of 2, 4-dichlorophenoxyacetic acid (2,4-D) in adult crl:cd(sd) rats and their offspring: I. Toxicokinetics.	2009			X					EN
97	Saghir SA, Mendrala AL, Bartels MJ, Day SJ, Hansen SC, Sushynski JM, and Bus JS.	Strategies to assess systemic exposure of chemicals in subchronic/chronic diet and drinking water studies.	2006			X					MET
98	Schubert S. et al.	Sensitivity of Brown Trout Reproductio to Long-Term Estrogenic Exposure.	2008							X	ECO

Appendix A: Data Summary

Study Identification				Summary of Citation Use							Bin
*	Study Authors	Study Name	Study Year	Original NRDC Petition, Nov 6, 2008	Supplement to NRDC Petition, Feb 23, 2009	Task Force Comments on Petition, Feb 23, 2009	Beyond Pesticides Comment, Feb 23, 2009	Newton Comment, Feb 15, 2009	NY State Comment, Feb 23, 2009	NCASI Comment, Feb 20, 2009	
99	Soloneski S, Gonzalez NV, Reigosa MA, Larramendy ML.	Herbicide 2,4-dichlorophenoxyacetic acid (2, 4-D induced cytogenetic damage in human lymphocytes in vitro in presence of erythrocytes	2007	X							MUT
100	Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO.	The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants.	1995			X					EN
101	Spiteri ID, Guillette LJ Jr, Crain DA.	The functional and structural observations of the neonatal reproductive system of alligators exposed in ovo to atrazine, 2,4-D, or estradiol.	1999			X					ECO
102	Sturtz N, Bongiovanni B, et al.	Detection of 2, 4-dichlorophenoxyacetic acid in rat milk of dams exposed during lactation and milk analysis of their major components.	2006	X		X					NEU
103	Sturtz N, Evangelista de Duffard AM, Duffard R.	Detection of 2,4-dichlorophenoxyacetic acid (2,4-D) residues in neonates breast fed by 2,4-D exposed dams.	2000	X		X					NEU
104	Sturz N, Deis RP, Jahn GA, Duffard R, and Evangelista de Duffard AM.	Effect of 2,4-Dichlorophenoxyacetic acid on rat maternal behavior.	2008		X		X		X		NEU
105	The Herbicide Resistance Action Committee (HRAC)	Classification of Herbicides According to Mode of Action.	2005				X				NR
106	Ton C, Lin Y, Willett C.	Zebrafish as a model for developmental neurotoxicity testing.	2006	X							NEU
107	Tripathy NK, Routray PK, Sahu GP, Kumar AA,	Genotoxicity of 2,4-dichlorophenoxyacetic acid tested in somatic and germ-line cells of Drosophila	1993	X							MUT
	Tuschl h, Schwab C.	Cytotoxic effects of the herbicide 2, 4-dichlorophenoxyacetic acid in HepG2 cells.	2003	X							MUT

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Study Identification				Summary of Citation Use							
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	US Federal Register	Order Denying Objections to Issuance of Tolerances.	2005			X					NR
	US Federal Register	Order Denying Petition To Revoke Substances	2006			X					NR
	US Federal Register	Proposed Tolerance Actions.	2007a			X					NR
	US Federal Register	2,4-D Tolerance Actions	2007b			X					NR
	US Federal Register	2,4-D Tolerance Actions	2007c			X					NR
	US Federal Register	Order Denying NRDC's Petition to Revoke All DDVP Tolerances.	2007d			X					NR
	USEPA	2, 4-D. HED's Human Health Risk Assessment for the Reregistration Eligibility Decision (RED) Revised to Reflect Error-only comments from Resgistrants.	2004	X							NR
	USEPA	2, 4-D. HED's Human Health Risk Assessment for the Reregistration Eligibility Decision (RED) Revised to Reflect Public Comments	2005	X							NR
	USEPA	2,4-D RED Facts.	2005				X				NR
	USEPA	Acssessing Pesticide Cumulative Risk In: Pesticides: Health and Safety.	2008				X				NR
	USEPA	Fact Sheet: Protecting Children from Pesticides	2002	X							NR
	USEPA	Generic and Product Specific Data Call-in Notice	2005			X					NR
	USEPA	Reregistration Eligibility Decision for 2,4-D	2005	X		X	X				NR
	USEPA	Reregistration Eligibility Determination for 2, 4-D		X							NR
	USEPA	Response to comments	2005	X							NR
	USEPA	The National Pesticide Retrieval System (NPIRS)	1998-2009				X				NR

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	USEPA Memorandum	Office of Prevention, Pesiticides, and Toxic Substances, Updated Human Health Risk Assessment.	2002			X					NR
	USEPA Memorandum	2,4-D: Response to Public comments	2004	X		X					NR
	USEPA Memorandum	HED's Human Health Risk Assessment for the Reregistration Eligibility Decision (RED).	2004			X					NR
	USEPA Memorandum	2,4-D: Response to Phase 5 Public comments.	2005			X					NR
	USEPA Memorandum	Review of Recent 2,4-D Cancer Epidemiology Studies	2004							X	NR
	van Ravenzwaay B, hardwick TD, Needham D, Pethen S, and Lappin GJ.	Comparative metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) in rat and dog.	2003			X					MET
	Venkov P, Topashka-Ancheva M, Georgieva M, Alexieva V, Karanov E.	Genotoxicity of 2,4-dichlorophenoxyacetic acid tested in somatic and germ-line cells of Drosophila.	1993	X							MUT
	Vighi m, Altenburger R, Arrhenius A, Backhaus T, Bodeker W, Blanck H, Consolaro F, Faust M, Finizio A, Froehner K, Gramatica P, Grimme LH, Gronvall F, Hamer V, Scholze M, and Walter H.	Water quality objectives for mixtures of toxic chemicals: problems and perspectives.	2003				X				ECO
	Vonier PM, CD Andrew, JA Mclachlan, LJ Guillete Jr; SF Arnold.	Interaction of environmental chemicals with the estrogen and progesterone receptors from the oviduct of the American alligator.	1996			X					ECO

Appendix A: Data Summary

Study Identification				Summary of Citation Use							Bin
*	Study Authors	Study Name	Study Year	Original NRDC Petition, Nov 6, 2008	Supplement to NRDC Petition, Feb 23, 2009	Task Force Comments on Petition, Feb 23, 2009	Beyond Pesticides Comment, Feb 23, 2009	Newton Comment, Feb 15, 2009	NY State Comment, Feb 23, 2009	NCASI Comment, Feb 20, 2009	
	Wester RC, J Melendres, L Sedic, H Maibach, and JE Riviere.	Percutaneous absorption of salicylic Acid, Theophylline, 2,4-D Dimethylamine, Diethyl Hexyl Phthalic Acid, and p-Aminobenzoic Acid in the isolated perfused procine skin flap compared to man in vivo.	1998			X					DA
	WSSA.	Herbicides: Chemical Structures	2007				X				NR
	Xie L, Thrippleton k, Irwin MA, et al.	Evaluation of estrogenic activities of aquatic herbicides and surfactants using an rainbow trout vitellogenin assay.	2005	X		X	X			X	ECO
	Yeary RA.	Urinary excretion of 2,4-D in commersial lawn specialists.	1986			X					PL
	Zeljezic D, Garaj-Vrhovac V.	Chromosomal abberrations, micronuclei and nuclear buds induced in human lymphocytes by 2, 4-dichlorophenoxyacetic acid pesticide formulation.	2004	X		X					MUT

* studies numbered alphabetically

Appendix B:
Hazard Data Summaries*

*(Study #) refers to the number listed in Appendix A Data Summary table

BIN: NEUROTOXICITY

*(Study 7) **Citation**: Bortolozzi, A.; Duffard, R; and Evangelista de Duffard A (2003). Asymmetrical Development of the Monoamine Systems in 2, 4-Dichlorophenoxyacetic acid (2,4-D) Treated Rats. NeuroToxicology 24: 149-157. **[Human Studies Considerations Apply: No]**

Principal Endpoint: [Bin: Neurotoxicity]

Methods Summary: Pregnant Wistar rats administered 2,4-D *via* diet (0, 70 mg 2,4-D/kg/day) from gestation day (GD) 16 to post partum day (PND) 23; litters culled to 8/litter on PND 0; after weaning, 2,4-D pups were administered untreated diet (T1) or maintained on 2,4-D diet up to PND 90 (T2). Neonates sacrificed by decapitation; brain areas removed were prefrontal cortex (PFc), striatum (St), nucleus accumbens (NAc), substantia nigra (SN), ventral tegmental area (VTA), midbrain (Mb, which did not involve separate analysis of SN and VTA) and cerebellum (Cer), which was separated from the pons medulla. After the cerebellum had been removed from the brainstem, the midbrain was separated by a cut at the level of the inferior colliculus; levels of endogenous monoamines and their metabolites were measured [noradrenaline (NA), dopamine (DA), 3,4-dihyrdoxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindolacetic acid (5-HIAA).

Results Summary: 5-HT was increased in several brain areas (both sexes) in both groups (PFc, striatum, midbrain) and in 2,4-D post weaning group (SN, cerebellum). Alterations (↑↓) in NA, DA, DOPAC, HVA were observed in various brain areas and in both sexes (with and/or without 2,4-D post weaning treatment).

Table 1. Effects of pre-/postnatal exposure on NA, DA, DOPAC, and HVA of 90-day-o□d rat brain areas

Group	NA		DA		DOPAC		HVA	
	male	female	male	female	Male	Female	male	Female
PFc								
T1	-	-	↓45%	↓32%*	↓42%**	↓55%**	-	-
T2	-	-	↓54%	↓45%**	↓58%**	↓77%**	↓43%**	↓46%**
Striatum								
T1	-	-	-	-	-	-	-	-
T2	-	-	-	↑30%*	-	↑63%**	-	-
Nac								
T1	-	-	-	-	-	-	-	-
T2	-	-	-	-	↑30%*	↑30%*	↑71%**	↑64%**
Mid								
T1	-	-	-	↓41%*	↓38%*	↓41%*	-	-
T2	-	-	↓26%*	↓62%**	↓43%*	↓60%**	-	-
SN								
T1	-	-	-	-	-	-	-	-
T2	↑43%*	↑50%*	↑57%**	↑30%*	-	-	-	-
VTA								
T1	-	-	-	-	-	↓30%*	-	-
T2	-	-	↓37%*	↓58%**	↓48%*	↓72%**	↓59%**	↓59%**
Cerebellum								
T1	-	-	-	↓46%**	-	-	-	-
T2	↓29%*	↓□1%**	↑□0%**	↓69%**	↓40%*	↓47%**	-	-

% ↑↓; * p<0.05; ** p<0.01

Table 2. Effect□ of pre- and postnatal exposure on 5-HT and 5-HIAA levels of 90-day-old rat brain areas

	5-HT		□-HIAA	
	male	Female	Male	Female
PFc				
T1	↑23%*	↑49%*	-	-
T2	↑23%*	↑37%*		-
Striatum				
T1	↑24%*	↑34%*	-	-
T2	↑35%*	↑51%*	↑43%**	↑37%**
Nac				
T1	-	-	-	-
T2	-	-	-	-
Midbrain				

T1 T2	↑30%* ↑39%*	- ↑29%*	- -	- ↑29%*
SN				
T1 T2	- ↑36%*	- ↑69%**	- -	- -
VTA				
T1 T2	- -	- -	- -	- -
Cerebellum				
T1 T2	- ↑36%*	- ↑36%*	- -	-

% ↑↓; * p<0.05; ** p<0.01

CONCLUSIONS: according to authors, results support view that during the critical period in which monoamines can influence cell development, neonatal exposure to 2,4-D produces alterations on these neurotransmitter systems; equated to previous findings expressed in spontaneous circling activity towards the right side by adult offspring of dams exposed to 2,4-D, indicating a correlation between monoamines brain level alterations and this behavior. Subchronic 2,4-D exposures modify neurotransmitter levels in cerebral brain areas that are involved in neurodegenerative diseases like Parkinson and schizophrenia.

COMMENT/HED**:** The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. Study does not provide any dose-response information, since only dose was tested (70 mg/kg/day) and it exceeds rat renal clearance levels (overwhelms the body's ability to excrete 2,4-D). It does not provide information regarding potential effects of 2, 4-D on neurotransmitters at dose levels below the saturation level for renal clearance. An extended 1-generation reproductive toxicity study on 2,4-D was submitted to Agency in February of 2010, in response to the data requirements identified in the 2005 RED, included a developmental neurotoxicity assessment. The young rat was exposed during gestation, lactation *via* the milk, and directly *via* the diet following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), landing foot splay, grip strength, motor activity, habituation, and acoustic startle response (ASR). The young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry, and a special stain was used to evaluate brain myelination. Three dose levels were tested. At the highest dose (82 mg/kg bw/day in males/59 mg/kg bw/day in females), there were no treatment-related adverse effects on any of the numerous parameters evaluated to assess the developmental neurotoxicity potential of 2, 4-D. The parental rats were assessed for clinical signs of toxicity and maternal behavior, among other parameters. Maternal behavior towards the offspring was comparable among the groups, and there were no clinical signs, such as circling to the right, observed in the dams or offspring. Although monoamine levels were not monitored in this study, neonatal exposure was similar to that in the cited study, and the three dose levels (intended dose levels of 5, 15, 30/40 mg/kg/day) provide dose-response information and were adequate (approaching saturation of renal clearance). The extended 1-generation reproductive toxicity study of 2, 4-D addresses the neurotoxicity/developmental neurotoxicity concerns raised by NRDC.
The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

CITED BY: NYS DOH – cited as additional studies that should be considered in any new assessment along with those identified in the NRDC petition.

(Study 10) **Citation**: Bortolozzi, A.; Duffard, R; and Evangelista de Duffard A (1999). Behavioral Alterations Induced in Rats by a Pre- and Postnatal Exposure to 2, 4-Dichlorophenoxyacetic Acid. NeuroToxicology and Teratology 21 (4): 451-465. [**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**

Methods Summary: Pregnant Wistar rats administered 2,4-D *via* diet (0, 70 mg 2,4-D/kg/day) from gestation day (GD) 16 to post partum day (PND) 23; litters culled to 8/litter on PND 0; after weaning, 2,4-D pups were administered untreated diet (T1) or maintained on 2,4-D diet up to PND 90 (T2). Parameters procedures: physical/sexual development (fur growth, eye opening, testes descent, vaginal opening); surface righting (on PND 1, 3, 5, 6, 8, 10; considered a reflection of subcortical maturation); negative geotaxis (tests labyrinthine and cerebellar integration, from PND 2-16); hindlimb support (tests neuromuscular and locomotor development. PND 9-23); open field tests (measures activity and habituation, PND 12, 15, 19, 21; PND 30, 45, 60, 75, 90; # crosses, rearings, vertical head movements, grooming episodes); 5-HT syndrome behaviors [forepaw movements (treading, excessive placement or tapping), hunching (back arching), sprawling of limbs, backing, hindlimb abduction, mobility (ability for locomotion

despite gait abnormalities, including paddling), abnormal head movements (vertical or lateral side-to-side repetitive movements), wet-dog shakes (including head shakes), lying on side, limb extension dystonic, and flat body posture]; circling behavior (PND 60, 75, 90); measurement of catalepsy (PND 30, 60, 90).

Results Summary: ontogeny of righting reflex delayed (days 1, 3, 5, 6 only); negative geotaxis (longer mean latency of 2,4-D pups from PND 1-14); hindlimb support times significantly decreased (days 11-15 only); open field (exploratory activity) behavior: # of crosses, vertical head movements, rearings, groomings increased in 2,4-D pups compared to controls (PND 12-21); during PND 60-90, # crosses and rearings were decreased compared to control (both sexes), and males showed increased # grooming PND 60-90, compared to control. 5-HT syndrome behaviors: 2,4-D induced distinctive motor syndrome: increased forepaw tapping, stiff or elevated tail, mobility, and sprawling of limbs (PND 1-7; not observed in adults PND 60 and 90 except stiff tail);

Table 1. 5-HT Syndrome Behaviors

	Male	Female
Flat body posture		
PND 30		
C	0	0
T1	0	0
T2	0	0
PND 60		
C	0	0
T1	0	0
T2	1.10±0.25*	0.82±0.20*
PND 90		
C	0.25±0.08	0.15±0.09
T1	0.22±0.13	0.12±0.09
T2	2.60±0.28*	1.58±0.58*
Hunching		
PND 30		
C	0.50±0.12	0.58±0.18
T1	0.46±0.10	0.54±0.11
T2	1.12±0.33*	0.98±0.25*
PND 60		
C	0	0
T1	0	0.75±0.19
T2	0.91±0.33*	1.57±0.22*
PND 90		
C	0	0
T1	0	0
T2	1.15±0.54*	2.19±0.39*
Stiff tail		
PND 30		
C	0.32±0.10	0.29±0.05
T1	0.39±0.08	0.41±0.19
T2	2.32±0.65*	1.98±0.33*
PND 60		
C	0.29±0.02	0.33±0.01
T1	0.55±0.03	0.45±0.12
T2	1.95±0.23*	2.06±0.42*
PND 90		
C	0.30±0.09	0.28±0.05
T1	0.41±0.15	0.55±0.10
T2	2.10±0.25	2.22±0.39*
Forepaw treading		
PND 30		

C	0.31±0.09	0.28±0.10
T1	0.35±0.05	0.32±0.01
T2	0.52±0.07	0.58±0.07
PND 60		
C	0	0
T1	0	0
T2	0	0
PND 90		
C	0	0
T1	0	0
T2	0	0
Head movement		
PND 30		
C	1.25±0.38	0.95±0.15
T1	1.38±0.58	1.52±0.60*
T2	2.00±0.32*	1.89±0.25*
PND 60		
C	0	0
T1	0	0
T2	0	0
PND 90		
C	0	0
T1	0	0
T2	0	0

* $p<0.05$; maximum score of 4; mean±SEM; data from Table 2 (page 459)

CONCLUSIONS: 2,4-D exposure at a dose producing no overt signs of toxicity may cause long-term alterations in functional state of rat CNS, detected through a neurobehavioral test battery; exposure during pre- and postnatal development induces behavioral abnormalities that seem to disappear in adulthood after 2,4-D cessation**;** mechanism of 2,4-D neurotoxicity not yet known

COMMENT/HED**:** The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. Study does not provide any dose-response information. The only dose used (70 mg/kg/day) exceeds rat renal clearance levels and does not provide information regarding potential effects of 2, 4-D on neurotransmitters at dose levels below the saturation level for renal clearance. An extended 1-generation reproductive toxicity study on 2,4-D was submitted to Agency in February of 2010, in response to the data requirements identified in the 2005 RED, which included a developmental neurotoxicity assessment. Offspring were exposed to 2, 4-D during gestation, lactation *via* the milk, and directly *via* the diet through PND 22, 70, 139, and the developmental neurotoxicity component of the study included neurobehavioral tests at dose levels that do not saturate metabolic processes. Parameters evaluated in the study include an assessment of developmental neurotoxicity (including FOB observations, landing foot splay, grip strength, motor activity, acoustic startle response, habituation, and a neuropathological assessment). Also evaluated were potential effects in offspring on survival, growth, development of immune and reproductive systems; endocrine and selected systemic toxicity parameters, estrous cycle evaluation, evaluation of sperm parameters, thyroid hormone assessment; anogenital distance, nipple retention and puberty onset (vaginal opening and preputial separation. There were no treatment-related adverse effects on any of the numerous parameters evaluated to assess neurotoxicity potential, behavior, physical development and sexual development following 2, 4-D exposure. Most of the same parameters evaluated in the cited study were evaluated in the extended 1-generation reproductive toxicity study at dose levels (intended dose levels of 5, 15, 30/40 mg/kg/day) considered appropriate (approaching saturation of renal clearance). The cited study does not identify a more sensitive endpoint for human risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

CITED BY NYS DOH – cited as additional studies that should be considered in any new assessment along

with those identified in the NRDC petition.

(Study 9) **Citation**: Bortolozzi, AA; Evangelista de Duffard A.; Duffard, R; and Antonelli, MC (2004). Effects of 2, 4-Dichlorophenoxyacetic acid Exposure on Dopamine D_2–Like Receptors in Rat Brain. Neurotoxicology and Teratology 26: 599-605. [**Human Studies Considerations Apply: No**]
Principal Endpoint: **[Bin: Neurotoxicity]**
Methods Summary: Pregnant Wistar rats administered 2,4-D *via* diet (0, 70 mg 2,4-D/kg/day) from gestation day (GD) 16 to post partum (PND 23); litters culled to 8/litter on PND 0; after weaning, 2,4-D pups were administered untreated diet or maintained on 2,4-D diet up to PND 90. Neonates sacrificed at PND 6, 15, 30, 45, or 90 for membrane receptor binding assays employing [^{3} H] nemonapride.
Results Summary: subchronic oral exposure increased DA D_2-type receptor 40% in Cpu; DA D_2-type receptor levels increased in PFc (15 and 30 days) and Cer (30 and 90 days); females more affected than males. When 2,4-D exposure stopped (T1), DA D_2–type receptor density was apparently recovered and stabilized to control level; suggest reversible vulnerability of D_2–type receptors to 2,4-D exposure.
CONCLUSION: Regional increases in D_2–type receptor density may explain certain behaviors, such as catalepsy and right-turning preference in 2,4-D exposed rats reported earlier.
COMMENT/HED**:** The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. Study does not provide any dose-response information. The only dose used (70 mg/kg/day) exceeds rat renal clearance levels and does not provide information regarding ontogeny of dopaminergic D_2 –type receptors in prefrontal cortex (PFc), striatum (CPc), hippocampus (H), and cerebellum (Cer) and potential effects of 2, 4-D on neurotransmitters at dose levels below the saturation level for renal clearance. An extended 1-generation reproductive toxicity study on 2,4-D (MRID 47972101) was submitted to Agency in February of 2010, in response to the data requirements identified in the 2005 RED, which included a developmental neurotoxicity assessment. Offspring were exposed to 2, 4-D during gestation, lactation *via* milk, and directly *via* the diet through PND 22, 70, 139, and the developmental neurotoxicity component of the study included neurobehavioral tests at dose levels that do not saturate metabolic processes. Parameters evaluated in the study include an assessment of developmental neurotoxicity (including FOB observations, landing foot splay, grip strength, motor activity, acoustic startle response, habituation, and a neuropathological assessment). The neuropathology/histopathological assessment included cross-sections of the olfactory bulb, cerebrum (frontal, parietal, temporal and occipital lobes), thalamus/hypothalamus, midbrain, pons, medulla oblongata, and cerebellum; sections were prepared from the trigeminal ganglion and nerve, pituitary gland, eyes with optic nerves, spinal cord (cervical and lumbar), olfactory epithelium, and skeletal muscles (gastrocnemius and anterior tibial); and sections containing representative sections of the cerebellum and corpus callosum were stained to assess myelin. Spinal nerve roots (cervical and lumbar), dorsal root ganglia (cervical and lumbar), and peripheral nerves (sciatic, tibial (proximal and distal (muscular) and sural) were examined also. There were no treatment-related adverse effects on any of the numerous parameters evaluated to assess neurotoxicity potential. The behaviors being addressed in the cited study were not replicated in the extended 1-generation reproductive toxicity study at dose levels (5, 15, 30/40 mg/kg/day) considered appropriate (approaching saturation of renal clearance). The cited study does not identify a more sensitive endpoint for human risk assessment than already identified by the Agency. Additionally, the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D.
CITED BY NYS DOH – cited as additional studies that should be considered in any new assessment along with those identified in the NRDC petition.

(Study 8) **Citation**: Bortolozzi, A.; Evangelista de Duffard A.; Dajas, F.; Duffard, R; and Silveira, R. (2001). Intracerebral Administration of 2, 4-Dichlorophenoxyacetic acid Induces Behavioral and Neurochemical Alterations in the Rat Brain. NeuroToxicology 22: 221-232. [**Human Studies Considerations Apply: No**]
Principal Endpoint: **[Bin: Neurotoxicity]**
Methods Summary: ***Intrastriatal injection***: male Wistar rats received 100 µg of 2,4-D in 1 µL DMSO into one striatum; Accumbens (NAc) injection: 100 µg of 2,4-D in 0.5 µL DMSO into one NAc; Medial Forebrain Bundle (MFB) injection: 50 µg of 2,4-D in 0.5 µL DMSO into one MFB or 8 µg of 6-OHDA dissolved in 0.5 µL saline into one MFB; estimated 2,4-D concentrations in each brain area (striatum, Nac,

and MFB) were 2-4 mM, 40-100 fold the concentration after systemic treatment (although concentration in each brain area seems high, 2,4-D penetration rate into the brain is low)
Results Summary: 2,4-D injected into 1 striatum produced a marked depression in locomotor activity and elicited a moderate circling towards the ipsilateral side at 6 and 24 hours post dose; these changes were accompanied by a ↓ of 5-HT and an ↑ in homovanillic acid (HVA) levels; administration into the nucleus accumbens induced similar behavioral and neurochemical patterns, although rats did not present notorious turning
CONCLUSION: neurotoxic effects of 2,4-D on basal ganglia by interacting with the monoaminergic system depended not only on the exact location of injection, but also on the dose and time period post-injection; toxicity produced by 2,4-D appears to be different in monoaminergic terminals, axonal fibers, and cell bodies
COMMENT/HED**:** The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. The route of exposure is not appropriate; human exposure would not be by direct injection into the brain; unknown relationship between the dose injected into various areas of the brain and that attained following oral/dermal/inhalation exposures; provides potential mode of action information (mechanistic assessment). An extended 1-generation reproductive toxicity study on 2,4-D was submitted to Agency in February of 2010, in response to the data requirements identified in the 2005 RED, which included a developmental neurotoxicity assessment. Offspring were exposed to 2, 4-D during gestation, lactation, and directly *via* the diet through PND 22, 70, 139, and the developmental neurotoxicity component of the study included neurobehavioral tests at dose levels that do not saturate metabolic processes. Parameters evaluated in the study include an assessment of developmental neurotoxicity (including FOB observations, landing foot splay, grip strength, motor activity, acoustic startle response, habituation, and a neuropathological assessment). Also evaluated were potential effects in offspring on survival, growth, development of immune and reproductive systems; endocrine and selected systemic toxicity parameters, estrous cycle evaluation, evaluation of sperm parameters, thyroid hormone assessment; anogenital distance, nipple retention and puberty onset. There were no treatment-related adverse effects on any of the numerous parameters evaluated to assess neurotoxicity potential, behavior, physical development, and sexual development following 2, 4-D exposure post-natally. Although monoamine levels were not monitored in this study, the route of exposure (oral) was appropriate for human health risk assessment and the dose levels (5, 15, 30/40 mg/kg/day) were adequate (approaching saturation of renal clearance). The circling behavior noted in the cited study was not replicated in the extended 1-generation reproductive toxicity study. The cited study does not identify a more sensitive endpoint for human risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.
CITED BY NRDC (35) – rodent studies have revealed a region-specific neurotoxic effect on basal ganglia of the brain, resulting in an array of effects on critical neurotransmitters and adverse effects on behavior. NRDC cited the study, among others, as evidence that postnatal exposures to 2, 4-D during the critical period of development of the infant brain raise serious scientific concerns.
CITED BY Task Force – The Task Force states that this study cited by NRDC was not a developmental neurotoxicity study but a very high dose adult toxicity study where 2,4-D was directly administered into the rat's brain, which is a route not relevant to human exposures. They also pointed out that the estimated concentrations of 2, 4-D in each brain area were 2-4 mM, which the author defined as 40- to100-fold the concentration in brain after systemic treatment.

(Study 15) **Citation**: Brusco, A.; Saavedra, JP; Garcia, G.; Tagliaferro, P.; Evangelista de Duffard A.; and Duffard, R (1997). 2, 4-Dichlorophenoxyacetic Acid Through Lactation Induces Astrogliosis in Rat Brain. Molecular and Chemical Neuropathology 30: 175-185. [**Human Studies Considerations Apply: No**]
Principal Endpoint: **[Bin: Neurotoxicity]**
Methods Summary: Female Wistar rats (dams) administered 2,4-D i.p. (DMSO, 70 mg 2,4-D/kg/day) from PND 9-25; litters culled to 10/litter on PND 0; neonates sacrificed at PND 25; brain and brainstem removed. Immunocytochemical staining was performed using an anti-GFAP and anti-S-100 protein as

primary antibodies.

Results Summary: Comparison of astroglial immunoreactivity in mesencephalon, cerebellum, and hippocampus of 25-day old pups exposed to 2,4-D through mother's milk was made using a quantitative immunohistochemical analysis. A glial reaction was detected at the level of serotonergic nuclei and extreme astrogliosis in the hippocampus and cerebellum. Specific markers were GFAP and S-100 protein. There was a significant increase in number, size, number of processes, and density of immunostaining in 2,4-D exposed pups.

►goal of study was to detect the effect of 2,4-D on astrocytes in different CNS regions of rats exposed to 2,4-D during lactation using 2 rat astroglial markers; paper indicates that one of the most widely documented cellular reactions following nervous system damage has been hypertrophy of astrocytes, often referred to as a reactive gliosis or astrogliosis.; it is characterized by rapid synthesis of glial fibrillary acidic protein (GFAP) intermediate filament, which is a sensitive and early biomarker of neurotoxicity; another rat astroglial marker, the S-100 protein, appeared confined to the cytosol of both normal and reactive astrocytes

Mean Values of Area and F-Shape of Astrocytes Immunolabeled w/ Anti-GFAP or Anti-S-100 Protein Hippocampus			
Immunostaining for	# astrocytes	Area, μm^2 of each astrocyte	F-shape
GFAP Control 2,4-D	 14 31	 39.9±15.0 53.3±21.5**	 0.30±0.14 0.43±0.12**
S-100 Control 2,4-D	 9 22	 26.5±8.4 29.8±12.6	 0.60±0.12 0.68±0.11*
Mean Values of # of Astrocytes/Field (Ast/A), Maximum Diameter (MD) in Granular Layer and Distance Between Bergmn Fibers (DBf) in Molecular Layer - Cerebellum			
	DBf, µm	Ast/A	MD
Control 2,4-D	15.12±4.82 (n=25) 9.40±4.09*** (n=20)	4±1.41 (n=8) 12±1.41** (n=21)	10.31±2.94 (n=8) 13.47±3.6□♪ (n=21)

♪ p<0.025; * p<0.05; ** p0.001; *** p<0.0001;

CONCLUSION: exposure to 2,4-D during first days of life modifies the astroglial cytoarchitecture in parallel with previous neuronal changes observed

COMMENT/HED**:** The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. The study does not provide any dose-response information (i.p. to dams, pups exposed *via* milk), and the dose to pups is unknown. The only dose tested (70 mg/kg/day) would be expected to exceed rat renal clearance levels. The extended 1-generation reproductive toxicity study on 2,4-D, as described previously, in which offspring were exposed to 2, 4-D during gestation, lactation, and directly *via* the diet through PND 22, 70, 139, includes an assessment of developmental neurotoxicity (neurobehavioral tests) at dose levels up to those that saturate metabolic processes. The young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluations and brain morphometry, and a special stain was used to evaluate brain myelination. There were no treatment-related adverse effects on any of the numerous parameters evaluated to assess neurotoxicity and neuropathology potential following 2, 4-D.

The cited study does not identify a more sensitive endpoint for human risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

CITED BY NYS DOH: identified as additional studies that should be considered in any new assessment along with those identified in the NRDC petition.

(Study 24) **Citation**: Duffard, R.; Bortolozzi, A; Ferri, A.; Garcia, G.; and Evangelista de Duffard A. M. (1995). Developmental Neurotoxicity of the Herbicide 2,4-Dichlorophenoxyacetic Acid. Neurotoxicology 16 (4): 764. [**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**
Methods Summary: offspring of dams treated (i.p) with 70 mg/kg/day 2,4-D/kg/day from GD 16 to weaning (PND 21). Offspring treated with control or 2,4-D via diet until adult life. Vaginal smears monitored, prolactin and progesterone measured. Citation is an ABSTRACT (few details of methods).
RESULTS: both pup groups exposed to 2,4-D *via* milk from GD 16-PND 21; then to control diet or to 2,4-D via diet. Seraprolactine and progesterone levels slightly increased in both groups; increased number and size of corpus luteum on the ovary of both 2,4-D treated groups, but only in the 2,4-D fed rats was the estrus cycle arrested in diestrous, judged from the presence of an assortment of the cell types (predominantly leucocytes and nucleated cells) in the vaginal smears.
CONCLUSION: Authors stated that these preliminary results demonstrate a 2,4-D adverse effect on the endocrine system.
COMMENT/HED: ***only an abstract***. Study was binned according to citation title; however, effects described relate to endocrine issues (see discussion under Endocrine BIN). Dose to pups *via* milk during this time interval unknown. Study does not provide any dose-response information. The only dose used (70 mg/kg/day) would be expected to exceed rat renal clearance levels and does not provide information regarding potential effects of 2, 4-D on estrus cycle, hormone levels at dose levels below the saturation level for renal clearance. The extended 1-generation reproduction study on 2, 4-D examined potential effects on female reproductive function, including endocrine and systemic toxicity parameters, such as estrous cyclicity (female adult rats and offspring), puberty onset, ovarian weight, histopathology of the ovary. No treatment-related effects were observed. The cited study does not identify a more sensitive endpoint for human risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.
CITED BY NRDC (25) – one of several studies cited as demonstrating endocrine disrupting effects of 2,4-D
CITED BY Task Force – cited as one of a series of studies that NRDC contends provides evidence of 2,4-D endocrine activity; TF does not consider study as providing compelling evidence of potential environmental or human endocrine health concerns; was conducted at high dose (well above saturation of renal clearance) and thus of limited value for risk assessment.

(Study 25) **Citation**: Duffard, R.; Garcia, G.; Rosso, S.; Bortolozzi, A; Madariaga, M.; diPaolo, O; and Evangelista de Duffard A. (1996). Central Nervous System Myelin Deficit in Rats Exposed to 2,4-Dichlorophenoxyacetic Acid Throughout Lactation. NeuroToxicology and Teratology 18 (6): 691-696. [**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**

Methods Summary: offspring (8 pups/dam) of dams treated with 100 mg/kg/day 2,4-D/kg/day once a day (i.p.) (1) for 16 days (PND 9-25); (2) for 6 days (PND 9-15) then treated with DMSO until PND 25; (3) for 10 days (PND 15-25); from PND 9-15, dam treated with DMSO; (4) control treated with DMSO for 16 days (PND 9-25). Neonates were sacrificed on PND 25; brains were removed and weighed, and myelin markers (monohexosylceramide, phospholipids, free fatty acids, cholesteryl esters) were assessed.

RESULTS: Brain weight was not affected but there was a decrease in body weight in groups exposed before PND 15. A decrease in total lipids was observed in PND 9-25 pups, and changes were noted in lipid brain composition (Tables 1 and 2); pups exposed *via* milk

Table 1. Body and Brain Weights; Lipids and Protein in Brain

Control	**PND 9-25**	**PND 9-15**	**PND 15-25**
Body Weight (grams)			
43.94±1.55	28.56±0.95 (↓35%)	34.02±1.09 (↓23%)	45.3±1.23
Brain (wet weigh□,□g)			
1.26±0.01	1.18±0.02	1.25±0.02	Nd
Total lipids (mg/g w.wt.) in Brain			
0.07±0.01	0.03±0.02** (↓58%)	0.06±0.03	Nd
Total proteins (mg□g w.wt) □n Brain			

58.42±1.1	□7.32±2.56	53.97±4.70	Nd

*p <0.01; mean±SEM; nd no data provided

Tab□e □. Individual □ipids Content in Br□in of 25-Day-old Pups

chemical assay♪	Control	PND 9-25	PND 9-15
Total cholesterol	9.58±1.01	11.60±0□88	7.56±□.52
Cholesterol	9□3□±0.79	8.30±0.88	7.58±□.23
Cholesterol esters	0.65±0.013	2.36±0.32** (↑363%)	0.54±0.1
F□tty acids, pho□pholipids	13.10±0.3	9.87±0.69** (↓25%)	11.28±0.95
Phospholipids	314.09±18.92	192.67±36.45** (↓39%)	362.00±23.37
Free fatty acids	340.00±35.90	128.45±10.86** (↓62%)	148.13±61.21** (↓56%)

*p <0.01; mean±SEM; ♪mg/g wet weight;

Histochemical study demonstrated a decreased myelin staining in corpus callosum, cortical grey matter, and cerebellum of 25-day old rats exposed to 2,4-D from PND 15; myelin staining appeared normal in rats exposed before PND 15. Concentration of galactolipids (cerebrosides and sulphatides, both sensitive and quantitative chemical markers for myelin sheaths in CNS development) was significantly reduced in PND15-25 pups compared to control (Table 3).

Table 3. Myelin Marker Lipid Content in Brain of 25-day-old Pups Treated PND 15-25

Group	Monohexosylceramide	Sulphatides
Control	766.56±12.55	433.17±15.20
2,4-D (15-25 days)	633.60±27.48* (↓17%)	195.64±6.17** (↓45%)

* p<0.05; ** p<0.01; mean±SEM; µg/g wet weight

CONCLUSION: Myelin deficit in pups nursed from PND 9-25 or PND 15-25; in latter group, body and brain weight were not affected, so deficit in myelin cannot be attributed to undernourishment. In both groups, there was a decrease in brain total lipids, phospholipids, FFA content, and appearance of cholesteryl esters (presence of cholesteryl esters usually indicates myelin breakdown); a clear deficit in myelin staining and myelin chemical markers.

COMMENT/HED: The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. The only dose tested (100 mg/kg/day) was administered i.p. to dams, which is not relevant for human health pesticide risk assessment (dose *via* milk to pups), exceeds rat renal clearance levels and does not provide information regarding potential effects of 2, 4-D on myelination at dose levels below the saturation level for renal clearance. An extended 1-generation reproductive toxicity study on 2, 4-D, in which offspring were exposed to 2, 4-D during gestation, lactation *via* the milk, and directly *via* the diet through PND 22, 70, 139, includes an assessment of developmental neurotoxicity and neuropathology (including an evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation). The cited study was previously considered by the Agency in its rationale for additional toxicology data (DNT) in the RED. In the extended 1-generation reproductive toxicity study, there were no treatment-related adverse effects on myelination following 2, 4-D exposure at dose levels (5, 15, 30/40 mg/kg/day) considered appropriate (approaching saturation of renal clearance). A NOAEL of 59 mg/kg/day (actual intake) was identified for effects on myelination, which is 2-11 times greater than the toxicity endpoints utilized for the human health risk assessment supporting the 2, 4-D RED. The changes in myelination in the cited study were not replicated in the extended 1-generation reproductive toxicity study.

The cited study does not identify a more sensitive endpoint for human risk assessment than already identified by the Agency, and the results of the cited study do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

CITED BY NRDC (37) – cited as showing potential effects on the brain of neonatal rats exposed postnatally; 2,4-D appears to impair normal deposition of myelin in developing brain; cites additional studies that identify adverse effects in offspring after lactational exposure and states that EPA failed to include any lactational exposure in its aggregate risk assessment; concludes that effects of 2,4-D make it

likely that fetuses, infants, and children will be more susceptible to long-term adverse health effects from exposure to 2, 4-D.
CITED BY Task Force – study conducted at high doses (well above pharmacokinetic nonlinearity) and included use of an unrepresentative route and mode of administration (intraperitoneal), which would further amplify nonlinear pharmacokinetic performance. The Task Force also considered this paper and other NRDC cited papers from the same group of investigators to be methodologically flawed [litter was not used as the unit for statistical analyses (to avoid bias due to litter effects); the reporting of histopathological evaluation limited, etc.].
CITED BY NCASI – referenced as one of the studies cited in the petition as supporting claim that infants are at risk; argues that doses used in study are extremely high and likely exceed renal clearance levels and are in range known to produce frank maternal toxicity, which is inappropriate dose selection for developmental toxicity studies; considers that fact that high doses are needed to elicit the reported effects supports previous EPA conclusion that 2,4-D use according to label direction does not pose a risk to humans

(Study 26) **Citation**: Evangelista de Duffard A.; Bortolozzi, A.; and Duffard RO (1995). Altered Behavioral Responses in 2,4-Dichlorophenoxyacetic Acid Treated and Amphetamine Challenged Rats. *NeuroToxicology* 16 (3): 479-488. [**Human Studies Considerations Apply: No**]
Principal Endpoint: **[Bin: Neurotoxicity]**
Methods Summary: Wistar rats (i.p.); doses: 2,4-D: 0, 50, and 100 mg/kg; 0, 5, 10 mg/kg amphetamine (acute); 0.9% saline solution; each 2,4-D dose group challenged with both levels of amphetamine and groups of both dose levels of each compound alone
RESULTS: various components of serotonin syndrome (behavioral response induced by stimulation of 5-HT receptors) observed in rats after acute dose of 2,4-D plus amphetamine; *not with 2,4-D alone*.
CONCLUSION: 2,4-D appears to act through serotonergic and dopaminergic mechanisms
COMMENT/HED: route of exposure not relevant for human health pesticide risk assessment; dose levels used (50 and 100 mg/kg/day) are at or exceed rat renal clearance levels following oral exposure. The study is one among several studies cited from the same researchers that were performed to assess the mechanism of 2, 4-D neurotoxicity. An extended 1-generation reproductive toxicity study on 2, 4-D, in which the parental rats and/or offspring were exposed to 2, 4-D during the pre-mating period, gestation, lactation *via* the milk, and directly *via* the diet through PND 22, 70, 139, includes an assessment of developmental neurotoxicity (including neurobehavioral tests) and neuropathology at dose levels that do not saturate metabolic processes (as described previously). No treatment-related adverse effects were observed on parental behavior or on any of the numerous parameters evaluated to assess developmental neurotoxicity potential following oral exposure to 2, 4-D.The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results of the cited study do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.
CITED BY NRDC (34) – cited as evidence that 2,4-D results in delays in brain development and abnormal behavior patterns, including apathy, decreased social interactions, repetitive movements, tremors, immobility; females more sensitive
CITED BY TF – study does not assess brain development; finding could be non-specific effects rather than any selective neurological effect
CITED BY NCASI - petition fails to mention that dose levels likely exceed renal clearance levels and route of exposure is not appropriate

(Study 28) **Citation**: Ferri A, Duffard R, Evangelista de Duffard A. (2007). Selective Oxidative Stress in Brain Areas of Neonate Rats Exposed to 2,4-Dichlorophenoxyacetic Acid Through Mother's Milk. *Drug and Chemical Toxicology* 30: 17-30. [**Human Studies Considerations Apply: No**]
Principal Endpoint: **[Bin: Neurotoxicity]**
Methods Summary: PND 0 pups (8 pups/dam); exposed pups of (1) dams treated with 100 mg 2,4-D/kg/day once a day (i.p.) for 16 days (PND 9-25); (2) dams treated with DMSO once a day (i.p.) for 16 days (PND 9-25); (3) dams treated with DMSO and saline once a day (i.p.) for 16 days (PND 9-25).

Neonates sacrificed by decapitation; brain areas were removed and weighed (prefrontal cortex, hypothalamus, hippocampus, striatum, and midbrain, which involved neither separate analysis of the substantia nigra not ventral tegmental area and cerebellum, which was separated from the pons medulla). After the cerebellum had been removed from the brain stem, the midbrain was separated by a cut at the level of the inferior colliculus).

Enzyme and Other Assays: superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities; catalase activity; glutathione content; assay for reactive oxygen species (ROS).

RESULTS: after 16 days of exposure *via* mother's milk, ROS levels increased in the prefrontal cortex (15.7%), striatum (11.1%), and midbrain (17.2%); GSH content decreased in striatum (37.4%) and midbrain (34.0%) of 2,4-D pups compared with basal levels of 25-day-old pups.

Table 1. Reactive Oxygen Species/Glutathione Content/Protective Enzymes

Group	Prefrontal cortex	Striatum	Cerebellum	Hippocampus	Midbrain	hypothalamus
Reactive oxygen species levels (Fl/mg protein) in brain areas of 25-day-old pups lactationally exposed to 2,4-D						
C	18.1±0.6	21.8±0.9	24.3±1.2	19.3±1.4	19.9±1.1	20.9±1.3
VC	17.8 ±0.7	22.6±0.8	24.0±0.9	18.0±1.0	20.3±1.0	22.6±1.2
2,4-D	20.6±□.5*(↑16)	25.1±0.7* (↑11)	23.7±1.1	18.1±1.1	23.8±0.7*(↑17)	21.1±□.3
Glutathione content (µg GSH/mg protein) in brain areas of 25-day-old pups lactationally exposed t□ 2,4-D						
C	1.25±0.35	1.23±0.33	0.74±0.24	0.86±0.24	0.98±0.16	0.99±0.36
VC	1.23±0.29	1.31±0.24	0.79±0.19	0.88±0.19	1.06±0.12	1.07±0.41
2,4-D	1.29±0.20	0.82±0.18*(↓37)	0.80±0.22	0.94±0.24	0.70±0.15*(34)	1.08±0.30
Protective enzymes (mU/mg protein) in brain areas of 25-day-old pups lactationally exposed to 2,4-D						
(Zn,Cu)SOD						
C	2000±180	2430±175	2690±310	2290±180	2100±80	2410±150
VC	1950±200	2460±150	2730±330	2330±200	1950±120	2320±160
2,4-D	2450±3108(↑26)	2540±220	2610±240	2980±320*(↑28)	2140±90	2400±120
Catalase						
C	3100±190	2800±130	2440±165	2480±180	2400±180	2680±160
VC	2950±250	2740±200	2300±130	2580±160	2360±200	2740±150
2,4-D	2200±200*(↓25)	2250±150*(↓18)	2530±170	2690±210	1850±120*(↓22)	2810±210
(Se)GSh-Px						
C	30.5±1.6	28.8±2.3	25.3±1.5	28.3±0.9	32.3±1.0	28.4±1.3
VC	30.0±1.4	28.2±2.1	25.6±9.1	29.1±1.1	31.9±1.1	28.9±1.9
2,4-D	24.2±1.9*(↓19)	22.3±2.9*(↓21)	29.5±2.1*(↑15)	32.3±1.0*(↑11)	27.6±1.0*(↓14)	28.0±1.6
(noSe)GSH-Px						
C	20.5±0.9	18.6±0.7	18.3±1.3	20.9±0.7	22.6±0.9	15.6±1.0
VC	20.8±1.1	18.6±0.6	18.2±1.0	20.9±0.7	22.7±0.8	15.8±0.7
2,4-D	19.0±1.0	15.7±0.5*(↓16)	18.0±1.0	19.9±0.9	19.4±0.8*(↓15)	17.0±0.7

Mean±SEM; VC vehicle control; * $p<0.05$; data from Tables 2 and 3 (pages 24-25)

CONCLUSION: 2,4-D induces disparate alterations on enzymatic activities of defensive mechanisms and/or modifications of the reactive oxygen species levels in specific neonate brain regions. The midbrain, striatum, and prefrontal cortex were the areas where the alterations were more remarkable and with similar tendency. The hippocampus did not suffer many alterations, and the hypothalamus was the area where no changes were observed. Results suggest that the developing brain areas have different susceptibilities to the adverse effect of 2,4-D, especially those areas related to the dopaminergic system, and that oxidative stress is one 2,4-D mechanism of toxicity.

COMMENT/HED: The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. Dams exposed i.p., which is not a relevant mode of administration for human health pesticide risk assessment; pups were exposed *via* milk; do not know dose to the pup. 100 mg/kg/day *via* the oral route exceeds renal clearance levels; identifies possible oxidative stress in pup brain areas following lactational exposure. The extended 1-generation reproductive toxicity study on 2,4-D, as described previously (MRID 47972101), in which offspring were exposed to 2, 4-D during gestation, lactation *via* the milk, and directly *via* the diet through PND 22, 70, 139, includes an assessment of developmental neurotoxicity [functional operational battery (FOB), motor activity, and acoustic startle response (ASR)] at dose

levels up to those that saturate metabolic processes. The young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluations and brain morphometry, and a special stain was used to evaluate brain myelination. There were no treatment-related adverse effects on any of the numerous parameters evaluated to assess neurotoxicity and neuropathology potential following 2, 4-D exposure at dose levels (5, 15, 30/40 mg/kg/day) considered appropriate (approaching saturation of renal clearance). The no observed adverse effect level (NOAEL) for developmental neurotoxicity [(calculated intake: 82 mg/kg/day (males)/59 mg/kg/day (females)] is greater than the PODs utilized for the human health risk assessment supporting the 2,4-D RED. The PODs used in the RED risk assessment are protective of chronic effects identified in the extended 1-generation reproductive toxicity study and of those identified in the cited study.

CITED BY NRDC (61)– cited as support for need to consider lactational exposure to 2,4-D in aggregate RA; lactational exposure of neonatal rats to 2,4-D in maternal milk showed indications of oxidative stress in certain regions of brain (midbrain, striatum, prefrontal cortex); one mechanism of toxicity

CITED BY TF – study conducted at high dose (well above pharmacokinetic nonlinearity) and included use of an unrepresentative route and mode of administration (intraperitoneal), which would further amplify nonlinear pharmacokinetic performance.

(Study 32) **Citation**: Garcia, G.; Tagliaferro, P.; Ferri A, Evangelista de Duffard A.; Duffard, R.; and Brusco, A. (2004). Study of Tyrosine Hydroxylase Immunoreactive Neurons in Neonate Rats Lactationally Exposed to 2,4-Dichlorophenoxyacetic Acid. *NeuroToxicology* 25: 951-957. [**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**

Methods Summary: PND 0 pups (8 pups/dam); exposed pups of (1) dams treated with 70 mg 2,4-D/kg/day once a day (i.p.) for 16 days (PND 9-25); (2) dams treated with 100 mg 2,4-D/kg/day once a day (i.p.) for 16 days (PND 9-25); (3) dams treated with DMSO once a day (i.p.) for 16 days (PND 9-25). Neonates were anaesthetized and sacrificed by perfusion-fixation; brains were removed and weighed; immunohistochemical staining.

RESULTS: Brain and body weight were not affected in the 25-day-old pups nursed by the 70 mg/kg/day dams; decreased body (↓27.5%) and brain (↓13.8%) weights were observed in 25-day-old pups nursed by the 100 mg/kg/day dams. A similar decrease (25%) in TH-IR (tyrosine hydroxylase immunoreactivity) was observed in neurons of the SN (substantia nigra) at both dose levels; VTA (ventral tegmental area) TH neurons showed a decreased immunoreactivity only in 100 mg/kg/day pups (33%). Decreased 5-HT fiber density in SN and VTA was observed at both dose levels

Relative Optical Density of TH neurons in SN and VTA		
Control	70 mg/kg/day	100 mg/kg/day
Relative optical density (ROD) of TH neurons in substatia nigra		
3.21±0.75	2.40±0.71** (↓25%)	2.40±0.62** (↓25%)
Relative optical density (ROD) of TH neurons in VTA		
1.95±0.28	-	1.31±0.25 (↓33%)
Relative Area of 5-HTT-Immunolabeled Fibers in SN and VTA		
Relative area of 5-HTT-immunolabeled fibers in substantia nigra		
0.48±0.012	0.31±0.021 (↓36%)	0.33±0.023 (↓32%)
Relative area of 5-HTT-immunolabeled fibers in VTA		
0.50±0.006	0.36±0.014 (↓28%)	0.39±0.023 (↓22%)
Body weight (g)		
49.3±2.8[A]	45.6±2.6 [A]	35.7±3.0* [A] (↓27.5%)
Brain weight (g)		
1.38±0.05 [A]	1.34±0.06 [A]	1.19±0.05* [A] (↓13.78%)

mean±s.d. or [A] SEM; ** $p<0.01$

CONCLUSION: TH-IR is significantly decreased, probably as a consequence of an inhibitory 5-HT effect. This 2,4-D inhibitory effect on the dopaminergic neurons probably mediated by 5-HT may lead to biochemical and functional consequence like alterations in DA biosynthesis, storage, release, feedback control systems or receptor sensitivity.

COMMENT/HED: The cited study is one of several research studies from the same group of investigators

looking into possible mechanism of 2, 4-D neurotoxicity. In substantia nigra and in ventral tegmental area, a decreased neuron tyrosine hydroxylase immunostaining along with diminution in serotonin (5-HT) fiber density were reported in 2,4-D (lactationally-exposed) pups. Dams were exposed i.p., which is not a relevant mode of administration for human health pesticide risk assessment; pups were exposed *via* milk; do not know dose to the pup. Both dose levels (70 and 100 mg/kg/day) *via* the oral route exceed renal clearance levels. Study identifies effects on pup brain following lactational exposure. The extended 1-generation reproductive toxicity study/DNT study on 2,4-D provides information on 2, 4-D levels in the milk and plasma of the offspring exposed during gestation and lactation *via* the milk, then directly *via* the diet up to PND 139 at dose levels up to those that saturate metabolic processes. There were no treatment-related adverse effects on any of the numerous parameters evaluated to assess neurotoxicity and neuropathology potential following 2, 4-D exposure. The no observed adverse effect level (NOAEL) for developmental neurotoxicity was established at 59 mg/kg/day. This NOAEL is greater than the PODs utilized for the human health risk assessment supporting the 2,4-D RED. The PODs used in the RED risk assessment are protective of chronic effects identified in the extended 1-generation study and of those identified in the cited study.
The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. An exposure pathway identified in the NRDC petition is lactational exposure to 2, 4-D. The Agency currently is addressing the issue of a lack of information regarding the levels of 2, 4-D that can occur in human breast milk. Preliminary studies performed to determine the doses to be used in the extended 1-generation reproductive toxicity study provide data on the levels of 2, 4-D in the milk of the dams and plasma levels of both the dams and offspring. Currently, it cannot be determine what the actual dose (*via* milk) to each pup was due to the lack of information on the amount of milk ingested by the pups.

CITED BY NRDC (62) – study cited as support for need to consider lactational exposure to 2,4-D in aggregate RA; cited as showing potential effects on the brain of neonatal rats exposed lactationally

CITED BY TF – study conducted at high doses (well above pharmacokinetic nonlinearity) and included use of an unrepresentative route and mode of administration (intraperitoneal), which would further amplify nonlinear pharmacokinetic performance.

(Study 33) **Citation**: Garcia, G.; Konjuh, C.; Duffard RO, Evangelista de Duffard A. (2006). Dopamine-β-Hydroxylase immunohistochemical Study in the Locus Coeruleus of Neonate Rats Exposed to 2,4-Dichlorophenoxyacetic Acid Through Mother's Milk. *Drug and Chemical Toxicology* 29, 435-442. [**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**

Methods Summary: PND 0 pups (8 pups/dam); exposed pups of (1) dams treated with 70 mg 2,4-D/kg/day once a day (i.p.) for 14 days (PND 9-22); (2) dams treated with 100 mg 2,4-D/kg/day once a day (i.p.) for 14 days (PND 9-22); (3) dams treated with DMSO once a day (i.p.) for 14 days (PND 9-22). On PND 22, neonates were anaesthetized and transcardially perfused-fixed; brains were removed and weighed; relative optical density (ROD) of the DβH stained LC (locus coeruleus) sections in all rats was measured.

RESULTS: Brain and body weight were not affected in the 22-day-old pups nursed by the 70 mg/kg/day dams; decreased body (20%) was observed in 25-day-old pups nursed by the 100 mg/kg/day dams (brain weight not affected). There was a significant decrease (73% to 75%) in D-β-H immunoreactivity (D-β-Hir) in exposed neonates at both dose levels. Morphological data confirmed these observations: ROD of D-β-H immunoreactivity was 0.200 ±0.025 and 0.172 ± 0.029 at 70 and 100 mg/kg/day, respectively compared to control (0.752 ±0.387).

CONCLUSION: TH-IR is significantly decreased, probably as a consequence of an inhibitory 5-HT effect. This 2,4-D inhibitory effect on the dopaminergic neurons probably mediated by 5-HT may lead to biochemical and functional consequence like alterations in DA biosynthesis, storage, release, feedback control systems or receptor sensitivity; both doses produced similar effect implies that it was maximal

COMMENT/HED: The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. The dams were exposed i.p., which is not a relevant mode of administration for human health pesticide risk assessment; pups were exposed *via* milk; do not know dose to the pup. 70 mg/kg/day and 100 mg/kg/day *via* the oral route exceed renal clearance

levels; identifies effects on pup brain following lactational exposure. An extended 1-generation reproductive toxicity study on 2,4-D was submitted to Agency in February of 2010, in response to the data requirements identified in the 2005 RED, which included a developmental neurotoxicity assessment. The young rat was exposed during gestation, lactation *via* the milk, and directly *via* the diet following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), landing foot splay, grip strength, motor activity, habituation, and acoustic startle response (ASR). The young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry, and a special stain was used to evaluate brain myelination. Three dose levels were tested. At the highest dose (calculated intake: 82 mg/kg bw/day in males/59 mg/kg bw/day in females), there were no treatment-related adverse effects on any of the numerous parameters evaluated to assess the developmental neurotoxicity potential of 2, 4-D. The parental rats were assessed for clinical signs of toxicity and maternal behavior, among other parameters. Maternal behavior towards the offspring was comparable among the groups, and there were no clinical signs such as circling to the right observed in the dams or offspring. Although neurotransmitter levels and brain enzymes were not monitored in the extended 1-generation reproduction study, the dose levels were adequate (approaching saturation of renal clearance), and the no observed adverse effect level (NOAEL) for developmental neurotoxicity is greater than the PODs utilized for the human health risk assessment supporting the 2,4-D RED. The PODs used in the RED risk assessment are protective of chronic effects identified in the extended 1-generation study and of those identified in the cited study.

The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. Additionally, the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. An exposure pathway identified in the NRDC petition is lactational exposure to 2, 4-D. The Agency currently is addressing the issue of a lack of information regarding the levels of 2, 4-D that can occur in human breast milk. Preliminary studies performed to determine the doses to be used in the extended 1-generation reproductive toxicity study provide data on the levels of 2, 4-D in the milk of the dams and plasma levels of both the dams and offspring. Currently, it cannot be determine what the actual dose to each pup was due to the lack of information on the amount of milk ingested by the pups.

CITED BY NRDC (63) – cited as showing potential effects on the brain of neonatal rats exposed lactationally; alterations in brain enzymes levels and neurotransmitters in pups exposed to 2,4-D through maternal milk and as support for the need to consider lactational exposure to 2, 4-D in aggregate RA.

CITED BY TF – study conducted at high doses (well above pharmacokinetic nonlinearity) and included use of an unrepresentative route and mode of administration (intraperitoneal), which would further amplify nonlinear pharmacokinetic performance. The Task Force commented that EPA was aware that 2, 4-D may be present in breast milk (discussed in the 2, 4-D RED). Compared to the high doses used in the animal studies, the Task Force noted that EPA estimated of the maximum dietary exposure from food to human females ages 13-50 to be 0.01018 mg/kg/day and the average exposure to be 0.000642 mg/kg/day (USEPA memorandum, 2002). These values range from 4900 to 1 million times lower than the values in the cited rat study. Additionally, EPA's manner of doing risk assessment for infants is protective of any pesticide exposure to infants from human breast milk because the exposure values for pesticides in cow's milk greatly exceed the values that could be present in breast milk (US Federal Register 2005 at 46706, 46735).

(Study 37) **Citation**: Giasson, BI, Lee, VM. (2000). A New Link Between Pesticides and Parkinson's Disease. Environ Hlth Perspect. 103: 113. [**Human Studies Considerations Apply: No**]

Principal Endpoint: [**Bin: Neurotoxicity]**

Methods Summary: not applicable; paper discusses another paper (Betarbet et al., 2000; rats exposed chronically (i.v.) to rotenone, a mitochrondrial inhibitor), which reports that chronic administration of rotenone can induce the major features of Parkinson's disease in rats; states that this "remarkable observation not only provides a new animal model for the study of PD, but will also reinvigorate interest in the possible role of exposure to pesticides and other toxins as a cause of human neurodegenerative disease."

Results Summary: in the Betarbet paper discussed, rats develop neuropathological and behavioral symptoms of Parkinsonism following *iv* exposure;

CONCLUSION: Giasson concludes that this (Betarbet) study will revitalize the search for environmental toxins, including other pesticides that may contribute to the etiology of this disease.

COMMENT/HED**:** paper is not 2, 4-D specific and i.v. exposure is not a normal route of human exposure; the extended 1-generation reproductive toxicity study (discussed previously) addresses developmental neurotoxicity endpoints, including assessment of motor activity, FOB, myelination. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. Additionally, the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D.

CITED BY: 2,4-D TF: cited in response to NRCD's use of the Ton, et al., 2006 zebrafish paper to support their claim that 2,4-D is associated with developmental neurotoxicity in mammals, including decreased motor activity and Parkinson's-like tremors. TF states that the support for the Ton claim is a "news and view commentary" by Giasson and Lee, 2000, who discuss using the pyrethroid insecticide and pesticide rotenone as a model for Parkinson's disease.

(Study 59) **Citation**: Konjuh, C.; Garcia, G.; Lopez, L.; Evangelista de Duffard A.; Brusco, A.; and Duffard, R. (2008) Neonatal hypomyelination by the herbicide 2,4-dichlorophenoxy acetic acid. Chemical and ultrastructural studies in rats. Toxicol. Sciences 104 (2), 332-340.
[**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**

Methods Summary: Pregnant Wistar rats administered 70 mg 2,4-D/kg/day i.p. (DMSO) to dams on PND 9-22; litters of 14 pups (UN - undernourished) vs litters of 8 pups (WN – well nourished) per litter**;** pups exposure *via* maternal milk (oral)

Results Summary: myelin proteins (analyzed by Western blot and/or immunohistochemical study) showed a significant decrease in WN70, UN, and UN70 with respect to control; UN hypomyelination expressed by a diminution of the number of sheets and the MDLt, whereas 2,4-D hypomyelination had a normal number of sheets but presented altered compaction of myelin sheaths, increased ID and the presence of a higher number of blebs.

Table 1. Myelin Chemical Composition

Treatment	Total proteins (mg/g brain)	Total lipids (mg/g brain)	Cholesterol (mg/g brain)	Galactolipids (mg/g brain)	Phospholipids P (nm P/g brain)
WN	6.0±0.7	683.6±117.2	3.2±0.2	1.00.3	9.1±1.6
WN70	4.3±0.3**	478.6±71.2*	2.0±0.3*	0.6±0.1*	4.9±1.4**
UN	4.5±0.6**	526.2±94.6	1.9±0.2*	0.5±0.2**	6.8±2.1
UN70	3.4±1.0***#	218.3±121.1***#	1.7±0.2*	0.3±0.2**	4.4±1.9***

N=5; * p<0.05; ** p<0.01; *** p<0.001 (WN); #0.001 (UN)

Table 2. Morphometric Study at ultrastructural Level of Myelin around Corpus Callosum Axons

Treatment	# of sheets	ID (nm)	MDL_t (nm)
WN	7.90±1.90	9.12±0.39	2.70±0.12
WN70	6.00±1.50	8.01±0.68	2.18±0.30**
UN	5.60±2.00**	8.82±1.00	2.30±0.17**
UN70	5.90±1.50**	10.83±0.96***	1.78±0.34***

** p<0.01 (WN); *** p<0.001 (UN); MDL_t thickness of major dense line; ID interperiod distance

CONCLUSION: 2,4-D and undernourishment are two independent hypomyelinating factors.

COMMENT/HED: The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. Study does not provide any dose-response information **(**do not know dose to pups). The only dose used (70 mg/kg/day) exceeds rat renal clearance levels following oral exposure and does not provide information regarding potential effects of 2, 4-D on myelination at dose levels below the saturation level for renal clearance. An extended 1-generation reproductive toxicity study on 2, 4-D, in which offspring were exposed to 2, 4-D during gestation, lactation *via* the milk, and

directly *via* the diet through PND 22, 70, 139, includes an assessment of developmental neurotoxicity and neuropathology (including an evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation) at dose levels that do not saturate metabolic processes**.** There were no treatment-related microscopic changes in the central or peripheral nervous systems in the perfused offspring, no exposure-related changes on myelin deposition in the offspring, or changes in microscopic measurements of structures in the cerebral cortex, cerebellum, thalamus, or hippocampus. The no observed adverse effect level (NOAEL) for developmental neurotoxicity is greater than the PODs utilized for the human health risk assessment supporting the 2,4-D RED. The PODs used in the RED risk assessment are protective of chronic effects identified in the extended 1-generation study and of those identified in the cited study.
The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D.

As noted by the Task Force, EPA was aware that 2, 4-D may be present in milk. There was a discussion of this issue in the 2, 4-D RED. Compared to the high doses used in the animal studies, the EPA estimates of the maximum dietary exposure from food to human females ages 13-50 is 0.01018 mg/kg/day and the average exposure is 0.000642 mg/kg/day (USEPA memorandum, 2002). These values range from 4900 to 1 million times lower than the values in the cited rat study. Additionally, EPA's manner of doing risk assessment for infants is protective of any pesticide exposure to infants from human breast milk because the exposure values for pesticides in cow's milk greatly exceed the values that could be present in breast milk (US Federal Register 2005 at 46706, 46735). The Agency currently is addressing the issue of a lack of information regarding the levels of 2, 4-D that can occur in human breast milk. Preliminary studies performed to determine the doses to be used in the extended 1-generation reproductive toxicity study provide data on the levels of 2, 4-D in the milk of the dams and plasma levels of both the dams and offspring. Currently, it cannot be determine what the actual dose to each pup was due to the lack of information on the amount of milk ingested by the pups.

CITED BY NRDC (38)– study referenced as one that shows that 2,4-D appears to impair normal deposition of myelin in developing brain; effects of 2,4-D make it likely that fetuses, infants, and children will be more susceptible to long-term adverse health effects from exposure to 2. 4-D

CITED BY TASK FORCE: excessive doses (ip); only one dose (70 mg/kg/day); addressed undernutrition (14 pups/litter vs 8/litter); small sample size

CITED BY NCASI: petition fails to mention that dose levels likely exceed renal clearance levels and route of exposure is not appropriate

(Study 94) **Citation**: Rosso, SB; Garcia, GB; Madariaga, MJ; Evangelista de Duffard A.; and Duffard, R. (2000). 2, 4-Dichlorophenoxyacetic Acid (2,4-D) in Developing Rats Alters Behaviour, Myelination and Regions Brain Gangliosides Pattern. *NeuroToxicology* 21 (1-2): 155-164. [**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**

Methods Summary: Wistar rats: pups administered 2,4-D (0, 70, or 100 mg/kg/day) intermittently (sc**)**, every 48 hours, 8 pups/dam; exposure from PND 7-25 (100 mg/kg) or PND 12-25 (70 mg/kg); control at PND 25 injected with vehicle (water:ethanol). Neurobehavioral test performed each 48 hrs, 4 hrs before dose (surface righting reflex, negative geotaxis, forelimb support, open field). Histological studies: myelin staining (histochemical and immunohistochemical); gangliosides analyses (GM_1 GD_{1a} GD_{1b} GT_{1b})

Results Summary: at 100 mg/kg, decreased body and brain weight at PND 25; surface righting reflex (took more time to return to normal position from PND 11-21); negative geotaxy (showed major latency from PND 11-21; decreased motor activity (both groups) and rearing frequency (100 mg/kg); alterations in forelimb support (both group); decreased myelin staining; changes in ganglioside levels (Table 2) in various brain areas (both groups).

Table 1. Brain Weight of PND 25 Pups Treated with 100 mg/kg 2,4-D (g)

Control	70 mg/kg	100 mg/kg
1.41±0.03	1.34±0.04	1.19±0.03 ** (↓16%)

** p<0.01; mean±SE

Table 2. Gangliosides Levels in Different CNS Regions

Ganglioside	Control	100 mg/kg	70 mg/kg
GT_{1b}			
Olfactory bulbs	222.4±9.7	174.6±10.3** (↓21%)	23□.3±6.6
Prefrontal cortex	249.5±11.	257.4±9□7	220.0±20.0 (↓12%)
Striatum	254.4±7.77	269.8±12.2	237.0±13.2
Hippocampus	260.1±10.4	282.7±16.8	297.2±21.2
Cerebellum	341.7±13.8	280.8±9.43** (↓18%)	238.0±4.6** (↓30%)
GD_{1b}			
Olfactory bulbs	161.8±9.5	157.□±7.1	196.9±6.7** (↑22%)
Prefrontal□□ortex	2□6.□□13.9	237.2±19.1	284.7±9.4
Striatum	297.9±15.2	268.6±15.1	369.3±14.5* (↑24%)
Hippocampus	234.5±7.2	207.9±17.2	256.9±1□.9
Cerebellum	120.7±10.4	132.9±14.7	□23.4±8.5
GD_{1ab}			
Olfactory bulbs	394.4±28.8	321.6±13.5* (↓18%)	342.0±8.1
Prefrontal c□rtex	51□.0□□4.1	527.3±21.2	542.9±12.4
Striatum	759.6±28.6	740.8±29.1	865.3±25.1
Hippocampus	370.1±12.1	459.1±19.6** (↑24%)	398.6□6.8
Cerebellum	236.7±9.0	210.4±15.5	□40.7±9.4
GM_{1a}			
Olfactory bulbs	191.4±12.1	145.2±10.5* (↓24%)	130.9±17.8* (↓32%)
Pref□ontal cortex	363.6±10.4	263.7±7.6** (↓28%)	329.2±10.8* (↓10%)
Striatum	338.5±7.1	286.0±11.1** (↓16%)	305.2±10.7** (↓10%)
Hippocampus	243.7±12.6	226.3±17.5	210.2±7.3
Cerebellum	76.8±6.9	49.8±7.4* (↓35%)	53.3±2.8* (↓31%)

nmol ganglioside/g tissue; * $p<0.05$;* *$p<0.01$; mean±SE

CONCLUSION: early exposure of pups to high dose of 2, 4-D resulted in a deficit in myelin deposition and behavioral alterations together with GM1 reduction, which could be reflecting a 2,4-D CNS injury, although a weak undernutrition could be involved also; when 2,4-D dose was lower, body and brain weight effects were not observed, but changes in GM1 contents and in myelin deposition, and alterations in muscular force and motor activity were still observed.

COMMENT/HED**:** The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. The dose levels are above level for saturation of renal clearance mechanism (oral route) and the route of exposure (sc) of pups is not relevant to human pesticide exposure. However, the reported deficit on myelin deposition was also reported following pup exposure *via* milk (Duffard, 1996). The extended 1-generation reproductive toxicity study on 2, 4-D, in which offspring were exposed to 2, 4-D during gestation, lactation *via* the milk, and directly *via* the diet through PND 22, 70, 139, includes an assessment of developmental neurotoxicity and neuropathology (including an evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation) at dose levels that do not saturate metabolic processes (described previously)**.** There were no treatment-related adverse effects on myelination at dose levels approaching renal saturation levels. Additionally, there were no treatment-related microscopic changes in the central or peripheral nervous systems in the perfused offspring, or changes in microscopic measurements of structures in the cerebral cortex, cerebellum, thalamus, or hippocampus. The no observed adverse effect level (NOAEL) for developmental neurotoxicity is greater than the PODs utilized for the human health risk assessment supporting the 2,4-D RED. The PODs used in the RED risk assessment are protective of chronic effects identified in the extended 1-generation study and of those identified in the cited study.

The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D

CITED BY NRDC (36) – study referenced as one that shows that 2,4-D appears to impair normal deposition of myelin in developing brain; effects of 2,4-D make it likely that fetuses, infants, and children

will be more susceptible to long-term adverse health effects from exposure to 2, 4-D.
CITED BY TASK FORCE – excessive doses (sc); litter not stat unit; effect may be due to undernutrition
CITED BY NCASI: petition fails to mention that dose levels likely exceed renal clearance levels and route of exposure is not appropriate

(Study 92) **CITATION**: Rosso, SB; Caceres, AO.; Evangelista de Duffard AM.; Duffard, R. and Quiroga, S. (2000). 2, 4-Dichlorophenoxyacetic acid disrupts the cytoskeleton and disorganizes the Golgi apparatus of cultured neurons. Toxicological Sciences 56, 133-140. [**Human Studies Considerations Apply: No**]

Principal Endpoint: [**Bin:** Neurotoxicity]

Methods Summary: cultured cerebellar granule cells shown to be excellent model system for studying neurite outgrowth and the expression of cytoskeletal proteins involved in nerve cell morphogenesis;

Results Summary: An *in vitro* study (cultured neurons) cell cultures; examined effects of 2,4-D in primary cultures of cerebellar granule cells; 24-hr exposure: dose-dependent inhibition of neurite extension; paralleled by a significant reduction in cellular content of both dynamic and stable microtubules, a disorganization of Golgi apparatus, and inhibition in synthesis of complex gangliosides.

Table 1. Gangliosides Biosysthesis in Cultured Cerebellar Granule Cells Treated with 2,4-D (24 hours)

Ganglisides	Control	0.2 mM	0.5 mM	1.0 mM	2.0 mM
GT1b	1.00	0.96	1.10	0.79*	0.13***
GD1b	1.00	1.16	1.10	0.57**	0.10***
GD1a	1.00	0.96	0.87	0.79*	0.24***
GD3	1.00	1.05	2.29***	2.87***	4.56***
GM1	1.00	1.07	1.26	1.11	0.15***
GM3	1.00	0.83	0.91	1.49**	13.27***

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

CONCLUSION: primary toxic effects of 2,4-D on neurons are most probably due to inhibition of microtubule polymerization; observations raise possibility that at least one basic mechanism underlying 2,4-D neurotoxicity involves an inhibition of microtubule assembly.

COMMENT/HED**:** The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. The mechanistic information (*in vitro*) is not directly applicable to human risk assessment. Agency identified the need for additional data on neurotoxicity. An extended 1-generation reproductive toxicity study on 2, 4-D, in which offspring were exposed to 2, 4-D during gestation, lactation *via* the milk, and directly *via* the diet through PND 22, 70, 139, includes an assessment of developmental neurotoxicity and neuropathology (including an evaluation of myelin in the PND 22 and PND 60 offspring following exposure during gestation and lactation) at dose levels that do not saturate metabolic processes (described previously). There were no treatment-related microscopic changes in the central or peripheral nervous systems in the perfused offspring or changes in myelin, or changes in microscopic measurements of structures in the cerebral cortex, cerebellum, thalamus, or hippocampus. Do not know how mM dose relates to levels attained in the brain *in vivo.* The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D

CITED BY: NYS DOH: identified as additional studies that should be considered in any new assessment along with those identified in the NRDC petition.

(Study 93) **Citation**: Rosso, SB; DiPaolo, OA; Evangelista de Duffard A.; and Duffard, R. (1997). Effects of 2, 4-Dichloro phenoxyacetic Acid on Central Nervous System of Developmental Rats. Associated Changes in Ganglioside Pattern. Brain Research 769: 163-167. [**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**

Methods Summary: Wistar rats: pups administered 2,4-D (0, 70, or 100 mg/kg/day) intermittently (sc**)**, every 48 hours, 10 pups/dam; exposure from PND 7 to PND 17 or PND 25; control at PND 17 or PND 25 injected with vehicle (water:ethanol). Body and brain weights, brain DNA and protein levels, and gangliosides (GM_1 GD_{1a} GD_{1b}

GT_{1b}) were assessed.

Results Summary: no changes in body weight and DNA/protein content of brain at PND 17; decreased body and brain weights, protein and DNA levels were observed at PND 25; decreased brain weight was observed on PND 17 at both dose levels when exposure started on PND 7 and at 100 mg/kg/day when initiated at PND 12.

Table 1. Effects of 2, 4-D on Body/Brain Weights; Protein/DNA Content; Total/Individual Brain Gangliosides Levels

		Dosed from PND 7		**Dosed from PND 12**	
Parameter	**Control**	**70 mg/kg/day**	**100 mg/kg/day**	**70 mg/kg/day**	**100 mg/kg/day**
17 days old					
Body weight (g)	30.11±0.65	30.31±0.89	28.31±2.40	29.59±0.63	28.21±0.28
Brain weight (g)	1.29±0.02	1.16±0.03**(10)	1.08±0.02**(16)	1.22±0.04	1.15±0.01**(11)
Protein (mg/brain)	150.46±6.03	155.29±14.89	134.43±5.67	133.72±12.46	148.65±35.42
DNA (mg/brain)	1.84±0.12	1.95±0.07	1.97±0.14	2.17±0.02	2.13±0.00
25 days old					
Body weight (g)	57.50±2.45	49.81±1.65*(14)	44.9±2.34**(22)	52.71±2.10	48.77±0.85*(16)
Brain weight (g)	1.41±0.03	1.24±0.02**(12)	1.19±0.03**(16)	1.34±0.04	1.33±0.01
Protein (mg/brain)	143.21±8.33	125.17±12.21	111.1±2.9*(22)	137.56±4.35	150.06±10.55
DNA (mg/brain)	2.30±0.07	2.34±0.11	1.84±0.08*(20)	2.14±0.05	2.55±0.06**(11)
17 days old					
GT_{1b}	201.97±7.2	206.1±17.94	211.78±8.99	206.73±13.07	219.43±14.81
GD_{1b}	302.68±11.9	321.3±16.38	276.45±15.30	318.17±15.58	185.43±12.37**(39)
GD_{1a}	427.36±13.7	484.6±20.6*(13)	410.97±17.98	498.43±24.56	446.79±15.43
GM_1	222.9±11.4	197.7±10.1	187.69±9.09	227.53±3.75	215.35±5.48
25 days old					
GT_{1b}	428.14±17.6	363.25±17.4*(15)	338.2±1.2*(21)	298.71±8.02**(30)	354.42±17.49*(17)
GD_{1b}	362.69±14.2	364.56±17.73	275.7±29.79**	501.56±24.75**	346.48±48.73
GD_{1a}	559.09±15.	505.4±13.15*	422.0±27.03**	633.95±40.72*	513.53±16.98
GM_1	298.39±11.8	217.54±20.9**(27)	197.5±8.63	283.73±20.56	261.23±8.9

Data from Tables 1 and 2 (pages 165-166); * $p<0.05$; ** $p<0.01$; (%↑↓); gangliosides levels: nmol/brain; n=10

CONCLUSION: results suggest a delay in CNS development when pups exposed to 100 mg/kg/day (sc); at lower dose, brain capable of triggering biochemical mechanisms producing a plasticity response, which is expressed as changes in ganglioside content and composition.

COMMENT/HED**:** The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. The dose levels would be expected to exceed the threshold level for saturation of renal clearance mechanism; route of exposure (sc) to pups is not relevant to human health pesticide risk assessment. An extended 1-generation toxicity study on 2, 4-D, in which offspring were exposed to 2, 4-D during gestation, lactation *via* the milk, and directly *via* the diet through PND 22, 70, 139, includes an assessment of developmental neurotoxicity and neuropathology (including an evaluation of myelin in the PND 22 offspring following exposure during gestation and lactation) at dose levels that do not saturate metabolic processes (described previously). There were no treatment-related delays in CNS development at dose levels approaching renal clearance saturation. No treatment-related microscopic changes in the central or peripheral nervous systems in the perfused offspring or changes in myelin, or changes in microscopic measurements of structures in the cerebral cortex, cerebellum, thalamus, or hippocampus were observed. The no observed adverse effect level (NOAEL) for developmental neurotoxicity was established at 59 mg/kg/day. This NOAEL is greater than the Points of Departure (PODs) utilized for the human health risk assessment supporting the 2,4-D RED. The PODs used in the RED risk assessment are protective of chronic effects identified in the extended 1-generation study and of those identified in the cited study.

The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. The extended 1-generation reproduction study on 2, 4-D confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

CITED BY NYS DOH: identified as additional studies that should be considered in any new assessment along with those identified in the NRDC petition.

(Study 102) **Citation**: Sturtz N, Bongiovanni B, Rassetto, M.; Ferri, A.; Evangelista de Duffard A.; and

Duffard, R. (2006). Detection of 2, 4-dichlorophenoxy acetic acid in rat milk of dams exposed during lactation and milk analysis of their major components. Food & Chem Toxicology 44, 8-16. [**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**

Methods Summary: Wistar rats: 0, 15, 25, 50, or 70 mg/kg/day in diet to dams during PND 1-16; milk collected on PND 16; serum samples from dams and pups on PND 16; effect on milk components assessed

RESULTS: 2,4-D can affect the composition of milk; dose-dependent reduction of PUFAs; these significant changes in milk fatty acid composition indicate a disturbance in the supply of PUFAs to the neonate, which could alter normal development of the brain.

Dietary exposure of dams at 15, 25, 50, or 70 mg 2,4-D/kg/day resulted in comparable reductions in total lipids in PND 16 maternal milk at the 3 highest dose levels (↓29.6%, 27.8%, and 31.4% at 25, 50, and 70 mg/kg/day, respectively); ↓pup body weight gain at all dose levels (↓ 11.4%, 11.6%, 17.8%, 16.7%, respectively, on PND 16); decrease in BWG evident from PND 6-16; residues of 2,4-D in dam and pup serum and milk at each dose level were provided and show that milk content is greater than in maternal serum; pup serum levels are ≈30% (24-32.9%) of the dams' levels

amount to dam	amount in milk[A]	amount in dam serum [A]	amount in pup serum [A]	serum ratio d/p
15 mg/kg/day	28.92±3.04	26.09±4.24	6.34±1.68	4.12 (4.56) [B]
25 mg/kg/day	59.56±12.03	38.18±10.59	11.52±3.01	3.31 (5.17)
50 mg/kg/day	86.69±22.04	51.30±10.70	16.90±3.24	3.04 (5.13)
70 mg/kg/day	115.08±21.26	74.23±11.54	18.96±4.50	3.92 (6.07)

[A]µg /mL of sample; [B] (ratio of amount in milk to amount in pup serum)

CONCLUSIONS: Maternal dietary exposure during lactation results in damage to pups' nutritional status; 2,4-D produced a dose-related reduction of polyunsaturated fatty acids (PUFAs); significant changes in milk fatty acid composition indicating a disturbance in the supply of PUFAs to the neonate, which can alter normal developmental of the brain.

COMMENT/HED**:** The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. Provides data following oral doses to dam that are below, at, and above renal clearance capacity; do not know dose to pup, but provides information regarding oral dose to dam and amount in milk (dose to nursing pups) and resultant amount of 2,4-D in pup serum. Although a decrease in pup body weight gain was observed at all dose levels, the Agency notes that the cited paper only provides pup body weight gain data (in the form of a graph), and data on pup body weights were not provided. Additionally, the lack of body weight gain is not necessarily considered an adverse effect. It is a calculated value and can be misleading. For example, if the control and treated groups both show a body weight of 50.9 grams on day X and the control group gains 1.1 grams while the treated group gains 0.8 gram, this would show a 24% decrease in body weight gain for the treated group compared to the control. However, 52 grams vs 51.7 grams is a 1% difference in body weight. Preliminary studies performed in conjunction with the extended 1-generaion reproductive toxicity study also provide data with respect to the 2, 4-D levels in the plasma of pups and dams and levels in the milk. In the extended 1-generation reproductive toxicity study of 2, 4-D (described previously), there were no treatment-related delays in CNS development at dose levels approaching renal clearance capacity. No treatment-related microscopic changes in the central or peripheral nervous systems in the perfused offspring or changes in myelin, or changes in microscopic measurements of structures in the cerebral cortex, cerebellum, thalamus, or hippocampus were observed. The no adverse effect level (≈30 mg/kg/day) for decreased pup body weight identified in the extended 1-generation study is greater than in the cited study. An exposure pathway identified in the NRDC petition is lactational exposure to 2, 4-D. The Agency currently is addressing the issue of a lack of information regarding the levels of 2, 4-D that can occur in human breast milk. Preliminary studies performed to determine the doses to be used in the extended 1-generation reproductive toxicity study provide data on the levels of 2, 4-D in the milk of the dams and plasma levels of both the dams and offspring. Currently, it cannot be determine what the actual dose to each pup was due to the lack of information on the amount of milk ingested by the pups. However, the doses in the extended one-generation reproduction study were adjusted during the lactational period to prevent excessive dosing both to the maternal rat and to the pups in early lactation and due to a "double exposure" when pups are both nursing and starting to consume diet. Adjustments to the diet were also performed in the Sturtz study, although the procedures used were different and may, to some extent, explain the apparent differences in

the Sturtz study compared to the extended one-generation reproduction study. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency. Additionally, the results in the cited study do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D

CITED BY NRDC (60) – NRDC says study confirms lactational exposure; decreased offspring body weights where dam exposed to 15 mg/kg/day (LOAEL) for 16 post natal days; 2,4-D accumulated by about 1.6 fold in maternal milk; nutritional content of milk altered (fat content dropped significantly; altered some proteins in maternal milk); suggests a lower NOAEL than that being used for short-term (25 mg/kg/day).

CITED BY Task Force – results not duplicated in recent rat dietary life-stage study (Marty, 2009); demonstrated ↓ pup BW only when dams treated with 2,4-D ≥800 ppm in diet (premating through PND 21 (52, 61, 71-117 mg/kg/day); no effects at 400 ppm (25, 28, 37-57 mg/kg/day); at 800 ppm, 2,4-D PK behavior in lactating dams was distinctly non-linear (Saghir, 2009)

(Study 103) **Citation**: Sturtz, N.; Evangelista de Duffard A.; and Duffard, R. (2000). Detection of 2, 4-Dichlorophenoxy Acetic Acid (2,4-D) Residues in Neonates Breast-Fed by 2,4-D Exposed Dams. *NeuroToxicology* 21 (1-2): 147-154. [**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**

Methods Summary: Wistar rats: dams administered 2,4-D (0, 50, 70, or 100 mg/kg/day) intermittently (i.p.), every two days, to obtain a maintained 2,4-D neonates' exposure; litters culled to 8 pups/dam; exposure from PND 1-15 or PND 1-9 (then dams treated only with DMSO until pups were 15 days old). Measured levels of 2,4-D in kidney, blood, stomach, and brain; pups exposed *via* milk

Results Summary: Decreased pup body weight was observed at all dose levels by PND 7, and the 100 mg/kg/day pups failed to gain weight from PND 7-13 when body weight loss was evident. On PND 16, pup body weights were 70%, 65%, and 40% of control at 50, 70, and 100 mg/kg/day, respectively. Brain weights were decreased significantly at the 70 and 100 mg/kg/day dose levels on PND 16. The quantity of 2, 4-D in the blood of the dams on PND 16 (24 hours after last dose) was 4.21, 7.02, and 15.46 µg/mL at the 50, 70, and 100 mg/kg/day. A comparison of the magnitude of the residues of 2, 4-D in the pup brain between the 50 and 100 mg/kg/day dose levels on PNDs 4 and 8 shows a 5-fold difference, whereas the same comparison at PND 16 shows a 2.6-fold difference.

Table 1. Organ Weights of Neonates Breast Fed by 2,4-D Treated Dams

Tissue	Dose	Age of neonates (days)		
		4	8	16
Brain	50	-	↓13.5%	-
	70	-	↓16%	↓13.9%
	100	-	↓24%	↓32□3%
Kidney	50	-	↓33.4%	-
	70	-	↓39.5%	-
	100	-	↓28.6%	≈50%

N=16pups/group, data from Figure 1 (page 149)

Table 2. 2,4-D Residues (µg/g) in Neonates Breast Fed by 2,4-D Treated Dams

Tissue	Dose	Ag□ of neonates (days)		
		4	8	□6
Brain	50	0.58±0.16	1.27±0.41	2.28±0.88
	70	0.98±0.19	2.84±0.94	3.82±1.65
	100	2.94±0.81	6.45±1.33	5.95±3.24
Blood	50	2.56±0.64	2.01±0.33	8.06±5.92
	70	4.98±0.38	4.18±0.67	20.54±13.87
	100	6.77±2.3	25.33±7.86	30.65±10.30
Kidney	50	6.82±1.29	6.87±1.31	9.85±2.68
	70	18.54±1.41	15.84±2.78	18.62±6.76
	100	12.39±2.92	79.01±13.92	91.18±20.53
Stomach content	50	8.83±2.26	17.98±1.49	27.57±12.90
	70	19.17±6.15	57.73±1.19	77.03±37.91
	100	42.16±11.53	100.97±19.44	114.09±32.45

N=16 pups/group, data from Table 1 (page 150)

Table 3. 2,4-D Residues (µg/g) in Neonates Breast Fed by 2,4-D Treated Dams (100 mg/kg/day)				
Age of neonate	Tissue			
	Brain	Blood	Kidney	Stomach content
8	6.45±1.33 [100]	25.33±7.86 [100]	79.01±13.92 [100]	100.97±19.44 [100]
11 (2)	0.64±0.52 [9.92]	11.7±5.94 [46.2]	6.19±0.18 [7.83]	10.52±4.36 [10.4]
13 (4)	0.50±0.14 [7.64]	9.92±3.66 [39.2]	3.57±0.89 [4.52]	10.55±4.48 [10.44]
14 (5)	0.29±0.08 [4.49]	8.76±2.73 [34.58]	1.91±0.23 [2.41]	0.55±0.21 [6.49]
15 (6)	0.28±0.02 [4.34]	4.33±0.54 [17.1]	0.89±0.09 [1.12]	6.24±2.76 [6.18]
16 (7)	0.27±0.01 [4.18]	2.52±2.08 [9.95]	0.58±0.39 [0.73]	2.92±2.12 [2.89]

N=16 pups/group; (days after withdrawal); [% of 2,4-D content]; data from Table 2 (page 151)

CONCLUSION: Residues of 2,4-D in stomach content, blood, brain, kidneys of PND 4 pups and older; residues were dose- and exposure-time dependent; at 100 mg/kg/day, decreased pup BW and tissue weight and diminished stomach content; demonstrates that 2,4-D is transferred to neonates and decreased body and organ weights could be due to a diminished milk intake or/and to the direct 2,4-D toxic effect; when 2,4-D withdrawn from dams, 2,4-D residues remained in stomach content for at least a week

COMMENT/HED**:** Study provides dose-response information at dose levels at or above level for saturation of renal clearance mechanism. Although the dams were dosed i.p., pups were exposed *via* the milk, although the dose to the pups is not known; however, this study, in conjunction with another Sturtz study, may provide information for determining pup dose. Additionally, preliminary results submitted to the Agency to support the dose levels selected for the extended 1-generation reproduction study provide similar exposure values to the pups. At this time, it is not possible to calculate the dose to the pup from exposure to 2, 4-D *via* the milk during lactation, since the amount of milk each pup receives is not known. The extended 1-generation reproductive toxicity study provided numerous detailed measures on the offspring following exposure across all life stages, including *via* the milk during lactation. The LOAEL for PND 22 offspring is based on decreased body weight, which was observed at the highest dose tested (600 ppm-800 ppm; currently, the dose on a mg/kg/day basis has not been calculated). The intended mg/kg/day dose to the parental rats at these ppm levels was 30-40 mg/kg/day.

The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.

CITED BY NRDC (59) – cited as showing exposure to 2,4-D through maternal milk; when maternal exposure stopped, 2,4-D continued to be excreted in maternal milk for a week; EPA failed to include any lactational exposure in its aggregate risk assessment

CITED BY TASK FORCE – 2,4-D treatment rates to lactating rats were at doses in excess of saturation of renal clearance of 2,4-D; toxicity observed in the resulting non-linear pharmacokinetic range does not inform human risk assessment, because nonlinear doses are exceedingly unlikely to occur in humans.

CITED BY NCASI: petition fails to mention that dose levels likely exceed renal clearance levels and route of exposure is not appropriate

(Study 104) **Citation**: Sturtz, N.; Deis, RP; Jahn, GA; Duffard, R; and Evangelista de Duffard A (2008). Effect of 2, 4-Dichlorophenoxyacetic acid (2,4-D) on rat maternal behavior. *Toxicology* 247: 73-79. [**Human Studies Considerations Apply: No**]

Principal Endpoint: **[Bin: Neurotoxicity]**

Methods Summary: Wistar rats: dams administered 2,4-D *via* diet (0, 15, 25, 50 mg/kg/day); litters 8 pups/dam; exposure from PND 1-7; on PNDs 5 and 7, dams were tested for maternal behavior in home cage; maternal behavior parameters: nursing behavior with retrieval, crouching, licking (of whole body of pup), anogenital licking, time spent out of nest and eating; also measured latency and length of retrieval, defined as the time elapsed between the beginning of experiment and the retrieval of the first and last pups, respectively; latency of crouching (time elapsed between the beginning of experiment and the moment when mother assumed a crouching position with pups under her). Serum prolactin levels; endogenous monoamines (noradrenaline (NA), dopamine (DA), 3,4-dihyrdoxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindolacetic acid (5-HIAA) and their metabolites

determined.

Results Summary: maternal behavior impaired; pup care parameters, such as licking and grouping of pups were decreased and latencies of retrieval and crouching were increased, as was time spent out of nesting area; however, pups did not show body-weight loss during testing period, probably because milk supply was normal. Serum prolactin levels were diminished by 61%, 67%, and 71% at 15, 25, and 50 mg/kg/day, respectively; drastic decrease in indolamine levels (5-HT, 5-HIAA) in arcuate nucleus (AcN) of treated dams; increased catecholamine levels.

Table 1. Maternal Behavior Parameters

Parameter	**0 mg/kg/day**	**15 mg/kg/day**	**25 mg/kg/day**	**50 mg/kg/day**
	PND 5			
# pups retrieved	8	8	6.7±0.3	8
Latencies of retrieval	17.3	37.9±4.4*	40.2±4.4**	38.7±3.7*
Length of retrieval of 8 pups	54.0±2.9	80.8±4.2***	84.7±6.4***	88.0±2.9***
# pups in nest	6.4±0.9	5.1±1.0	4.9±1.0	4.2±1.1
Latencies of crouching (seconds)	216±30	348±32**	328±33*	528±29***
% dams licking pups	100	50***	42***	16***
% dams licking ano-genital region of pups	83	0***	0***	0***
% dams leaving nest	25	100***	100***	100***
Time spent out of nest	13±8	132±10***	148±8***	138±11***
% dams going out to eat	0	17	25	33
	PND 7			
# pups retrieved	8	8	7.6±0.2	8
Latencies of retrieval	26.2±4.8	59.3±6.1***	66.5±7.8***	78.7±7.3***
Length of retrieval of 8 pups	48.9±3.1	87.5±5.7**	95.3±5.2***	93.5±5.6***
# pups in nest	8	2.8±1.1*	1.7±0.9*	1.1±0.7**
Latencies of crouching (seconds)	222±26	332±40*	428±37***	600±0***
% dams licking pups	100	0***	0***	0***
% dams licking ano-genital region of pups	75	0***	0***	0***
% dams leaving nest	42	67*	83***	100***
Time spent out of nest	26±12	136±23***	141±24***	120±10***
% dams going out to eat	0	17	25	17

Data from Table 1 (page 76)

Table 2. AcN Catecholamines and Indoleamines Levels in Control and 2,4-D-Treated Dams on PND 7

	0	15 mg/kg/day	25 mg/kg/day	50 mg/kg/day
NA	72.0±6.5	49.9±4.5	50.6±4.5	51.1±8.4
DA	35.5±1.5	41.3±2.0	49.9±1.5***(↑40)	51.7±1.9***(↑46)
DOPAC	10.4±0.7	9.4±2.1	17.2±1.8*(↑65)	18.6±0.9**(↑79)
HVA	14.5±1.7	25.8±3.2* (↑78)	25.5±2.1*(↑76)	28.1±2.4 (↑94)
5-HT	252.1±3.0	71.1±1.9***(↓72)	40.4±1.7***(↓84%)	30.9±0.9***(↓88)
5-HIAA	85.8±3.5	59.7±2.5*** (↓30)	40.6±1.5***(↓53)	38.7±2.7*** (↓55)

* p<0.05; ** p<0.01; *** p<0.001; ↓↑ (%); fmol/mg wet weight; data from Table 2 (page 77)

CONCLUSIONS: differences observed at all dose levels (latencies of retrieval; latencies of crouching; increased time for retrieval of 8 pups; decreased % dams licking pups; none of 2,4-D dams licked anogenital area of pups; increased time spent out of nest); decreased 5-HT and 5-HIAA (all doses, DR); increased DA, DOPAC (25 and 50 mg/kg/day); decreased HVA (all doses).

COMMENT/HED: The cited study is one of several research studies from the same group of investigators looking into possible mechanism of 2, 4-D neurotoxicity. Doses of 15, 25, 50 mg/kg/day are all at or below level of renal clearance saturation. Duration of exposure to the dams was 7 days (PND 1-7), with an assessment of behavior and monoamines, etc. following 5 and 7 days of exposure *via* the diet. Prolactin levels have been related to the onset and maintenance of maternal behavior in rats. Serum levels of prolactin were measured on PND 7 and were found to be significantly reduced by 2,4-D treatment at all dose levels (61%, 67%, and 71% at 15, 25, and 50 mg/kg/day, respectively). Maternal behavioral

assessment is one parameter assessed in the extended 1-generation reproductive toxicity study (described previously) in which the dams were exposed pre-mating, throughout gestation and lactation *via* the diet at dose levels of 5, 15, and 40 mg/kg/day. No treatment-related adverse effects were observed on parental behavior or on any of the numerous parameters evaluated to assess reproductive toxicity potential following oral exposure to 2, 4-D. Monoamines were not monitored in the extended 1-generation reproductive toxicity study. The cited study identifies an effect of 2, 4-D on maternal behavior towards her offspring that were associated with decreased 5-HT and increased DA levels in the AcN and decreased circulating prolactin following exposure of the dams to 2, 4-D for 5 and 7 days after the birth of their litters. Maternal neglect was not observed in the extended 1-generation reproductive toxicity study at dose levels at or approaching renal clearance saturation. The cited study provides an endpoint for consideration in the overall weight of evidence, although it does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.
CITED BY Beyond Pesticides –Changes to maternal behavior in rats, along with increased catecholamine levels and decrease in indolamine levels are among effects that need to be considered along with the scientific literature, which abounds with evidence that 2,4-D is a dangerous chemical
CITED BY Supplement to NRDC petition indicates that study shows that even short-term exposure to 2, 4-D at dose levels below toxic levels severely inhibits normal maternal hormones in brain and thereby adversely affects maternal behavior.
CITED BY NYS DOH – one of studies cited as additional studies that have accumulated since the RED, in addition to those in NRDC petition, which should be considered in any new 2, 4-D assessment.

(Study 106) **Citation**: Ton, C.; Lin, Y.; and Willett, C. (2006). Zebrafish as a Model for Developmental Neurotoxicity Testing. Birth Defects Research (Part A) 76: 533-567. [**Human Studies Considerations Apply: No**]
Principal Endpoint: **[Bin: Neurotoxicity]**
Methods Summary: NA - screening tool assessment; assay model;
Results Summary: sublethal exposure to 2,4-D in fish tank water showed specific neurotoxicity; caused significant increase in brain cell death (apoptosis), disrupted motor neuron growth at high concentrations, decreased motility at moderate concentrations; slightly teratogenic in zebrafish;
CONCLUSION: 2,4-D showed specific neurotoxicity (induction of apoptosis or necrosis is an indicator of neurotoxicity) in zebrafish; zebrafish is a predictive animal model for neurotoxicity screening
COMMENT/HED**:** 2,4-D was used as a one of several well-characterized compounds to examine several parameters of neurotoxicity during development (screening tool to identify potential neurotoxic compounds). Study is a screening assay and not directly applicable to human health risk assessment. These data suggest that zebrafish may be an appropriate alternative model to study the mechanism(s) responsible for the developmental neurotoxicity of pesticides and aid in identification of compounds that should be further tested in mammalian systems. Neurotoxicity is already identified as a potential endpoint for 2, 4-D and more appropriate studies have been performed to assess 2, 4-D's developmental neurotoxicity potential. In the extended 1-generation reproduction study on 2,4-D (MRID 47972101), young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (via the diet) following weaning. Offspring were subjected to a developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). An evaluation of myelin in the PND 22 offspring following exposure during gestation and lactation was performed. On PND 60, the young rats were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation, myelination assessment, and brain morphometry. The extended 1-generation reproductive toxicity study on 2, 4-D was performed at three dose levels up to the saturation level for renal clearance. At the highest dose tested, there was no evidence of developmental neurotoxicity and no adverse effect on myelination.

The cited study does not identify a more sensitive endpoint for human health than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D. Additionally, the extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to neurotoxic effects.
CITED BY NRDC (32): evidence of neurotoxicity; sublethal exposure to 2,4-D in fish tank water significantly increased brain cell death, disrupted motor neuron growth, and decreased motility; in mammals, 2,4-D has been shown to exert related developmental neurotoxicity, including decreased motor activity and Parkinson's like tremors.
CITED BY Task Force: a method development exercise; lowest conc affecting motor neuron growth exceeded LC50 for zebrafish; not appropriate

BIN: ENDOCRINE

(Study 5) **Citation**: Blair, R.M., Fang, H., Branham, W.S., Hass,, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R., Sheehan, D.M. (2000). The Estrogenic Receptor Relative Binding Affinities of 188 Natural and Xenochemicals: Structural Diversity of Ligands. Toxicological Sciences 54: 138-154. [**Human Studies Considerations Apply: No**]

Principal Endpoint: [**Bin: Endocrine]**

Methods Summary: a validated (standardized) estrogen receptor (ER) competitive-binding assay was used to determine the ER affinity; uteri from ovariectomized Sprague-Dawley rats were the ER source for the competitive binding assay; chemicals were screened at high concentrations to determine whether chemical competed with [^{3}H]–estradiol for the ER. Those with affinity were assayed using a wide range of concentrations to characterize the binding curve and to determine each chemical's IC and relative binding affinity (RBA) values.

Results Summary: 2,4-D (99%) was reported as negative in this investigation

CONCLUSION: 2,4-D in this test system did not exhibit any estrogenic activity at the concentrations tested.

COMMENT/HED**:** Study examined the effects of 2, 4-D in a validated (standardized) estrogen receptor (ER) competitive-binding assay, which was used to determine the ER affinity. 2, 4-D was one of many chemicals that were screened at high concentrations to determine whether the chemical competed with [3H]–estradiol for the ER. 2, 4-D did not exhibit any estrogenic activity at the concentrations tested. The Agency used an exposure based approach to select the first list of chemicals to receive Test Orders for the Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery; 2,4-D was included in the list of chemicals. Additionally it should be noted that this study was cited by the Test Order recipient as Other Scientifically Relevant Information (OSRI) in response to the Agency's EDSP Test Order and was evaluated by the Endocrine Disruptor Review Team (TXR No. 0055461). The EDRT concluded that while 2, 4-D as reported was negative in this investigation, no data were supplied to substantiate this conclusion, and this study did not meet Test Order requirements (TXR No. 0055461, page 17). EPA waived the Tier 1 tests most relevant to human endocrine effects for 2,4-D due to the availability of the extended one generation reproduction study with 2,4-D. (Ref. Office of Chemical Safety and Pollution Prevention, EPA, Memorandum from Greg Ackerman to Katie Weyrauch, "**2, 4-Dichlorophenoxyacetic Acid (2,4-D) - Report of the Endocrine Disruptor Review Team - Test Order #: EDSP–031001-120" (December 20, 2010).**) EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D, which include an *in vitro* Estrogen Receptor Binding Assay (890.1250). The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.

CITED BY Task Force: This is one of several studies referenced by the Task Force that they identified as demonstrating that 2,4-D does not bind to or activate estrogen receptors, contrary to NRDC's assertions that 2, 4-D is a "potent" endocrine disruptor. 2, 4-D did not exhibit any estrogenic activity at the concentrations tested.

(Study 42) **Citation**: Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J, O'Heir CD, Mitchell ML, Hermos RJ, Waisbren SE, Faix JD, Klein RZ. (1999). Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. The New England Journal of Medicine 341 (8): 549-555. [**Human Studies Considerations Apply: Yes**

Principal Endpoint: [**Bin: Endocrine]**

Methods Summary: thyrotropin was measured in stored serum samples collected from 25216 pregnant women between January 1987 and March 1990; 47 women located with serum thyrotropin concentrations at or above the 99.7th percentile of values for all pregnant women, 15 women with values between the 98th and 99.6th percentiles, inclusive, in combination with low thyroxine levels, and 124 matched women with normal values; their 7-9 year old children were tested (15 tests) for intelligence, attention, language, reading ability, school performance, and visual-motor performance.

Results Summary: children of the 62 women with high serum thyrotropin concentrations performed

slightly less well on all 15 tests; their full-scale IQ scores on Wachsler Intelligence Scale for Children averaged 4 points lower than those of the children of the 124 matched control women; 15% ha scores of 85 or less, and compared with 5% of the matched control children; of the 62 women with thyroid deficiency, 48 were not treated during pregnancy, and the full-scale IQ scores of their children averaged 7 points lower than the 124 matched control children; 19% had scores of 85 or less. Eleven years after the pregnancy, 64% of the untreated women and 4% of matched control women had confirmed hypothyroidism.
CONCLUSION: undiagnosed hypothyroidism in pregnant women may adversely affect their fetuses; therefore, screening for thyroid deficiency during pregnancy may be warranted.
COMMENT/HED**:** This is a general paper (not 2,4-D specific); describes children of women who had thyroid deficiency during pregnancy; assessed for various abilities (15 tests of intelligence, attention, etc.); contrary to statement in NRDC petition, concern for potential thyroid effects were identified in the 2005 2, 4-D RED; the Data Call-In issued for a 2-generation reproduction study on 2, 4-D specifically required examination of the thyroid. The extended 1-generation reproductive toxicity study on 2,4-D (described previously) provides an assessment of the thyroid (thyroid weight, thyroid hormone levels, histopathology) of the adult and offspring. In the 2, 4-D extended 1-generation reproductive toxicity study in rats, the expected hormone response consistent with perturbation in thyroid function was seen in the GD 17 satellite dams. These findings correlated with the thyroid alterations histologically. There was a comparable increase (↑9%) in thyroid weights at the low and high dose levels but no change in thyroid weight at the mid dose. In the F1 PND 4 pups, there were decreases in T3 levels at the low (↓8%) and mid (↓13%) dose groups, but not at the high dose. Decreased T4 levels were seen at the mid (↓15) and high (↓12%) dose groups, but there was no dose response. TSH level was increased (↑19%) at the high dose. Thyroid weight and histopathology data were not collected for this group. In the F1 PND 22 offspring, there was a decrease in T4 (↓20%) only at the high dose. Thyroid weight and histopathology data were not collected for this age group. In the F1 PND 62-64 offspring, T3 level was increased (↑11%) only at the high dose. T4 levels were increased (↑19%) both at the mid and high dose levels. There was a dose-dependent increase in TSH levels at the mid (↑11%) and high (↑24%) dose levels. Female offspring (PND 70) displayed decreased absolute thyroid weights at the mid (↓9%) and high (↓5%) dose levels. No treatment-related histopathological lesions were seen in the thyroid glands of P1 rats or F1 offspring. The thyroid effects noted in the extended 1-generation reproductive toxicity study were considered to be adaptive and not adverse. Additionally, no robust effects on auditory startle or statistically significant brain morphometric changes were seen in the DNT component of the extended one-generation toxicity study; findings otherwise expected as sequelae to any marked thyroid hormone deficiency. The no observed adverse effect level (NOAEL) for thyroid toxicity was established at ≈40 mg/kg/day. This NOAEL is greater than the PODs utilized for the human health risk assessment supporting the 2,4-D RED. It is to be noted that 2, 4-D was tested in rats, which are much more sensitive to thyroid perturbations than are humans because the serum half-life of the thyroid hormone, thyroxine, is much shorter in rats (less than 1 day) than in humans (5-9 days). The 10X interspecies uncertainty factor applied to 2, 4-D to account for the possibility that humans are more sensitive than the test animals is therefore more than adequate to protect humans. The 10X intraspecies factor accounts for variability in sensitivity among species and gives protection for women with iodine deficiency.. The extended one-generation reproduction study confirms the protectiveness of the existing Points of Departure as to thyroid effects.
CITED BY NRDC (19): cited in support of their conclusion that EPA failed to incorporate information on the endocrine disrupting effects of 2, 4-D; NRDC states that thyroid hormone plays a critical role in development of brain; slight thyroid suppression adversely affects neurological development in fetus, with lasting effects on child learning and behavior

(Study 49) **Citation**: Hurst MR, Sheahan DA. (2003). The potential for oestrogenic effects of pesticides in headwater streams in the UK. The Science of the Total Environment 301: 87-96. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]**
Methods Summary: Yeast Estrogen Screen (YES) or yeast two-hybrid screen
Results Summary: negative

CONCLUSION: human YES negative
COMMENT/HED**:** This is an *in vitro* study, which cannot be directly extrapolated to the complex *in vivo* situation. The referenced study [yeast two-hybrid screen; Yeast Estrogen Screen (YES)] is a screening study in which 2, 4-D was reported as negative. The Task Force referenced this study to dispute NRDC's claim that 2, 4-D is an endocrine disruptor. Concern for endocrine disruption was identified in the RED. The Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2, 4-D was included in this list of chemicals. This citation was included in the Endocrine Disruptor Review Team assessment of 2, 4-D (see TXR No. 0055461). EDRT did not accept yeast-based assays as other scientifically relevant information (OSRI) primarily because some test chemicals are unable to penetrate the cell wall of the yeast cells and this may result in a false negative. Another issue with these assays is cytotoxicity. Tier 1 screening assays are limited to evaluating whether a substance is capable of interacting with the endocrine system and are not sufficient to determine whether a chemical may have an effect in humans that is similar to an effect produced by naturally occurring hormones. The fact that a substance may interact with a hormone system does not mean that when the substance is used, it will cause adverse effects in humans or ecological systems. EPA waived the Tier 1 tests most relevant to human endocrine effects for 2,4-D due to the availability of the extended one generation reproduction study with 2,4-D. (Ref. Office of Chemical Safety and Pollution Prevention, EPA, Memorandum from Greg Ackerman to Katie Weyrauch, "**2, 4-Dichlorophenoxyacetic Acid (2,4-D) - Report of the Endocrine Disruptor Review Team - Test Order #: EDSP–031001-120" (December 20, 2010).**) EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints**.**
CITED BY TF: one of several studies referenced by TF to dispute NRDC's claim that 2,4-D is an endocrine disruptor; states that Agency has on several occasions rejected the position that data gathered under Endocrine Disruptor Screening Program is a prerequisite to a safety determination.

(Study 54) **Citation**: Jung J, Ishida K, Nishihara T. (2004). Anti-estrogenic activity of fifty chemicals evaluated by *in vitro* assays. Life Sciences 74: 3065-3074. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]**
Methods Summary: Yeast two-hybrid assay system with rat estrogen receptor (ER) α and the coactivator, T1F2; introduced 2 expression plasmids, pGBT9-ERLBD and –GAD424-T1F2, into yeast cells (*Saccharomyces cerevisiae* Y190). The yeast two-hybrid system transfected with a rat ER receptor, a transcriptional activation assay using MCF-7 cells, and an ER binding assay using fluorescence polarization.
Results Summary: 2,4-D was negative
CONCLUSION: 2,4-D was not found to inhibit estrogen activity
COMMENT/HED**:** 2,4-D is among the group of 58 pesticide active ingredients on the initial list to be screened under EDSP (test orders issued). Concern for endocrine disruption was identified in the RED. The Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2, 4-D was included in this list of chemicals. This citation was included in the Endocrine Disruptor Review Team assessment of 2, 4-D (see TXR No. 0055461). EDRT did not accept yeast-based assays as other scientifically relevant information (OSRI) primarily because some test chemicals are unable to penetrate the cell wall of the yeast cells and this may result in a false negative. Another issue with these assays is cytotoxicity. This citation was included in the Endocrine Disruptor Review Team assessment of 2, 4-D (see TXR No. 0055461, page 17). EDRT did not accept yeast-based assays as OSRI primarily because some test chemicals are unable to penetrate the cell wall of the yeast cells and this may result in a false negative. Another issue with these assays is cytotoxicity. The test order recipient did not cite this study as OSRI. EPA waived the Tier 1 tests most relevant to human endocrine

effects for 2,4-D due to the availability of the extended one generation reproduction study with 2,4-D. (Ref. Office of Chemical Safety and Pollution Prevention, EPA, Memorandum from Greg Ackerman to Katie Weyrauch, "**2, 4-Dichlorophenoxyacetic Acid (2,4-D) - Report of the Endocrine Disruptor Review Team - Test Order #: EDSP–031001-120" (December 20, 2010).**) EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.

CITED BY TF: one of several *in vitro* studies referenced by TF to dispute NRDC's assessment of Xie, et al (2005) report that 2,4-D has "relatively potent estrogenic effects in fish"; states that Agency has on several occasions rejected the position that data gathered under Endocrine Disruptor Screening Program is a prerequisite to a safety determination.

(Study 55) **Citation**: Jungbauer A, Beck V. (2002). Yeast reporter system for rapid determination of estrogenic activity. J. Chromatography B 777: 167-178. [**Human Studies Considerations Apply: No**]

Principal Endpoint: [**Bin: Endocrine]**

Methods Summary: an *in vitro* test system for the determination of estrogens, xeno- and phytoestrogns, based on the activation of human estrogen receptor-α, was examined for ability in monitoring environmental estrogens; system consists of an expression plasmid for the human estrogen receptor-α and a reporter plasmid containing the lacZ under the control of the vitellogenin hormone response element. These plasmids have been transformed into S. cerevisiae. Cultivation of yeast in the presence of estrogenic substances leads to activation of the estrogen receptor and induces the expression of the reporter lacZ. B-Galactosidase activity of the translated gene lacZ is a measure of the estrogenic activity of a compound.

Results Summary: 2,4-D – no activity (reference cited is Soto et al., 1995);

CONCLUSION: 2,4-D no activity (E-Screen);

COMMENT/HED**:** paper cited data from Soto, et al., 1995. As noted above, 2,4-D was included on the list of chemicals to receive test orders for the Endocrine Disruption Screening Program (EDSP) Tier 1 battery. This citation and the paper by Soto, et.al., 1995 were included in the Endocrine Disruptor Review Team assessment of 2, 4-D (TXR No. 0055461, page 17). The concentrations were not identified nor were data provided in this citation. This study was not cited as OSRI by the test order recipient. EDRT did not accept yeast-based assays as OSRI primarily because some test chemicals are unable to penetrate the cell wall of the yeast cells and this may result in a false negative. Another issue with these assays is cytotoxicity. EPA waived the Tier 1 tests most relevant to human endocrine effects for 2,4-D due to the availability of the extended one generation reproduction study with 2,4-D. (Ref. Office of Chemical Safety and Pollution Prevention, EPA, Memorandum from Greg Ackerman to Katie Weyrauch, "**2, 4-Dichlorophenoxyacetic Acid (2,4-D) - Report of the Endocrine Disruptor Review Team - Test Order #: EDSP–031001-120" (December 20, 2010).**) EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.

CITED BY TF: listed in table of 11 assays that indicate that 2,4-D is negative for estrogenicity; one of several *in vitro* studies referenced by TF to dispute NRDC's assessment of Xie, et al (2005) report that 2,4-D has "relatively potent estrogenic effects in fish".

(Study 56) **Citation**: Kim HJ, Kim WD, et al. (2002). Mechanism of phenoxy compounds as an endocrine

disruptor. J Toxicology Public Health 18:331-339. (in <u>*Korean*</u>). [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]**
Methods Summary: Dose of 50 mg 2,4-D/kg po gavage for 10 days; dose of 100 mg DCP/kg po gavage for 10 days; an androgenicity assay system (Hershberger assay), to assess potential endocrine disruption; 2,4-D/DCP may act as androgenic endocrine disruptors by binding to androgen receptor, as well as by inhibiting metabolism of testosterone.
Results Summary: administration of both compounds to rats caused an increase in tissue weight of ventral prostate, Cowper's gland, and glands penis; these increases were additively potentiated when rats were simultaneously treated with low dose of testosterone (1 g/kg s.c.)
CONCLUSION: results collectively suggest that 2,4-D and DCP may act as androgenic endocrine disruptors by binding to the androgen receptor as well as by inhibiting the metabolism of testosterone.
COMMENT/HED**:** concern for endocrine disruption was identified in 2005 RED. This article was written in Korean and was not available to the Agency in English. The Agency identified concern for potential endocrine effects in the 2005 RED. In response to this and other concerns raised, the registrant conducted the extended one-generation reproductive toxicity study. This study measures several key endpoints. These endpoints include an evaluation of potential effects on parental male and female reproductive function offspring, survival, growth including endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring), evaluation of sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment. In the extended 1-generation reproductive toxicity study on 2,4-D (MRID 47972101) submitted in response to the data call-in, young adult rats were administered 2, 4-D *via* the diet for ≈ four weeks prior to mating and continuing through mating, gestation, and lactation at dose levels up to those that saturate metabolic processes. The young rat was exposed during gestation, lactation (*via* the milk), and directly (*via* the diet) following weaning. Parameters assessed include sperm parameters, sex gland weights and histopathology; AGD, nipple retention; puberty timing. There were no treatment-related, adverse effects on any of the parameters assessed, including prostate and other sex organ weights. The citation was included in the Endocrine Disruptor Review Team assessment of 2, 4-D (TXR No. 0055461). EDRT concluded that this citation cited as OSRI did not satisfy the requirement for the Androgen Receptor Binding Assay (Guideline 890.1150). The requirement for the Hershberger Assay is satisfied based on the extended 1-generation reproductive toxicity study (MRID 47972101) of 2, 4-D (TXR No. 0055461, page 37).
CITED BY NRDC (24) – paper cited as evidence of endocrine disrupting effects of 2,4-D; exposure to 2,4-D caused increases in prostate size in laboratory rats
CITED BY TF: Effect on prostate occurred at dose known to be above threshold for saturation of renal clearance; *in vitro* study of questionable value; effects of dietary 2,4-D treatment on prostate weight and pathology is being examined in the data call-in studies requested by EPA

(Study 57) **Citation**: Kim H-J, Park YI, Dong M-S. (2005). Effects of 2,4-D and DCP on the DHT-induced androgenic action in human prostate cancer cells. Toxicological Sciences 88 (1): 52-59. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]**
Methods Summary: To elucidate the mechanism of the synergistic effects of 2,4-D and DCP on the androgenicity of testosterone, the androgenic action of 2,4-D and DCP was characterized using a mammalian detection system in prostate cancer cell lines. 2,4-D (>98%); 2 human prostate cancer cell lines were used: AR expressed 22Rv1 and androgen-independent PC3; The 22Rv1 cells are derived from a relapsed tumor and have been characterized as a valuable tool for the study of prostate cancer progression; a representative androgen-insensitive cell line PC3 was used for validation;
Results Summary: 2,4-D treatment alone did not increase the proliferation rate of 22Rv1 cells; the combined effects of 2,4-D with DHT on prostate cell proliferation showed that whereas stimulation with DHT alone showed a 1.5-fold increase in cell growth rate, co treatment with 2,4-D exhibited a 2.5-fold increase in cell growth rate, which suggests to authors that 2,4-D acted either additively or synergistically with DHT on the proliferation of prostate cancer cells.
CONCLUSION: provides clear evidence for androgenic actions of phenoxy compounds using mammalian

system; enhance DHT-induced AR transcriptional activity; promotes proliferation of androgen-sensitive cells
COMMENT/HED**:** Kim et al., (2005) studied the ability of 2,4-D and its metabolite to increase the proliferation of 22Rv1 human prostate cancer cells. Negative results were reported. Trancriptional activation studies were conducted with various cell lines. All were reported negative, except 2,4-D was reported to increase the response when coadministered with testosterone. NRDC cited the paper as evidence of endocrine disrupting effects of 2, 4-D, stating that the combination of 2, 4-D and testosterone resulted in synergistic increases in the proliferation of prostate cancer cells. The Task Force commented that this *in vitro* mechanistic study cited by NRDC is based on the earlier report that 2, 4-D increased prostate weight in rats following oral (gavage) exposure at a high dose above the threshold for saturation of renal clearance, which they state renders the usefulness and interpretation of this *in vitro* study of questionable value to potential human risk The Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2, 4-D was included in this list of chemicals. This study was included in the Endocrine Disruptor Review Team assessment of 2, 4-D (TXR No. 0055461); it should be noted that due to the limitations of these studies, the submitter did not claim them as OSRI. Effects of dietary 2, 4-D treatment on prostate weight and pathology were examined in extended 1-generation reproductive toxicity study on 2, 4-D, and there were no treatment-related effects. The NOAEL (45 mg/kg/day) is greater than the PODs utilized for the human health risk assessment supporting the 2, 4-D RED. The objective of the EDSP Tier 1 battery assays is to screen for the potential to interact with the estrogen, androgen and thyroid hormone pathways. EPA waived the Tier 1 tests most relevant to human endocrine effects for 2,4-D due to the availability of the extended one generation reproduction study with 2,4-D. (Ref. Office of Chemical Safety and Pollution Prevention, EPA, Memorandum from Greg Ackerman to Katie Weyrauch, "**2, 4-Dichlorophenoxyacetic Acid (2,4-D) - Report of the Endocrine Disruptor Review Team - Test Order #: EDSP–031001-120" (December 20, 2010).**) EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints. The purpose of Tier 2 testing is to identify and establish a dose response relationship for any adverse effects that might results from interactions identified through Tier 1 screening. The Tier 2 mammalian study is currently the 2-Generation Reproduction and Fertility Effects Study (Note: the OECD Guideline for the Extended F1 One Generation Reproduction Test Guideline is also under consideration). The existing 2, 4-D hazard database includes an Extended F1 One Generation Reproduction Test Guideline.
CITED BY NRDC (23): paper cited as evidence of endocrine disrupting effects of 2,4-D; shows that the combination of 2,4-D and testosterone resulted in synergistic increases in the proliferation of prostate cancer cells, including up to a 32-fold increase in activation of these cancer cells.
CITED BY TF: this *in vitro* mechanistic study is based on their earlier report that 2,4-D increased prostate weight in rats at 50 mg/kg/day (oral gavage); use of high dose (above threshold for saturation of renal clearance) renders usefulness and interpretation of this *in vitro* study of questionable value to potential risk; states that effects of dietary 2,4-D treatment on prostate weight and pathology is currently being examined in extended 1-generation reproduction study

(Study 58) **Citation**: Kojima H, Katsura E, Takeuchi S, Niiyama k, Kobayashi K. (2004). Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. Environm Hlth Perspect 112 (5): 524-531. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]**

Methods Summary: CHO-K1 cells; reporter gene assays for human ERα (hER α) and hERβ and AR (hAR) expression
Results Summary: 2,4-D not identified as showing estrogen or androgen receptor activities
CONCLUSION: 2,4-D did not show estrogenic effects or antiestrogenic properties in the hER α transactivation assay; did not show androgenic transcriptional activity; did not show an inhibitory effect on the androgenic activity induced by DHT
COMMENT/HED**:** In an *in vitro* assay (hER α transactivation assay; reporter gene assays for human ERα (hER α) and hERβ and AR (hAR) expression), 2,4-D did not show estrogenic effects or antiestrogenic properties in the hER α transactivation assay; did not show androgenic transcriptional activity; and did not show an inhibitory effect on the androgenic activity induced by DHT. Concern for endocrine disruption potential was identified in the RED. The Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2,4-D was included in this list of chemicals. This is one of several studies cited as Other Scientifically Relevant Information (OSRI) in response to the EDSP Tier 1 Test Orders and by the Task Force to refute the NRDC evidence of the endocrine disrupting effects of 2,4-D. This citation was evaluated as OSRI in the Endocrine Disruptor Review Team assessment of 2, 4-D (TXR No. 0055461, Table 7, page 35). The citation does not provide any data to substantiate the reported negative results. Transcriptional assays do not measure receptor binding and involve post-binding events; therefore, they are not an adequate substitute for receptor binding assays. The F1-extended one generation reproductive toxicity study of 2,4-D evaluated several key endpoints. These endpoints include an evaluation of potential effects on parental male and female reproductive function, offspring survival, growth including endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring), evaluation of sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment. In addition the test guideline includes options to evaluate development of the immune and nervous systems. The findings for 2, 4-D for the extended one-generation reproductive toxicity study determined there were no adverse effects observed on any of the numerous parameters evaluated, and the NOAEL was established at 45 mg/kg/day in males and 40 mg/kg/day in females. Both NOAELs are greater than the Points of Departure utilized for the human health risk assessment supporting the 2, 4-D RED. The extended 1-generation reproduction study confirms the protectiveness of the existing Points of Departure.
CITED BY TF: one of several studies cited that indicate that 2,4-D is negative for estrogenicity; human estrogen receptor-alpha, estrogen receptor-beta transactivation: negative

(Study 66) **Citation**: Lin, N.; Garry, VF (2000). *In vitro* studies of cellular and molecular developmental toxicity of adjuvants, herbicides, fungicides, Minnesota. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]**
Methods Summary: YES: Yeast Estrogen Screen or yeast two-hybrid screen
Results Summary: commercial-grade 2,4-D LV4 & 2,4-D amine induced MCF-7 cells; adjuvants induced proliferation of MCF-cells but at lower concentration; reagent-grade 2,4-D inactive; did not induce proliferation of MCF-7 cells
CONCLUSION: 2,4-D negative in this assay
COMMENT/HED**:** The Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery; 2,4-D was included in this list of chemicals. This is one of several studies cited as Other Scientifically Relevant Information (OSRI) in response to the EDSP Tier 1 Test Orders and by the Task Force to refute the NRDC evidence of the endocrine disrupting effects of 2,4-D. This citation was evaluated as OSRI in the Endocrine Disruptor Review Team assessment of 2, 4-D (TXR No. 0055461; Table 4, page 18). EDRT noted that a cell proliferation assay does not measure receptor binding and may be affected by factors other than binding with the receptor. Study may be useful supplementary information. EPA waived the Tier 1 tests most relevant to human endocrine effects for 2,4-D due to the availability of the extended one generation reproduction study with 2,4-D. (Ref. Office of Chemical Safety and Pollution Prevention, EPA, Memorandum from Greg Ackerman to Katie Weyrauch, "**2, 4-Dichlorophenoxyacetic Acid (2,4-D) - Report of the Endocrine Disruptor Review Team - Test Order #: EDSP–031001-120" (December 20, 2010).**) EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be

considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.
CITED BY TF: one of several studies cited that indicate that 2,4-D is negative for estrogenicity; YES for 2,4-D ai was negative; YES formulated material, which may contain estrogenic surfactants was positive.

(Study 67) **Citation**: Liu, RCM; Hahn, C.; and Hurtt, ME (1996). The Direct Effect of Hepatic Peroxisome Proliferators on Rat Leydig Cell Function *in vitro*. Fundamental & Applied Toxicol. 30: 102-108. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]** (cancer MOA)
Methods Summary: Leydig cells, isolated from adult Crl:CDBR rats (12-16 weeks old), were treated with 2,4-D for 21 hours and medium was assayed for estradiol; function of treated Leydig cells was evaluated by measuring the release of testosterone in response to human chorionic gonadotropin (hCG); addressing MOA for Leydig cell adenomas and PP
Results Summary: 2,4-D did not significantly reduce the hCG-stimulated release of testosterone (70% of C); concentration that decreased response to 50% of control response (IC50) testosterone was 1147 µM; basal estradiol production: estimated concentration that increased control response to half-maximum EC50 (µM) estradiol was 221
CONCLUSION: results suggest that peroxisome proliferators, as a class of compounds, directly modify the steroidogenic function of Leydig cells *in vitro.* Further investigations necessary to address the mechanism for the *in vitro* effects on Leydig cells and to clarify the apparent relationship between peroxisome proliferator-induced changes in Leydig cell function and the development of Leydig cell tumors.
COMMENT/HED**:** The referenced study is an *in vitro* study designed to address the mode of action for Leydig cell adenomas and peroxisome proliferation. The results suggest that peroxisome proliferators, as a class of compounds, directly modify the steroidogenic function of Leydig cells *in vitro*. Investigators stated that further investigations were necessary to address the mechanism for the *in vitro* effects on Leydig cells and to clarify the apparent relationship between peroxisome proliferator-induced changes in Leydig cell function and the development of Leydig cell tumors. This study is one of several studies cited by NRDC as the basis for their concern for potential endocrine disrupting effects of 2, 4-D. The Task Force stated that no significant effects in hCG-stimulated release of testosterone were seen in rat Leydig cell cultures. 2,4-D is a known weak peroxisome proliferator and effects on reproductive organs/hormones were identified in RED; 2, 4-D is not associated with Leydig cell tumors. The citation is of ongoing research into the possible relationship between peroxisome proliferators and changes in Leydig cell function; 2, 4-D, as a known peroxisome proliferators , was used in this MOA assessment of peroxisome proliferators. Effects on reproductive hormones were identified in the RED and were addressed in extended 1-generation reproductive toxicity study (MRID 47942101). The F1-extended one generation reproductive toxicity study of 2,4-D evaluated several key endpoints. These endpoints included an evaluation of potential effects on parental male and female reproductive function, offspring survival, growth including endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring), evaluation of sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment. There were no adverse effects observed on any of the numerous parameters evaluated in the extended 1-generation reproductive study (TXR No. 0055341), and the NOAEL was established at 45 mg/kg/day in males and 40 mg/kg/day in females. Both NOAELs are greater than the Points of Departure utilized for the human health risk assessment supporting the 2, 4-D RED.
CITED BY NRDC (22): one of several studies cited that NRDC believes establishes the dangerous endocrine disrupting effects of 2,4-D that requires that 2,4-D be banned; 2,4-D causes slight decrease in testosterone release, significant increase in estrogen release from testicular cells;

CITED BY TF: states that n**o** significant effects in hCG-stimulated release of testosterone in rat Leydig cell cultures; indicates that NRDC fails to mention minimal effect concentration for effect on estrogen was 500 µM, which translates to ≈110 µg/mL; is > max 2,4-D plasma conc reported in rats treated w/ 100 mg/kg/day (above saturation of renal clearance) (Saghir, et al, 2006); given 100 mg/kg is well above saturation of renal clearance, these in vitro findings are not considered indicative of an endocrine risk by TF.

(Study 70) **Citation**: in *Russian* – Malysheva LN, and Zhavoronkov AA. (1997). Morphological and histochemical changes in the thyroid gland after a single exposure to 2,4-DA herbicide. Biull Eksd Biol Med 124 (12),676-677. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]**
Methods Summary: not available for review
Results Summary: as cited in BP: decreases in thyroid gland transport and production functions and impairment of hormone iodination in thyroid were observed after acute exposure to rats
CONCLUSION: don't have translation (in Russian).
COMMENT/HED**:** The study is in Russian and no translation is available. There are no details regarding test methods or dose levels used, and no data are available to substantiate the reported effects. As cited by Beyond Pesticides, decreases in thyroid gland transport and production functions and impairment of hormone iodination in thyroid were observed after acute exposure to 2, 4-D. Thyroid effects were identified in the 2, 4-D RED and a Data Call-In was issued for a 2-generation reproduction study that specifically required examination of the thyroid. The F1-extended one-generation reproductive toxicity study of 2, 4-D provides an assessment of the thyroid following repeat exposure at adequate dose levels; *i.e.,* an evaluation of thyroid hormones (T3, T4 and TSH), thyroid weight and histopathology of the thyroid across life stages, and an evaluation of auditory startle and brain morphometry data (reported to be sensitive to thyroid disruption). The thyroid toxicity NOAEL was established at ≈40 mg/kg/day, the highest dose tested. This NOAEL is greater than the Points of Departure utilized for the human health risk assessment supporting the 2, 4-D RED. The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D
CITED BY BP - additional evidence that 2,4-D poses unreasonable risks to human health and environment; its registration must be revoked

(Study 73) **Citation**: McKinlay, R.; Plant, JA; Bell, JNB; *et al.* (2008). Endocrine Disrupting Pesticides: Implications for Risk Assessment. Environment International 34, 168-183. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]**
Methods Summary: NA – review article
Results Summary: cited paper by Kim, *et al.* (2005); Synergistic androgenic effects when combined with testosterone (Kim 2005) promotes proliferation of androgen-sensitive cells
CONCLUSION: more data and research needed to quantify exposure
COMMENT/HED**:** The extended 1-generation reproductive toxicity study on 2,4-D includes a detailed assessment of endocrine endpoints [thyroid, estrus cyclicity, sexual maturation (delays in vaginal opening and preputial separation), andrology, ovarian staging]. 2,4-D was included on the list of chemicals to receive test orders for the Endocrine Disruption Screening Program (EDSP) Tier 1 battery. Anti-androgenicity or androgenicity sensitive parameters were evaluated in the F1-extended 1-generation reproductive toxicity study of 2, 4-D, which included: balano-preputial separation, anogenital distance, quantitative nipple retention, evaluation of sperm parameters, male reproductive organ weights and detailed histopathological evaluation of male reproductive organs**.** There were no treatment-related adverse effects on any of the numerous parameters assessed across life stages. The no observed adverse effect level (NOAEL) for androgenic effects was established at 45 mg/kg/day. This NOAEL is greater than the Points of Departure utilized for the human health risk assessment supporting the 2,4-D RED.

The cited study does not identify a more sensitive endpoint for human health risk assessment than already identified by the Agency, and the results do not yield information that would cause the Agency to modify its conclusions from the 2005 RED for 2, 4-D
CITED BY BP: cited study as showing that 2,4-D promotes the proliferation of androgen-sensitive cells by acting synergistically with its main metabolite, DCP, also known for its endocrine disrupting effects

(Study 79) **Citation**: Nishihara T, J Nishikawa, T. Kanayama, F. Dakeyama, K. Saito, M. Imagawa, S. Takatori, Y. Kitagawa, S. Hori, h. Utsumi. (2000). Estrogenic Activities of 517 Chemicals by Yeast Two-Hybrid Assay. Journal of Health Science 46 (4): 282-298. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]**
Methods Summary: yeast two-hybrid assay system with estrogen receptor (Erα and the coactivator, T1F2; introduced 2 expression plasmids, pGBT9-ERLBD and –GAD424-T1F2, into yeast cells (*Saccharomyces cerevisiae* Y190), which carry a β-galactosidase reporter gene and require tryptophan and leucine for growth; results evaluated by relative activity, expressed as REC10 (10% relative effective concentration), that is the concentration of test compound showing 10% of agonist activity of 10^{-7} M E2, which is the optimum concentration for E2. when the activity of test compound was higher thanREC10 within the concentration tested, chemical judged as positive.
Results Summary: 2, 4-D negative (REC10 2,4-D >1 x 10^{-3})
CONCLUSION: positive chemicals demonstrate a common structure: phenol with a hydrophobic moiety at the para- position without bulky groups at the ortho-position. 2, 4-D was reported as negative (page 296)
COMMENT/HED**:** The referenced study is a yeast two-hybrid assay system, in which 2, 4-D was reported to be negative. When the Agency used an exposure-based approach to select the first list of chemicals to receive Test Orders for the EDSP Tier 1 screening battery, 2,4-D was included in the list of chemicals. This study was referenced in a document submitted to the Agency by the 2,4-D Consortium in response to the EDSP Tier 1 Test Order, however it was not cited as Other Scientifically Relevant Information (OSRI). This citation was included in the Endocrine Disruptor Review Team assessment for 2, 4-D (TXR No. 0055461, page 18). In yeast-based assays some test chemicals are unable to penetrate the cell wall and this failure may result in a false negative. Another issue with these assays is cytotoxicity. EPA waived the Tier 1 tests most relevant to human endocrine effects for 2,4-D due to the availability of the extended one generation reproduction study with 2,4-D. (Ref. Office of Chemical Safety and Pollution Prevention, EPA, Memorandum from Greg Ackerman to Katie Weyrauch, "**2, 4-Dichlorophenoxyacetic Acid (2,4-D) - Report of the Endocrine Disruptor Review Team - Test Order #: EDSP–031001-120" (December 20, 2010).**) EPA has received all required final study reports and data from the Tier 1 battery of tests for 2,4-D. The submitted EDSP Tier 1 tests will be considered with regard to potential environmental effects and the need for Tier 2 in vivo studies for effects in wildlife. Although the submitted Tier 1 *in vitro* studies may inform EPA on mechanistic issues in mammalian systems (e.g., whether 2,4-D can bind to the estrogen or androgen receptor in mammals), the studies will not affect EPA's conclusions on the quantitative endocrine risks posed by 2,4-D for humans given the availability of the extended one-generation reproduction study (an *in vivo* study in rats) that comprehensively examined the risks to human health from 2,4-D's interaction with endocrine system endpoints.
CITED BY: 2,4-D Task Force: one of several *in vitro* studies referenced by TF to dispute NRDC's assessment of Xie, et al (2005) report that 2,4-D has "relatively potent estrogenic effects in fish"; states that Agency has on several occasions rejected the position that data gathered under Endocrine Disruptor Screening Program is a prerequisite to a safety determination; yeast two-hybrid screen: negative (states that Xie, *et al.* , 2005, incorrectly indicated 2,4-D "estrogenic at a concentration of ≈0.2 g/L").

(Study 83) **Citation**: Oakes, *et al.* (2002) A Study of the Potential for a Herbicide Formulation Containing 2,4-D and Picloram to Cause Male-Mediated Developmental Toxicity in Rats. Toxicological Sciences 68: 200-206. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]** (Reproductive/Developmental)
Methods Summary: male Sprague-Dawley rats gavaged for 5 days/week for 9 weeks with mixture (0.125,

0.25, or 0.5 mL/kg/day 2,4-D and Picloram); 3 dose levels (150, 75, 37.5 mg/kg/day 2,4-D; distilled water); + control: cyclophosphamide; each male mated with 2 untreated females during weeks 2 and 4, 4 and 5, and 8 and 9 of treatment, and with 4 untreated females after an 11-week recovery period; females sacrificed on GD 20 and fetuses were weighed and examined for either structural malformations or skeletal development.
Results Summary: mean # corpora lutea, preimplantation loss (except 2-3 week exposure HD 15.2% vs 6.1% control), postimplantation loss, litter size, fetal weight, and malformation rate (except 2-3 week treatment 5.8%* HD vs 1.2% control) were unaffected.
CONCLUSION: results did not show any evidence that 2,4-D repeat exposure of male rats is likely to cause male-mediated birth defects in offspring or other adverse reproductive outcomes.
COMMENT/HED**:** Study examines the effects of 2, 4-D on male rats following 9 weeks of exposure with a mixture of 2,4-D and picloram. The treated males were mated with untreated female rats, and the females were sacrificed on GD 20, and fetuses were weighed and examined for either structural malformations or skeletal development. The results did not show any evidence that repeated exposure of male rats was likely to cause male-mediated birth defects in offspring or other adverse reproductive outcomes. Information provided in the report includes 2,4-D serum levels attained at 150 mg/kg/day dose level [daily dose Cmax values of 249-284 µg 2,4-D/mL serum]; serum half-life of ~6 hours; commercial applicators and forestry workers (no protection) estimated exposures ranging from 0.9 to 160 µg/kg/day, which represents exposure to 0.1-11 mg for 70 kg man (referenced Draper and Street, 1982; Kolmodin-Hedman and Erne, 1980; Lavy, et al, 1987; Nash, et al, 1982); in terms of estimated serum levels, men given an oral dose of 5 mg 2,4-D/kg (=350 mg for 70-kg man) had a Cmax of 40 µg/mL (Kohli, et al., 1974); an 11-mg intake would give an estimated plasma level of 1 µg/mL; negative study at doses up to 150 mg/kg/day 2,4-D, which exceeds renal excretion mechanism. This citation was included in the Endocrine Disruptor Review Team assessment of 2, 4-D (TXR No. 0055461, page 94), but was rejected in the EDRT's weight of evidence. Endocrine/reproductive concerns were identified in the RED and are addressed in the extended 1-generation reproductive toxicity study. The F1-extended one generation reproductive toxicity study of 2, 4-D evaluated several key endpoints. These endpoints included an evaluation of potential effects on parental male and female reproductive function, offspring survival, growth including endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring), evaluation of sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment. In addition the test guideline includes options to evaluate development of the immune and nervous systems. There were no adverse effects on any of the numerous parameters monitored in the extended 1-generation reproductive toxicity study of 2, 4-D (reproductive performance, sperm parameters assessed, sex gland weights and histopathology; AGD, nipple retention; puberty timing). The NOAEL was established at 45 mg/kg/day, which is greater than the Points of Departure utilized for the human health risk assessment supporting the 2, 4-D RED. As noted above, EPA has received all required study reports and data from the Tier 1 battery of test for 2, 4-D.
CITED BY: 2, 4-D Task Force: study cited as providing evidence that exposure to a herbicide formulation containing 2,4-D and picloram is not likely to cause male-mediated birth defects or other adverse reproductive outcomes; no effects characteristic of endocrine-mediated response.

(Study 100) **Citation**: Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. (1995). The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. Environ Hlth Perspect. 103: 113. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin: Endocrine]**
Methods Summary: E-screen is a quantitative assay that compares the cell number achieved by similar inocula of MCF-7 cells in the absence of estrogens (negative control) and in the presence of 17β-estradiol (positive control) and a range of concentrations of chemicals suspected to be estrogenic.
Results Summary: negative for 2,4-D
CONCLUSION: 2, 4-D was not identified as estrogenic
COMMENT/HED**:** This citation was included in the Endocrine Disruptor Review Team assessment of 2, 4-D (see TXR No. 0055461). The results of the MCF-7 cell proliferation assay were described as negative,

but no data were provided to substantiate this result. An extended 1-generation reproductive toxicity study on 2,4-D, in which offspring were exposed to 2, 4-D during gestation, lactation, and directly through PND 22, 70, 139, includes an assessment of 2, 4-D on several key endpoints. These endpoints include an evaluation of potential effects on parental male and female reproductive function offspring survival, growth including endocrine and systemic toxicity parameters such as estrous cyclicity (female adult rats and offspring), evaluation of sperm parameters, anogenital distance, nipple retention, puberty onset (vaginal opening and balano-preputial separation), and thyroid gland weight, hormone and histopathology assessment. In addition the test guideline includes options to evaluate development of the immune and nervous systems. In the extended one generation reproductive toxicity study, there were no treatment-related adverse effects on any of the measured parameters evaluated to assess the estrogen, androgen or thyroid endocrine systems in intact rats following 2, 4-D exposure at dose levels approaching saturation of renal clearance. As noted above, EPA has received all required study reports and data from the Tier 1 battery of test for 2, 4-D.
CITED BY: 2,4-D Task Force: cited to dispute NRCD's claim that 2,4-D has extensive hormone disrupting activity that may occur at low doses; 2,4-D not found to exhibit estrogenic properties; MCF7 cell proliferation: negative

BIN: DERMAL PENETRATION

(Study 11) **CITATION**: Brand, RM and Jendrzejewski, JL (2008). *The Effect of Chronic Ethanol Ingestion on Skin Penetration of 2,4-D* – title cited (could not find paper with this title). Chronic Ethanol Ingestion Alters Xenobiotic Absorption Through the Skin: Potential Role of Oxidative Stress. Food & Chemical Toxicol 46: 1940-1948. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin:** Dermal Penetration]
Methods Summary: rats pair-fed a diet containing 36% ethanol for 12 weeks; then switched to a non-alcohol diet and monitored for 4 weeks; non-invasive measurements for changes in dermal blood flow using laser Doppler velocimetry (LDV), damage to skin barrier via transepidermal water loss (TEWL), and changes in skin moisture content were performed; at 0, 1 day, or 1, 2, 3, 4 weeks after alcohol removal, rats sacrificed and their skin analyzed for alcohol and aldehyde dehydrogenase, and lipid peroxidation; transdermal penetration of DEET and 2,4-D was determined also.
Results Summary: Transdermal absorption, LDV, TEWL, skin alcohol and aldehyde dehydrogenase (enhancement ratio 1.6), and lipid peroxidation (↑2.3 fold) were significantly increased after continuous ethanol exposure; factors remained elevated for up to 4 weeks after alcohol consumption stopped; reflect fundamental changes in the skin
CONCLUSIONS: alcohol ingestion is correlated with several skin disorders; link between ethanol ingestion and skin disorders associated with alcohol use
COMMENT/HED**:** Alcohol is known to alter the skin, which can alter dermal function (barrier) and lead to increased dermal absorption (not unique to 2, 4-D). Alcohol shown to trigger increases in levels of enzymes associated with ethanol metabolism, induce physical changes in the skin leading to increased uptake of xenobiotics (e.g., 2,4-D); higher generation of reactive oxygen species; ethanol induces changes in the skin that mimic those seen in the liver, including increases in enzymes related to alcohol metabolism, oxidative stress and blood flow. The exposure conditions are questionable, especially with regard to the regimens used for ethanol administration. Dose level appears very high (36% of diet) compared to what would be expected for those drinking alcohol and then attempting to treat their lawns or work in their treated lawns. Many factors can alter dermal function (barrier) and lead to increased dermal absorption. Several human dermal absorption studies were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A mean-percent absorbed value of 5.7% of the applied dose was derived from four different human dermal absorption studies. Ultimately, a value of 10% was used by the Agency based on these data and given the variability noted in the results. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, in spite of using a dermal absorption value from a human study, the full 10X UF_H was retained. It should also be noted that the UF_H factor is partially intended to account for uncertainties associated with the relative sensitivity to a pesticide within a population, which includes factors such as differences in dermal absorption that could possibly be attributed to the use of

sunscreen, clothing, or occlusion. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective of any potential enhancement to dermal absorption from sunscreen/alcohol use.
CITED BY: **NRDC** (supplement; provided only authors names and year of publication). One of several studies cited by NRDC by the same investigators to support concern for enhanced dermal penetration of 2, 4-D following alcohol consumption; states that study showed an average of a doubling of absorption of 2,4-D through skin with acute and chronic ethanol intake; suggested that homeowner applying a "weed and feed" type product on a Saturday afternoon after drinking a beer would likely have a skin penetration of 2,4-D at double the rate of EPA's estimate and using DEET would enhance dermal absorption even more; concludes that EPA should use a dermal absorption factor of at least 30% to protect public.

(Study 12) **CITATION**: Brand RM, Charron AR, Dutton L, Gavlic TL, et al. (2004). Effects of Chronic Alcohol Consumption on Dermal Penetration of Pesticides in Rats. J. Toxicol Environ Health A 67, 153-161. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin:** Dermal Penetration]
Methods Summary: male Wistar rats fed Lieber & DeCarli liquid diets (35% fat, 18% total protein,, 36% ethanol, remainder as carbohydrate) for 6-8 weeks; skin shaved, removed, frozen; skin placed in an *in vitro* flow-through diffusion cell system; surface area 0.79 cm^2 (circular chamber 1 cm diameter); epidermal side exposed to 100 µL of [^{14}C]-2,4-dichlorophenoxyacetic acid (specific activity 92.6 µCi/mg) diluted 1:40 with water and 50000 dpm [^{14}C]-2,4-D; commercial formulation (Agriliance) used instead of neat herbicides because previous studies have demonstrated that the inert ingredients lead to enhanced transdermal penetration and are probably more realistic predictor of human exposure; 2,4-D consists of 47.3% 2,4-D DMA salt (473 g/L, 2.1 M) and 52.7% inert ingredients
Results Summary: feeding rats with ethanol (36% of calories) for 6-8 weeks leads to enhanced (by a factor of 2.2) transdermal penetration
CONCLUSIONS: if ethanol consumption has same effect on human skin, it could potentially have adverse health effects on people exposed to 2,4-D.
COMMENT/HED**:** this is a generic issue; EPA agrees that *in vitro* studies indicate that ethanol consumption could potentially enhance the dermal absorption of 2, 4-D under the test conditions. Alcohol consumption has been shown to adversely affect the skin itself (Brand & Jendrzejewski, 2008, Fd & Chem Tox 46: 1940-1948), which can alter dermal function (barrier) and lead to increased dermal absorption. The exposure conditions in the study cited are questionable, especially with regard to the regimen used for ethanol administration (36% of the diet). The Agency is already aware of the kinds of variables that impact the magnitude of the dermal absorption factor (e.g. anatomical site of application, presence or absence of penetration enhancers in the pesticide formation, whether liquid formulations are aqueous or organic, species differences, magnitude of dose, and duration of exposure). The human volunteer dermal absorption studies that were considered in the 2005 risk assessment addressed several of these variables (skin site, dose level, organic or aqueous dose vehicles) and a conservative, upper-bound estimate of 10% was selected that accounts for these variables. As such, the Agency considers the 10% DAF adequately protective for a wide range of the variables known to impact the magnitude of the dermal absorption factor. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, in spite of using a dermal absorption value from a human study, the full 10X UF_H was retained. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective of any potential enhancement to dermal absorption from alcohol use.
CITED BY NRDC: (66) one of several research papers from the same investigators cited by NRDC to show that the 2007 Brand study confirms prior research showing alcohol consumption enhances 2,4-D

penetration.

(Study 13) **CITATION**: Brand, RM; McMahon, L; Jendrzejewski, JL; et al. (2007). Transdermal absorption of the herbicide 2, 4-dichlorophenoxyacetic acid is enhanced by both ethanol consumption and sunscreen application. Food and Chemical Toxicology 45: 93-97. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin:** Dermal Penetration]
Methods Summary: Skin from rats ingesting 1.5 g/kg, 4.3 g/kg, or 6 g/kg of ethanol (control saline) was treated with a commercially available sunscreen containing titanium dioxide and octyl methoxycinnimate and transdermal absorption of 2,4-D amine [^{14} C] was monitored by excising the skin and placing it into a diffusion chamber.
Results Summary: Ethanol ↑ penetration by a factor of 1.9, 2.0, and 2.5, respectively, demonstrating an ethanol-induced dose response; sunscreen application to skin from ethanol-gavaged rats caused 2,4-D absorption above that induced by ethanol alone by an additional factor of 1.3, 2.1, and 2.9, respectively;
CONCLUSION: Results emphasize the importance of limiting ***excessive*** alcohol consumption in individuals with potential herbicide exposure rather than discouraging the use of sunscreens; states that studies demonstrate that personal behavior must be accounted for when estimating potential exposure in both occupational and recreational settings.
COMMENT/HED**:** study indicates that the use of sunscreens and ethanol can enhance dermal absorption of 2, 4-D. This is a generic issue; alcohol consumption has been shown to adversely affect the skin itself; exposure of skin to the sun has its own adverse effects and the use of sunscreens is important (Brand & Jendrzejewski, 2008, Fd & Chem Tox 46: 1940-1948). Many factors can alter dermal function (barrier) and lead to increased dermal absorption. Several human dermal absorption studies were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A mean-percent absorbed value of 5.7% of the applied dose was derived from four different human dermal absorption studies. Ultimately, a value of 10% was used by the Agency based on these data and given the variability noted in the results. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, inspite of using a dermal absorption value from a human study, the full 10X UF_A was retained. Therefore, the Agency is confident that the dermal absorption value used does not underestimate dermal risk and is adequately protective of any potential enhancement to dermal absorption from sunscreen/alcohol use. The exposure conditions are questionable, especially with regard to the regimens used for ethanol administration. Dose levels appear very high compared to what would be expected for those drinking alcohol and then attempting to treat their lawns or work in their treated lawns (dosed with ethanol at 1.5 g/kg to 6 g/kg BW). [Note: For a 60 kg woman, these dose rates equate to the consumption of 6 to 24 one ounce shots of 100-proof vodka.] Also, any dermal absorption enhancements associated with ethanol consumption is moot when considering children's risk since alcohol consumption by minors is illegal.
CITED BY NRDC: (64) states that EPA ignored data showing that dermal absorption is enhanced by alcohol consumption, sunscreen and DEET (didn't incorporate into risk assessment or adopt risk mitigation measures); alcohol consumption ↑ skin penetration by 1.9-2.5 fold; sunscreen further enhanced skin penetration of 2,4-D; one of several papers by same investigators cited to support concern.

(Study 14) **CITATION**: Brand, RM; Spalding, M; Mueller, C. (2002). Sunscreens can increase dermal penetration of 2, 4-dichlorophenoxyacetic acid. Journal of Toxicology Clinical Toxicology 40 (7): 827-832. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin:** Dermal Penetration]
Methods Summary: Hairless mouse (CRL:SK1) skin (dorsal) pretreated with commercially-available sunscreens; 2,4-D placed on top of epidermis in an *in vitro* diffusion chamber for 24 hours
Results Summary: total penetrating through skin in 24 hours ranged from 39.1%±1.7% for no sunscreen control to 81.0%±2.8% for Neutrogena Oil Free Sunscreen

Table 1. Effect of Sunscreen Pretreatment on Transdermal Penetration of 2,4-D

	Peak (% dose/1.5 hr)	Time to Peak (hr)	Cumulative (% dose/24 hr)	Lag time (hr)	Mean enhancement ratio
C to Go	12.9±1.1	7.5±0.9	56.8±6.6**	4.0±0.5	1.5
C Sport	8.8±2.3	7.8±1.2	48.1±6.3	3.9±0.6*	1.2
C Oil Free	13.0±1.4	7.5±0.9	68.0±1.5**	3.1±0.1**	1.7
NoAd	12.5±2.2	8.0±1.5	57.6±1.3**	3.3±0.6**	1.5
Neutrogena S	7.7±0.5	9.5±2.2	59.2±4.9**	3.6±0.5	1.5
Neutrogena OF	14.1±2.7	9.8±2.3	81.0±2.8**	4.0±0.2	2.1
Banana Boat	7.3±2.3	9.5±2.0	39.6±3.4	4.1±0.2	1.0
Off Skin (DEET)	14.4±1.2	6.0±0	46.8±2.6	3.1±0.0**	1.2
Off Deep (DEET)	17.9±2.5***	6.5±0.5	62.4±5.1**	2.8±0.2**	1.6
Control	8.4±0.3	8.8±0.4	39.1±1.7	4.6±0.1	1.0

mean±SEM; * p<0.05; ** p<0.01; *** p<0.001; lag time = x-intercept of line extending from the linear portion of cumulative penetration curve; enhancement ratio = 2,4-D cumulative transport after sunscreen treatment/mean 2,4-D cumulative transport for control

CONCLUSION: Commercially-available sunscreens can enhance dermal penetration of the moderately hydrophilic herbicide 2,4-D. Careful selection of sunscreens during pesticide application could reduce potential exposure.

COMMENT/HED**:** previous EPA response on this issue suggested that one means of dealing with this particular issue would be to add a statement to the label of such products informing users that use may enhance dermal absorption of various substances encountered in everyday life, including pesticides. Dermal absorption factor being used is already conservative: human average % dermal absorption (5.8%) vs 10% dermal absorption factor being used. Many factors can alter dermal function (barrier) and lead to increased dermal absorption. Several human dermal absorption studies were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A mean-percent absorbed value of 5.7% of the applied dose was derived from four different human dermal absorption studies. Ultimately, a value of 10% was used by the Agency based on these data and given the variability noted in the results. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, in spite of using a dermal absorption value from a human study, the full 10X UF_H was retained. It should also be noted that the UF_H factor is partially intended to account for uncertainties associated with the relative sensitivity to a pesticide within a population, which includes factors such as differences in dermal absorption that could possibly be attributed to the use of sunscreen, clothing, or occlusion. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective of any potential enhancement to dermal absorption from sunscreen/alcohol use.

CITED BY NRDC: (67) one of several research papers cited to show that 2007 Brand study confirms prior research showing alcohol consumption and sunscreens enhance 2,4-D penetration

(Study 27) **CITATION**: Feldmann R.G., Mailbach, H. I.. (1974). Percutaneous penetration of some pesticides and herbicides in man. [**Human Studies Considerations Apply: Yes**]

Principal Endpoint: [**Bin:** Dermal Penetration]

Methods Summary: [^{14}C] 2,4-D applied to forearm skin of 6 male humans (vehicle acetone); treated area washed after 24 hours.

RESULTS: 2,4-D absorption of 5.8%±2.4% (range 3.4-8.2%)

CONCLUSION: 2,4-D absorption of 5.8%±2.4% (range 3.4-8.2%)

COMMENT/HED: Several human dermal absorption studies, which included this study, were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A mean-percent absorbed value of 5.7% of the applied dose was derived from four different human dermal absorption studies. Ultimately, a value of 10% was used by the Agency based on these data and given the

variability noted in the results. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, in spite of using a dermal absorption value from a human study, the full 10X UF_H was retained. It should also be noted that the UF_H factor is partially intended to account for uncertainties associated with the relative sensitivity to a pesticide within a population, which includes factors such as differences in dermal absorption that could possibly be attributed to the use of sunscreen, clothing, or occlusion. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective.
CITED BY: NRDC: petition stated that EPA used a dermal absorption factor of 10% based on a 1974 study of 6 adult men, which they indicate is inappropriate, "especially considering the synergistic effects of other exposures." states that the dermal absorption factor of 10% in aggregate risk assessment is woefully insufficient.
CITED BY Task Force – regarding NRDC's allegation of enhanced dermal absorption with alcohol/sunscreen use, cites this study as one considered by EPA (5.8%±2.4; range: 3.4%-8.2%); indicates that study was an extreme test using 2,4-D applied with acetone vehicle, which tends to denature the skin, a process in which proteins or nucleic acids of the skin lose their 3-D structure, thus allowing for increased potential dermal absorption; skin not protected and area not washed for 24 hours for maximum absorption. Task Force states that EPA has previously considered and rejected NRDC's claim that dermal absorption may be enhanced through other factors (sunscreen/DEET/alcohol consumption)

(Study 43) **CITATION**: Harris SA, Solomon KR. (1992). Percutaneous penetration of 2, 4-dichlorophenoxyacetic acid and 2, 4-d dimethylamine salt in human volunteers. J Toxicol Environ Health 36 (3): 233-240. [**Human Studies Considerations Apply: Yes**]
Principal Endpoint: [**Bin:** Dermal Penetration]
Methods Summary: 2, 4-D and 2, 4-D dimethylamine salt (DMA)**;** five male volunteers (each dosed on separate occasions with both chemicals); urine samples, collected for 144 hours following dermal application of 10 mg to dorsum of the hand (9 cm^2), were analyzed for 2,4-D.
RESULTS: an average of 4.46±0.85% was recovered in urine following 2,4 –D exposure, and 1.76±0.57% following DMA; greater amounts of DMA (7.68±0.49%) were washed off at 6 hours compared to 2,4-D (5.35±0.38); average ≈half-lives for excretion were 39.5±8.1 hours (2,4 –D) and 58.5±13.2 hours (DMA).
CONCLUSION: 2,4-D was applied as a solution in acetone, which can be expected to increase penetration into corneum where it is resistant to removal by washing; thus overestimating dermal penetration under field conditions
COMMENT/HED: These data were evaluated previously by HED (TXR# 010044, 2/23/1993; several publications including bystander exposure, professional applicators); results were considered in previous assessments.
CITED BY Task Force – one of studies cited; stated that EPA's decision regarding glove use consistent with robust peer-reviewed literature, which repeatedly demonstrates that use of a protective rubber gloves alone dramatically reduces total absorbed dose of 2,4-D

(Study 74) **CITATION**: Moody RP, Franklin CA, and Ritter L, (1990). Dermal absorption of the phenoxy herbicides 2,4-D, 2,4-D isooctyl, and 2,4,5-T in rabbits, rats, rhesus monkeys, and humans: a cross-species comparison. J. Toxicology and Environmental Health 29: 237-245. [**Human Studies Considerations Apply: Yes**]
Principal Endpoint: [**Bin:** Dermal Penetration]

Methods Summary: Male New Zealand rabbits and male Sprague-Dawley rats (shaved middorsal region), male rhesus monkeys (shaved middorsal forearm or forehead regions); constant dose rate of µg/ cm^2; nonocclusive gauze patches used to protect dosed areas for 24 hours (except forehead); male human volunteers (0.7µCi of ^{14}C radiolabel dissolved in either acetone or Esteron LV96 was applied to a 45-cm^2 area of the forehead); ^{14}C ring-labeled 2,4-D (>98%), dimethylamine, and isooctyl ester: ^{14}C-2,4-D amine in acetone; 2,4-D isooctyl in acetone or Esteron LV96. Urine samples (animals) collected at 4 and 8 hrs on first day, 24-hr intervals for 14 days; human urine collected at 4, 8, and 12 hrs and daily until post-treatment day 7; dose site washed at 24 hrs post dose (all); % dermal absorption and excretion half-lives were calculated

RESULTS:

Table 1. Total Cumulative Dermal Absorption

Pesticide	Rabbit (back)	Rat (back)	Rat (tail)	Monkey (FA)	Monkey (FH)	Human (FA)	Human (FH)
2,4-D acid % rec±sd $T_{1/2}$ N	36±10.9 2.14 3	-	-	15±10.0 1.94 8	29±7.6 1.47 8	6±2.4♪ nr 6	-
2,4-D amine % rec±sd $T_{1/2}$ N	12±3.7 1.65 8	20±3.0 2.55 8	-	-	-	-	58±22.6 nr 6
2,4-D amine % rec±sd $T_{1/2}$ N	-	-	14±6.4 1.35 4	6±3.4 1.83 8	31±13.1 2.13 8	-	-
2,4-D isooctyl % rec±sd $T_{1/2}$ N	50±12.9 nr 6	-	-	40±24.2 2.07 8	56±15.2 2.04 8	-	3±3.0 1.33 4
2,4-D isooctyl % rec±sd $T_{1/2}$ N	34±7.6 0.74 8	-	-	-	-	-	6±2.6 1.63 4

Data from Table 1, page 241 of paper; FA forearm; FH forehead; ♪ data from Feldmann and Maibach, 1974; nr not reported

CONCLUSION: paper stated that anatomical site-related differences in skin permeability are one of a large number of variables known to affect permeability to chemicals; in Discussion section it states: it is noteworthy that the i.m. correction factor used for the animal data permits interspecies comparison of dermal absorption data and circumvents a potential problem that differential interspecies excretion of the pesticide radiolabel between urinary and fecal routes precludes direct extrapolation. They note that their human data were not corrected for non-urinary excretion since iv percent recovery was 100% for 2,4-D acid in humans (Feldmann, 1974). Investigators indicated that assuming a non-urinary excretion of the amine salt and isooctyl ester similar to that obtained in monkeys would tend to increase the human dermal absorption at most by 26-28%, a value consistent with the 20-25% estimated by Grover, et al. (1986) for non-urinary excretion and body storage of 2, 4-D acid ingested by humans (Grover, R., Cessna, AJ, Muir, NI, et al. 1986. Factors Affecting the Exposure of Ground-Rig Applicators to 2, 4-D Dimethylamine Salt. Arch Environ Contam Toxicol. 15: 677-686).

COMMENT/HED: The Agency is aware of the kinds of variables that impact the magnitude of the dermal absorption factor (e.g. anatomical site of application, presence or absence of penetration enhancers in the pesticide formation, whether liquid formulations are aqueous or organic, species differences, magnitude of dose, and duration of exposure). The human volunteer dermal absorption studies that were considered in the 2005 risk assessment addressed several of these variables (skin site, dose level, organic or aqueous dose vehicles) and a conservative, upper-bound estimate of 10% was selected that accounts for these variables. As such, the Agency considers the 10% DAF adequately protective for a wide range of the variables known to impact the magnitude of the dermal absorption factor.

CITED BY: Task Force – findings of study not quoted (not specifically addressed; just listed as a reference)

(Study 75) **CITATION**: Moody RP, Nadeau B. (1992). Effect of the mosquito repellent DEET and long-wave ultraviolet radiation on permeation of the herbicide 2, 4-D and the insecticide DDT in natural rubber gloves. Am Industrial Hygiene Assoc journal 53: 436-441. [**Human Studies Considerations Apply: No**]

Principal Endpoint: [**Bin:** Dermal Penetration]

Methods Summary: examined total ^{14}C-ring labeled 2,4-D permeability across a 1.5 cm^2 cut-out of natural rubber latex gloves incubated in laboratory apparatus at 37°C for 48 continuous hours; mean thickness 0.51±0.008 mm (n=4)

RESULTS: Deet itself rapidly permeated the glove, no significant effect of DEET was observed on glove permeability to 2,4-D (2.4±1.8% with DEET vs 3.2±3.46% without DEET); photoenhanced (long-wave ultraviolet (UVA) radiation) permeation of 2,4-D was observed in a natural rubber glove (6.2%±0.73% +UVA vs 0.3%±0.14% -UVA).

CONCLUSION: No significant effect of DEET on glove permeability to 2,4-D; glove permeability to 2,4-D enhanced when glove exposed to long-wave ultraviolet radiation

COMMENT/HED: As discussed elsewhere, Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective.

CITED BY: NRDC (71) – infers that glove use will not afford adequate dermal protection; study cited as focusing on rubber gloves commonly worn by farmers; NRDC cited study as showing that these gloves were highly permeable to 2,4-D penetration when there was simultaneous exposure to DEET and sunlight.

CITED BY: Task Force – states that study primarily intended as hypothesis-generating experiment; does not provide definitive conclusions for enhanced risk for following reasons: 1) authors concluded DEET did not significantly impact 2,4-D penetration through rubber (NRDC claims DEET alone enhanced 2,4-D permeability); 2) n=2; permeability of 2,4-D was ↑ to 6.2±0.73% when rubber was simultaneously exposed to UV lamp emitting UVA (not "sunlight"; non-UVA control 0.3±0.14% 2,4-D permeation); 3) neither DEET alone nor UVA alone rendered the rubber "highly permeable to 2,4-D despite the relatively severe experimental conditions; 4) only permeation of total [^{14}C] was measured and thus authors could not exclude that the postulated enhanced permeation with UVA_cotreatment was represented by 2,4-D photolysis product(s); and 5) authors stated the field relevance of observations remained to be established.

(Study 76) **CITATION**: Moody RP, Wester RC, Melendres JL, Mailbach HI. (1992). Dermal Absorption of the Phenoxy herbicide 2,4-D Dimethylamine in Humans: Effect of DEET and Anatomic Site. J. Toxicol Environ Health 36 (3): 241-250. [**Human Studies Considerations Apply: Yes**]

Principal Endpoint: [**Bin:** Dermal Penetration]

Methods Summary: ^{14}C-ring labeled 2,4-D-dimethylamine (>99%) (1) in water applied to left palmar region; (2) with DEET in water to left palmar region; (3) in water to left ventral forearm; (4) in acetone to left ventral forearm; 0.5µCi of ^{14}C radiolabeled 2,4-D amine (7 µg) dissolved in 10 µL water or acetone was applied topically to a 4.2 cm^2 area on palm or forearm (dose rate: 1.7 µg/ cm^2) ; for (2) 10 µL DEET was applied to treatment region on palm just prior to application of the 2,4-D amine; nonocclusive polypropylene chambers were affixed over treatment sites; after 24 hours, site washed vigorously with 5 successive gauze swabs wet, respectively with Radiac soap solution, water, Radiac soap solution, water, water; site protected after wash; urine samples collected for 5 days at 24-hr intervals; on day 5 treatment site tape stripped 10 times with cellophane tape, which was analyzed for residues

RESULTS:

Table 1. Average % Dose Recoveries of Soap and Water Washes

Wash	2,4-D-DMA -DEET (aq) on palm	2,4-D-DMA +DEET (aq) on palm	2,4-D-DMA -DEET (aq) on forearm	2,4-D-DMA -DEET (ac) on forearm
Soap	20±15.3	11±7.0	19±9.9	18±11.3
Water	7±4.9	5±2.3	7±4.6	10±8.1
Soap	3±1.6	3±2.0	4±3.4	3±2.7
Water	2±1.5	1±0.4	2±1.6	2±1.4
Water	2±1.0	1±0.5	2±1.7	2±1.4
Total	34±23.0	21±11.8	34±20.3	34±23.4
Tape strips	2±1.6	1±0.6	1±0.9	2±1.4

Chambers	22±3.4	37±7.6	18±3.4	19±4.9
Urine excretion	10±11.5	14±4.5	7±6.2	13±5.0

Data from Tables 1&2, pages 244-245 of paper; average % dose recoveries ± sd; aq (aqueous); ac (acetone)

Most of residue present in initial soap wash; plastic chambers covering treatment site showed 18%-37% recovery 24 hr post treatment; no significant difference between average % recoveries for any of the sample groups involving soap washes, tape strips, and urine samples between each of their respective treatment groups; significantly greater % recovery was obtained for the plastic chambers in sample group where DEET was employed; urinary data demonstrated that there was no significant difference between palmar and forearm skin permeability to 2,4-D-amine, that DEET had no significant effect on total cumulative palmar permeability, and there was no significant difference between % recoveries obtained with acetone or aqueous vehicles.

CONCLUSION: investigators indicated that, from regulatory viewpoint, these data should raise concern since 2, 4-D-amine has been shown to permeate the palm, and hands are the anatomical sites most often contaminated by pesticide applicators. Although this exposure may be somewhat reduced by wearing gloves, study shows significant dermal absorption even following rigorous soap and water washing at 24 hr post treatment.

COMMENT/HED: Several human dermal absorption studies were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A value of 10% was used by the Agency based on these data and given the variability noted in the results. Additionally, a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies) were applied. It is to be noted that although a dermal absorption value from human data was used, the full 10X UF_H was retained to account for uncertainties associated with the relative sensitivity to a pesticide within a population. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used (10%) does not under estimate dermal risk and is adequately protective.
It is also pointed out that if the results from the trial combining 2, 4-D and DEET (14%) are included in the overall average of dermal absorption values from the human studies (Ross, paper), the average dermal absorption only increases from 5.7% to 6.4%. Both numbers round to 6%.

CITED BY: NRDC (68) – cited as research demonstrating presence of DEET on skin significantly enhances absorption of 2,4-D (14% palmar absorption of 2,4-D after applying DEET to skin).

CITED BY: Task Force – states that EPA previously responded to this allegation (the conclusion that the data demonstrate a difference between exposure with (14±4.5%) and without (10±11.5%) DEET is not supported given the magnitude of the standard deviation); also points out that in the 2005 RED, the PPE changes implemented mitigated concerns for potential enhancement by DEET, etc.

(Study 84) **Citation**: Pelletier, O; Ritter, L; Caron, J; and Somers, D (1989). Disposition of 2,4-Dichlorophenoxyacetic Acid Dimethylamine Salt by Fischer 344 Rats Dosed Orally and Dermally. J. Toxicology and Environmental Health 28: 221-234. **[Human Studies Considerations Apply: No]**

Principal Endpoint: **[Bin: Dermal Penetration** (comparison of oral and dermal route)**]**

Methods Summary: dimethylamine salt of ^{14}C-ring-labeled 2,4-D administered to Fischer 344 rats orally (1 and 0.4 mg/kg) and dermally (10 mg/kg); designed to compare the pharmacokinetics of 2, 4-D administered orally and dermally to better understand the relationship between oral toxicity studies and typical dermal field exposure; stated that 2, 4-D is rarely commercially available in the acid form due to its poor water solubility and that commercial formulations are usually composed of alkali or amine salts or esters.

Results Summary: dermal dose (10 mg/kg) as within upper range that might be expected from typical agricultural use; oral dose (1 and 0.4 mg/kg) from preliminary work, which indicated a 4-10% absorption factor for 2,4-D amine through skin, as compared to almost complete absorption from oral route; 94-96% systemically available within 6 hours of oral dose/ 10% over 72 hours of dermal dose; peak concentrations in blood and kidneys achieved within 30 minutes (both routes); by 1.5 hrs after dosing, 2,4-D conc. in blood, muscle, liver, kidneys had decreased (both routes); plateau between 2-8 hrs (dermal) in these tissues

while urinary excretion increased; concentrations in orally-dose animals continued to decrease; at 7 hrs, skin wash removed 63% of applied dose; oral dose almost completely excreted within 24 hours
CONCLUSION: oral dose almost completely excreted within 24 hours; dermal exposure resulted in slower decline due to continued absorption from site of application (72-hour study)
COMMENT/HED**:** Based on urinary excretion values, systemic availability ≈ 10% of dermal dose, while that of the oral dose was virtually 100 % within 72 hours after exposure. In terms of 2, 4-D concentration, about 1 mg of 2, 4-D was excreted following either a dermal dose of 10 mg/kg or an oral dose of 1 mg/kg. Peak concentrations in blood and kidneys were achieved within 30 minutes (both routes). Oral dose almost completely excreted within 24 hours, whereas about 13% of the dermal dose was systemically available at 72 hours.
CITED BY: Task Force: in reference list, but did not find where it was discussed/cited

(Study 86) **CITATION**: Pont AR, Charron AR, Brand RM. (2004). Active ingredients in sunscreens act as topical penetration enhancers for the herbicide, 4-dichlorophenoxyacetic acid. Toxicology and Applied Pharmacology 195: 348-354. [**Human Studies Considerations Apply: No**]

Principal Endpoint: [**Bin:** Dermal Penetration]

Methods Summary: full-thickness dorsal skin of female hairless mice (CRL:SKH1); Kel-F Bronaugh-style flow through diffusion chamber (exposed surface area of 785 cm^2) with epidermal side facing donor chamber; 2,4-D amine in ethanol; 9 sunscreen formulations

Results Summary:

Table 1.

Sunscreen ingredient	Cumulative (% dose/24 hr)	Lag time (hr)
Hairless mouse skin *in vitro*		
Octyl methoxycinnamate	84.2±1.8**	1.7±0.2**
Octocrylene	65.6±4.3	3.7±0.3
Oxybenzone	74.9±4.8	3.7±0.4
Homosalate	72.5±3.5*	2.6±0.4
Octyl salicylate	74.1±3.1**	1.1±0.2**
DEET 9.5%	83.3±2.4**	2.6±0.2**
DEET 19.0%	82.8±3.4	2.4±0.1**
Padimate-o	86.9±2.5**	2.5±0.4**
Sulisobenzone	81.2±3.1**	1.5±0.2**
Control	54.9±4.7	4.4±0.2
Human skin *in vitro*		
Padimate-o	41.3±3.3**	1.9±0.4**
Octyl salicylate	35.7±11.5*	0.9±0.1**
Octocrylene	14.5±1.9	2.7±0.4*
Control	13.0±1.0	4.2±0.4

mean±SEM; * $p<0.05$; ** $p<0.01$

CONCLUSION: Most of the sunscreens increased the total amount of 2, 4-D flux through the skin vs control and decreased lag time vs control (both hairless mouse and human skin).

COMMENT/HED The existing dermal absorption value of 10% is based on the high end of the range of results in an extensive set of high quality human research results. The Agency considers this the best available data because these data eliminate the interpretative considerations necessary to extrapolate from a different species and an *in vitro* test methodology. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, inspite of using a dermal absorption value from a human study, the full 10X UF_H was retained. Additionally, the UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective of any potential enhancement to dermal absorption from sunscreen use.

Current OPP policy precludes using *in vitro* dermal absorption data as a stand-alone for quantitative use in risk assessment. Corroborating *in vivo* data is also required. This is because OPP does not consider *in vitro* methods to have been sufficiently validated for stand-alone regulatory use[1] Additionally, there are important limitations to *in vitro* models for dermal absorption, one of which is lack of an intact vasculature. NAFTA Dermal Absorption Group Position Paper on Use of In Vitro Dermal Absorption Data in Risk Assessment (2009).
CITED BY: NRDC– (65) one of several research papers by the same investigators cited to show that 2007 Brand study confirms prior research showing alcohol consumption and sunscreens enhance 2,4-D penetration; NRDC considers EPA's previous response to public comments (12/16/2004) urging EPA to consider these effects in the RA a cursory and highly deficient response

(Study 89) **CITATION**: Riviere JE, Baynes RE, Brooks JD, Yeatts JL, Monteiro-Riviere NA. (2003). Percutaneous absorption of topical N, N-diethyl-m-toluamide (DEET): effects of exposure variables and coadministered toxicants. J. Toxicol Environ Health A, 66(2): 133-151. [**Human Studies Considerations Apply: No**]
Principal Endpoint: [**Bin:** Dermal Penetration]
Methods Summary: isolated perfused porcine skin flaps (IPPSFs were prepared and perfused in a non-recirculating perfusion chamber; flow-through diffusion cells
Results Summary: an important finding is that vehicle and concentration seemed to have minimal effects on absorption, while exposure to systemic drugs or a topical toxicant such as subvesicating doses of sulfur mustard did increase absorption, thereby accentuating any role that DEET might play in a syndrome such as the Gulf War Illness; most obvious finding, and one easily addressable in an occupational environment, was that covering DEET dose areas with an occlusive dressing, or even fabric, enhances absorption. Care should be taken to avoid this scenario after DEET has been applied.
CONCLUSION: results suggest that co-exposure to a number of chemicals that potentially could be encountered in a military environment may modulate the percutaneous absorption of topically applied DEET beyond that seen for normal vehicles at typically applied concentrations.
COMMENT/HED**:** study is on DEET and cofactors that could modulate transdermal flux of topically applied DEET as it relates to contributory factor to the Gulf War Illness; a generic issue; Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, inspite of using a dermal absorption value from a human study, the full 10X UF_H was retained. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk. UF_H factor is partially intended to account for uncertainties with dermal absorption such as the use of sunscreen, clothing, or occlusion. Additionally, the UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. The Agency is aware that occlusive coverings increase skin hydration status by preventing water loss, and that hydrated skin is generally more permeable to pesticides than un-hydrated skin Therefore, occlusive coverings would be expected to increase exposure via the dermal route, resulting in increased risk. However, in EPA's evaluation of this study, it found that the Agency's risk assessment and margins of safety established through the application of uncertainty factors, already account for any uncertainty raised by NRDC relative to the findings in this study
CITED BY: NRDC – (70) states that occlusion can significantly enhance skin absorption of dermally-applied materials

(Study 91) **CITATION**: Ross RH, Driver JH, Harris SA, Maibach HI. (2005). Dermal absorption of 2, 4-D: a review of species differences. Regulatory Toxicology and Pharmacology 41: 82-91. [**Human Studies Considerations Apply: Yes**]
Principal Endpoint: [**Bin:** Dermal Penetration]
Methods Summary: comparison of dermal absorption studies in animals (rat, mouse, rabbit, Rhesus monkey) and humans
Results Summary: dermal penetration: rat 17.3%; rabbit 18.5%, monkey 10.5%; mouse 21%; references 5 human studies where either 2,4-D or DMA were applied to forearm or hand, which show consistent results

Table 1. Dermal Absorption Studies in Humans		
Reference (acid/salt)	Methods (site, n, dose, vehicle, time of wash, sample collection time	% absorbed (mean±sd)
Feldmann (acid)	Forearm; n=6; 4 µg/cm^2 ; acetone; 24 hours, at 120 hours	5.8±2.4
Maibach (DMA)	Forearm; n=6; 4 µg/cm^2 ; na; 4 hours, at 120 hours	3.7±na
Maibach (DMA)	Forearm; n=6; 4 µg/cm^2 ; na; 4 hours, at 120 hours	2.8±na
Moody (DMA)	Palm; n=4; 1.7 µg/cm^2 ; water; 24 hours; at 168 hours	10±11.57±6.2
Moody (DMA)	Forearm; n=4; 1.7 µg/cm^2 ; water; 24 hours, at 168 hours	13±5.0
Moody (DMA)	Forearm; n=4; 1.7 µg/cm^2 ; acetone; 24 hours, at 168 hours	4.5±0.9
Harris (acid)	Backhand; n=5; 1100 µg/cm^2 ; acetone; 6 hours, at 144 hours	1.8±0.7
Harris (DMA)	Backhand; n=5; 1100 µg/cm^2 ; water; 6 hours, at 144 hours	1.1±0.3
Wester (DMA)	Forearm; n=6; 39.7 µg/cm^2 ; ethanol; 24 hours, at 144 hours	
Weighted Average♪		5.7±3.4

♪sum of % absorption from each data set multiplied by the # of replicates in that data set divided by the total # of replicates (34) that excluded Maibach and Feldmann, 1974

CONCLUSION: For purposes of estimating potential human health risks associated with systemic absorbed doses, there is far less uncertainty in using carefully collected human data than in using dermal absorption estimates from small numbers of inbred laboratory animals. For purposes of developing simplistic, deterministic estimates of potential human (e.g., consumer or professional worker) daily absorbed doses of 2,4-D following skin exposures to liquid formulations across a range of applied dose densities (1-1000 µg of 2,4-D per cm^2 of skin), and for contact durations of 24 hours or less, a mean percent absorbed value of 5.7% of the applied dose (derived from 4 different human dermal absorption studies) is appropriate.

COMMENT/HED**:** Several human dermal absorption studies were considered in the Agency assessment as part of the weight of evidence for determining the dermal absorption factor. A mean-percent absorbed value of 5.7% of the applied dose was derived from four different human dermal absorption studies. Ultimately, a value of 10% was used by the Agency based on these data and given the variability noted in the results. It is to be noted that there is a 10X uncertainty factor applied for potential variation in sensitivity among members of the human population (UF_H interspecies) and a 10X uncertainty factor for extrapolation from animal to human (UF_A; intraspecies). Generally when an endpoint is derived from a human study, the 10X intraspecies factor can either be reduced to 1 or a 3X. In this case, in spite of using a dermal absorption value from a human study, the full 10X UF_H was retained. It should also be noted that the UF_H factor is partially intended to account for uncertainties associated with the relative sensitivity to a pesticide within a population, which includes factors such as differences in dermal absorption that could possibly be attributed to the use of sunscreen, clothing, or occlusion. The UF_A is typically used to account for the uncertainty associated with the extrapolation of animal testing results to humans for risk assessment, including dermal absorption. As such, the risk assessment results are inherently more protective in this case because this uncertainty has been eliminated. Therefore, the Agency is confident that the dermal absorption value used does not under estimate dermal risk and is adequately protective.

CITED BY: Task Force – cited in support of EPA selection of 10% dermal absorption factor as a protective value given study variation; review and summary of numerous dermal absorption studies; determined that human percutaneous absorption of 2,4-D has been well characterized; 5 published studies using human subjects showed remarkable reproducibility across a span of 3 decades and multiple laboratories, formulations, and methods; 5.7% dermal absorption from these studies in close agreement with findings from Feldmann and Maibach, 1974 (5.8%).

(Study 134) **CITATION**: Wester RC, J Melendres, L Sedic, H Maibach, and JE Riviere. (1998). Percutaneous Absorption of Salicylic Acid, Theophylline, 2,4-D Dimethylamine, Diethyl Hexyl Phthalic Acid, and p-Aminobenzoic Acid in the Isolated Perfused Procine Skin Flap Compared to Man *In Vivo*. Toxicology and Applied Pharmacology 151: 159-165. [**Human Studies Considerations Apply: Yes**]

Principal Endpoint: [**Bin:** Dermal Penetration]

Methods Summary: study compares percutaneous absorption in the isolated perfused porcine skin flap (IPPSF) system12 with that in man *in vivo*. **Human**: Six subjects (males, ages 18-85 years; postmenopausal women 50-65 years) topically dosed at 8.7 µg/cm^2 with [^{14} C] 2, 4-dichlorophenoxyacetic acid dimethylamine (99.9%; 0.022 mCi/mg; solubilized in 50 µL ethanol) on the ventral forearm (spread over a 10- cm^2 skin surface area. Ethanol vehicle was allowed to dry; site not occluded. Urine collected for day of dosing and subsequent 6 days; at 24 hr, site washed with 50% Liquid Ivory soap (1:1, v/v) and water; wash sequence: soap (50%), water rinse, soap, followed by 2 water rinses using a cotton swab. At 7 days after application, skin dosing site was cellophane tape-stripped 10, times for residual [^{14} C]-chemical. Percutaneous absorption determined from ^{14} C–urinary excretion. **IPPSF**: 5 IPPSF were surgically prepared, harvested and perfused in a nonrecirculating chamber and then dosed with [^{14} C] 2,4-D –

dimethylamine one hour after equilibrium; a 10- cm^2 area on the IPPSF was dosed in a manner identical to the *in vivo* human study. Skin flap viability was assessed by monitoring of glucose utilization; venous effluent was collected at 30-minute increments for 8 hours after which the perfusions were terminated; after termination, the skin flaps were weighed and the dosing area was swabbed 2 times with gauze dipped in a mild soap solution; dosed skin was they stripped 12 times with cellophane tape; treated area was then excised and removed from the subcutaneous tissue and weighed; the remainder of the skin flap was collected for mass balance purposes; total penetration after 8 hours was estimated as the radiolabel detected in perfusate plus treated skin samples (iv dosing of Rhesus monkey (Moody et al., 1990) to account for that part of dose excreted by other routes (feces) or retained in body).

RESULTS: Human: recovery of dose in urinary excretion was 0.77±0.21%; only 2.0±0.7% was recovered in soap and water wash; only 0.06±0.06% was recovered in tape stripping. IPPSF: dose in perfusate was 1.5±0.24% and skin content after tape stripping was 2.4±0.5%, giving a predicted absorption of 2, 4-dimethylamine of 3.8±0.6%; only 21.4% dose was recovered in the surface wash and 4.9±2.6% was recovered in tape strips, giving a material recovery of 31.2±5.6%.

CONCLUSIONS: human *in vivo* percutaneous absorption (% dose absorbed 1.1±0.3) compared to penetrated dose in IPPSF (% dose absorbed 3.8±2.4)

COMMENT/HED**:** IPPSF system tested as a model for predicting percutaneous absorption relative to man; appears to support a 10% dermal absorption factor

CITED BY: Task Force – in reference list but did not find where Task Force cited

BIN: METABOLISM

(Study 97) **Citation**: Saghir, SA; Mendrala, AL; Bartels, MJ; Day, SJ; Hansen, SC; Sushynski, JM;, and Bus, JS (2006). Strategies to Assess Systemic Exposure of Chemicals in Subchronic/Chronic Diet and Drinking Water Studies. Toxicology and Applied Pharmacology 211: 245-260. **[Human Studies Considerations Apply: No]**

Principal Endpoint: **[Bin: Metabolism]**

Methods Summary: dietary administration 2,4-D (97.3%); Fischer 344 rats (9 weeks old); 5 and 100 mg/kg/day

Results Summary: 2,4-D concentrations in plasma and AUC values increased in a distinctly nonlinear fashion between 5 and 100 mg/kg/day (4-week exposure); nonlinear increase was apparent from the 89-fold increase in C_{max} and a 69-fold higher plasmaAUC-24 hr, far exceeding the expected 20-fold difference between doses; higher than expected plasma AUC-24 hr was also supported by the saturation of urinary excretion at high dose; only 28-fold higher 2,4-D was recovered during 18 hr of collection instead of 69-fold as expected from difference in circulating levels;

Table 1. Data From Literature

Diurnal Blood/Plasma Levels after Steady-State Achieved (diet)			
Time	5 mg/kg/day	100 mg/kg/day	Ratio (100/5)
12 am	0.59±0.17	29.54±4.90	50
3 am	0.62±0.06	36.08±4.76	58
6 am	0.72±0.15	63.95±6.02	89
9 am	0.48±0.03	50.27±9.76	105
12 pm	0.41±0.08	35.65±8.18	87
3 pm	0.42±0.34	38.64±14.12	92
6 pm	0.44±0.08	19.28±8.51	44
9 pm	0.64±0.04	23.54±8.86	37
AUC (µg h mL^{-1})			
# time points♪	12.95	890.88	68
	13.08	1207.51	92
	13.92	998.79	72
	11.54	1206.49	105

n=3/time point (from literature)/2-7 animals♪

amount in diet	**amount in blood/plasma**
5 mg/kg/day	0.41-0.72 µg /mL
100 mg/kg/day	19-64 µg /mL

CONCLUSION: general - development of strategies to assess systemic exposure levels of chemicals to better design long-term studies
COMMENT/HED**:** demonstrates that a dose of 2, 4-D of 100 mg/kg/day can be considered an excessive dose; HED aware of saturation kinetics of 2,4-D.
CITED BY: Task Force: cited with respect to Sturtz (2000) citation by NRDC regarding lactational exposure at 50, 70, 100 mg/kg/day; TF states that toxicity observed in the resulting non-linear pharmacokinetics range does not inform human risk assessment because nonlinear doses are unlikely to occur in humans

(Study 96) **Citation**: Saghir, SA; Marty, MS; Clark, AJ; Zablotny, CL; Bus, JS; Perala, AW; Yano, BL; and Neal, BH (2009). A Dietary Dose Range-Finding and Toxicokinetic (TK) Study of 2,4-Dichlorophenoxyacetic Acid (2,4-D) in Adult Crl:CD(SD) Rats and Their Offspring. 1. Toxicokinetics. Abstract. **[Human Studies Considerations Apply: No]**
Principal Endpoint: **[Bin: Metabolism – Toxicokinetics]**
Methods Summary: dietary administration of 2,4-D (0, 100, 200, 400, 600, 800, 1200, or 1600 ppm) from pre-breeding through weaning; F1 offspring exposed through PND 35; 2,4-D AUC 24 h was calculated from 3 plasma samples collected at 6 am, 9 am, 5 pm on designated sample days; 2,4-D in milk determined on lactation days 4 and 14 and in plasma of nursing pups by collection of single 9 am sample on PND 4, 14, 21, and 28; due to saturation of renal clearance, nonlinear TK was observed in parental males at 1600 ppm (test day 28) and 1200 ppm (test day 71); onset of nonlinear TK in parental females was observed at <400 ppm (test day 29 and 95; AUC 100 ppm = 25; AUC 400 ppm = 201 $\mu g\ h\ mL^{-1}$); during gestation and lactation, female 2,4-D levels were higher due to higher diet consumption; nonlinear TK occurred at <400 ppm (GD 17 and LD 14) and > 400 ppm and <800 ppm (LD 4); a shift in nonlinear TK on LD 4 may have been due to excretion into milk (levels ≈50% of female plasma); plasma 2,4-D in pups 30-70% lower than dam on LD 4 (M/F TK nonlinear <800 ppm) but similar to or higher than dam on LD 14 (M/M TK nonlinear <400 ppm); maximal 2,4-D in pups was reached on PND 21 when pups were consuming 2,4-D diet in addition to nursing, but dropped to levels = to dams on test day 29 and 95 levels on PND 35
Results Summary: kinetically-derived maximum dose was 400 ppm (≈25 mg/kg/day) for females and 1200 ppm (≈65 mg/kg/day) for males; increased dam and pup AUCs during lactation supported dietary adjustment during lactation
CONCLUSION: TK data support a definitive study high dose for females and pups lower than that derived by traditional toxicity testing
COMMENT/HED**:** These dose selection data were discussed with Agency prior to extended 1-generation reproductive toxicity study initiation (MRID 47414902) and were included in the review of the extended 1-generation reproductive toxicity study of 2, 4-D (MRID 47972101). Study was evaluated by Agency and classified acceptable/non-guideline. The results do not yield information that would cause the Agency to modify its conclusions from the 2005 Reregistration Eligibility Document for 2, 4-D that pertain to hazard.
CITED BY: Task Force: cited with respect to Sturtz (2000) citation by NRDC regarding lactational exposure at 50, 70, 100 mg/kg/day; states that toxicity observed in the resulting non-linear pharmacokinetics range does not inform human risk assessment because nonlinear doses are unlikely to occur in humans

(Study 130) **Citation**: Van Ravenzwaay, B; Harwick, TD; Needham, D; Pethen, S. and Lappin, GJ (2003). Comparative Metabolism of 2,4-Dichlorophenoxyacetic Acid in Rat and Dog. Xenobiotica 33 (8): 805-821. **[Human Studies Considerations Apply: No]**
Principal Endpoint: **[Bin: Metabolism]**
Methods Summary: rats and dogs of both sexes dosed orally with either 5 or 50 mg/kg ^{14}C-2,4-D; rates and routes of excretion and plasma toxicokinetics and biotransformation were assessed
Results Summary: elimination from rat plasma significantly faster than in dog; $t_{1/2}$ were 1.3-3.4 hrs for rat and 99-134 hr for dog following 5 or 50 mg/kg, respectively; AUC for rat 21-57 $\mu\ eq.hg^{-1}$ and 4889-5298 $\mu\ eq.hg^{-1}$ for dog at 5 mg/kg; 122-2358 $\mu\ eq.hg^{-1}$ for rat and 34100-44296 $\mu\ eq.hg^{-1}$ for dog at 50 mg/kg; major route of excretion for rat was in urine; essentially complete after 24 hrs (low)/48 hrs (high) dose; elimination incomplete in dog over sampling period (50% of dose recovered); urine principal route at low

dose, but equal amounts excreted in urine and feces at high dose over 120 hrs; body burden of 2,4-D in dog significantly higher than in rat for an equivalent dose, which is consistent with the increased sensitivity of dog to 2,4-D toxicity.
CONCLUSION: In the case of certain organic acids (2, 4-D), the rat is a better model for assessing the relevance to man of the toxicity of these compounds than the dog.
COMMENT/HED**:** these data were considered previously; the toxicological endpoints for use in human risk assessment for 2,4-D were selected from the rat database, based on the determination that the rat is a better predictor than the dog of the potential toxicity of 2,4-D to man; the data at 50 mg/kg in the rat suggested the clearance mechanism was saturated.
CITED BY: Task Force: used this reference in discussion of lactational exposure (Sturtz 2006 study) where ↓pup BW observed at 15 mg/kg/day; TF contends that this effect level was not replicated in 2009 Marty dietary life-stage study, where BW decreases were not observed at 400 ppm (25, 28, 37-57 mg/kg/day); were observed at 800 ppm, which occurred under conditions in which 2,4-D pharmacokinetic behavior in lactating dams was distinctly non-linear, thus rendering these responses of limited relevance to human risk assessment; also, cited with respect to Sturtz (2000) citation by NRDC regarding lactational exposure at 50, 70, 100 mg/kg/day; toxicity observed in the resulting non-linear pharmacokinetics range does not inform human risk assessment because nonlinear doses are unlikely to occur in humans.

BIN: MUTAGENICITY

REEVALUATION of the GENETIC TOXICOLOGY PROFILE on 2, 4-D

Eighteen recent Genetic Toxicology publications were referenced by NRDC in the document submitted to the Agency. These articles have been retrieved and reviewed in conjunction with a revisit of all of the mutagenicity data presented in the fourth Cancer Peer Review of 2, 4-D. The new information as well as the revisit are discussed below:

A. NEW GENETIC TOXICOLOGY STUDIES on 2, 4-D

1. Genetic Toxicology assays of the Industry Task Force II on 2, 4-Dichlorophenoxyacetic acid (2, 4-D)

A series of Genetic Toxicology assays, which was supported by the Industry Task Force II on 2,4-Dichlorophenoxyacetic acid (2,4-D, 96.1%), were performed on 2,4-D acid and seven salts and/or esters listed as:

2, 4-D DEA = 2, 4-D diethanolamine salt (73.8%)
2, 4-D DMA = 2, 4-D dimethyamine salt (66.2%)
2, 4-D IPA = 2, 4-D isopropylamine (50.15%)
2, 4-D TIPA = 2, 4-D triisopropanolamine (70.9%)
2, 4-D BEE = 2, 4-D 2-butoxyethylester (95.6%)
2, 4-D EHE = 2, 4- D 2-ethylhexyl ester (98%)
2, 4-D IPE = 2, 4- D isopropyl ester (81.6%)

The test articles were evaluated in five studies (4 *in vitro:* gene mutation in bacterial and mammalian cells, chromosome aberrations in mammalian cells, and unscheduled DNA synthesis in primary rat hepatocytes and 1 *in vivo*: mouse bone marrow micronucleus assay). Only 2, 4-D IPA, 2, 4-D TIPA and 2, 4-D BEE were tested in the mammalian cell culture gene mutation and chromosome aberration assays. Only 2, 4-D was assayed in the second in vivo test system (*in vivo* rat hepatocyte UDS). All assays used the protocols in the US EPA pesticide guidelines (US EPA, 1991). The summarized results are presented in Table 1 and each study is discussed below.

Charles et al (1999a)

Charles et al (1999a) reported the results of the independently conducted Ames test plate incorporation and the preincubation modification to the plate incorporation assay. Additionally, independently performed the *in vitro* primary rat hepatocyte unscheduled DNA synthesis (UDS) assay and the *in vivo* test procedure with hepatocyte treated with 2,4-D acid were investigated. For the Ames assay, *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, and TA100 were exposed to 5 to 7 dilutions of the test articles in the presence or absence of Aroclor 1254-induce rat liver homogenates. Note: TA 1538, which is considered a duplicate of TA98, was not used for all test articles. All bacterial assays were performed for each test agent with a high concentration in excess of the limit dose for this test system (5000 µg/plate) or up to a cytotoxic concentration; negative (solvent) and the appropriate positive controls were included. A minimum of triplicate plates per dose per strain per condition was also included in each assay. Representative results are presented in Table 1. As shown none of the eight test articles induced a mutagenic response in any of the tester strains at any test article concentration. All negative and positive controls responded in the expected manner. **Accordingly, all bacterial assays were considered negative and fulfill the regulatory requirements for acceptable bacterial gene mutation assays**. *In vitro* UDS assays were conducted with isolate primary rat hepatocytes exposed to the five to six test article dilutions using solubility or cytotoxicity as the starting concentration. Hepatocytes were doses for 18-20 hours and scored for net nuclear grain (NNG) counts and the percentage of cells with ≥5 NNG. Data from these assays can be seen in Table 1 and clearly show that neither 2, 4-D acid nor its salts or esters induced a genotoxic effect. By contrast, the positive control induced marked increases in NNG and the percentage of cells with ≥5 NNG. **Accordingly, these assays are negative and fulfill the regulatory requirements for acceptable *in vitro* UDS assays.**

Only 2, 4-D acid was tested in the *in vivo* UDS assay conducted with male Wistar Han rats administered single oral gavage doses of 400 and 1000 mg/kg. Five animals per dose received the selected test material doses, vehicle (1% methylcellulose) or positive controls and were sacrificed at 2-4 and 12-14 hours after dosing. Hepatocytes were recovered from three animals per group, cultured with [^{3}H] thymidine and scored for NNG. Dose selection was based on the findings of a preliminary range finding study with 0, 600, 800 or 1000 mg/kg. Clinical signs of toxicity in the range finding study were loss of balance and red stains on the nose and mouth "shortly after dosing" with 600 mg/kg. At 800 mg/kg, lethargy, abnormal breathing, unsteady gait and rigid body tone were seen. Similar signs were observed at 1000 mg/kg within 5 hours and two rats were killed in extremis after 3 days (Bus, 2009). Accordingly, 1000 mg/kg was selected as the maximum tolerated dose (MTD). Findings from the main test indicated that 2, 4-D did not increase the frequency of NNG at any dose or treatment time.

Gollapudi et al., 1999

Gollapudi et al.(1999) continued the Industry Task Force II efforts on 2, 4-D and

investigated several 2, 4-D derivatives [2, 4-D BEE (95.6%), 2, 4-D IPA (50.15%) and 2, 4-D TIPA (70.9%)] in mammalian cells performing the Chinese hamster ovary (CHO) cell forward gene mutation assay at the HGPRT locus and the rat lymphocyte *in vitro* cytogenetic assay. For the CHO/HGPRT assay, concentrations ranges of 150 to 1400µg/mL +/-S9 (2, 4-D BEE), 500 to 3000 µg/mL +/-S9 (2, 4-D IPA), or 800 to 5000 µg/mL +/-S9 (2, 4-D TIPA) were tested. Duplicate cultures per concentration were exposed to the selected test material doses and the appropriate solvent and positive controls for 4-5 hours, allowed a 7-9 day expression time, selected and plated for mutants and survivors. Each experiment was independently repeated at least once. The highest concentration of each test agents was selected to represent either to the limit dose of 5000 µg/mL for the test system (2, 4-D TIPA), the limit of solubility (2, 4-D BEE) or severe cytotoxicity (2, 4-D IPA +/-S9). **None of the 2, 4-D derivatives induced a mutagenic effect in CHO cells in either the absence or presence of S9 activation at any concentration tested.**

Table 1. Results of US EPA –Pesticide Assessment Guideline Genetic Toxicology Studies Supported by the Industry Task Force II on 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and salts and esters

Test Assay	**2,4-D acid**	**2,4-D DEA [a]**	**2,4-D DMA [a]**	**2,4-D IPA [a]**	**2,4-D TIPA [a]**	**2,4-D BEE [a]**	**2,4-D EHE [a]**	**2,4-D IPE [a]**	**Reference**
Ames	Neg up to cytotoxic levels (≥6410 µg/plate –S9; (≥9610 µg/plate +S9	Neg up to 10,332 µg/plate +/–S9	Neg up to 6620 µg/plate +/–S9	Neg up to cytotoxic levels (≥5010 µg/plate +/–S9)	Neg up to 7090 µg/plate +/–S9	Neg up to a cytotoxic level (4780 µg/plate +/–S9)	Neg up to 9800 µg/plate +/–S9	Neg up to cytotoxic levels (≥2428 µg/plate –S9; (≥4855 µg/plate +S9	Charles et al., 1999
CHO/HGPRT Gene mutation (*in vitro*)	ND	ND	ND	Neg. up to a cytotoxic dose (3000 µL/mL+/-S9)	Neg. up to the limit dose (5000 µL/mL+/-S9)	Neg. up to the limit of solubility (1400 µL/mL +/-S9)	ND	ND	Gollapudi et al., 1999
Chromosome aberrations rat lymphocytes (*in vitro*)	ND	ND	ND	Neg. up to cytotoxic doses (3068 – S9 3000 µL/mL+S9)	Neg. up to a the limit dose (5000 µL/mL+/-S9)	Neg. up to the limit of solubility (1400 µL/mL – S9) or cytotoxicity (700 µL/mL +S9)	ND	ND	Gollapudi et al., 1999
Mouse Bone Marrow Micronucleus	Neg to the MTD (400 mg/kg)	Neg to the MTD (443 mg/kg)	Neg to the MTD (397 mg/kg)	Neg to the MTD (376 mg/kg)	Neg to the MTD (542 mg/kg)	Neg to the MTD (375 mg/kg)	Neg to the MTD (500 mg/kg)	Neg to the MTD (400 mg/kg)	Charles et al., 1999b
UDS 1°rat hepatocytes (*in*	Neg up to 96.9	Neg up to 369	Neg up to 66.2 µL/mL	Neg up to 250.5µL/mL	Neg up to 354.5µL/mL	Neg up to 478µL/mL	Neg up to 24.5 µL/mL	Neg up to 194.2µL/mL	Charles et al., 1999a

Test Assay	**2,4-D acid**	**2,4-D DEA [a]**	**2,4-D DMA [a]**	**2,4-D IPA [a]**	**2,4-D TIPA [a]**	**2,4-D BEE [a]**	**2,4-D EHE [a]**	**2,4-D IPE [a]**	**Reference**
vitro)[b]	µL/Ml	µL/mL							
Rat hepatocyte UDS (*in vivo*)	Neg at 400 & 1000 mg/kg, MTD	ND	ND	ND	ND	ND	ND	ND	Charles et al., 1999a

[a] 2, 4-D DEA = 2, 4-D diethanolamine salt
2, 4-D DMA = 2, 4-D dimethyamine salt
2, 4-D IPA = 2, 4-D isopropylamine
2, 4-D TIPA = 2, 4-D triisopanolamine
MTD = Maximum tolerated dose
[b] Tested to a cytotoxic level or the limit of solubility.

2, 4-D BEE = 2, 4-D 2-butoxyethylester
2, 4-D EHE = 2, 4-D 2-ethylhexyl ester
2, 4-D IPE = 2, 4-D isopropyl ester

For the *in vitro* chromosome aberration assay, duplicate cultures of rat lymphocytes were treated, 48 hours after culture initiation, for 4 hours with comparable levels of the three test articles and the appropriate solvent and positive controls for 4 hours. Cells were harvest 24 or 48 hours after the termination of treatment; slides were prepared, stained and coded. Fifty metaphases/duplicate cultures were score for structural chromosome aberrations; the Mitotic Index (MI) was determined based on the count of 1000 cells/replicate culture. Based on the MI, three concentrations of each agent were scored for aberrant chromosome morphology. The highest concentration of each test agents was selected to represent either the limit dose of 5000 µg/mL for the test system (2, 4-D TIPA), severe cytotoxicity, indicated by a ≥50% decrease in the MI, (2, 4-D IPA +/-S9) or the limit of solubility (2, 4-D BEE -/+ S9). **None of the 2, 4-D derivatives induced a clastogenic effect in rat lymphocytes in either the absence or presence of S9 activation at any concentration tested. Accordingly, these assays are negative and fulfill the regulatory requirements for acceptable *in vitro* mammalian cell gene mutation and chromosome aberration assays.**

Charles et al (1999b)

The final publication in this series of Industry Task Force II Genetic Toxicology investigations on 2, 4-D details the *in vivo* mouse bone marrow micronucleus assays conducted with 2, 4-D (96.1%), 2, 4-D DEA (73.8%) 2, 4-D DMA (66.2%), 2, 4-D IPA (50.15%), 2, 4-D TIPA (70.9%), 2, 4-D BEE (95.6%), 2, 4-D EHE (98%), and 2, 4-D IPE (97.1%). Doses selected for the assays were based on the findings of mortality in groups of three male and three female administered 3 to 5 oral gavage doses of each agent. In the main study, groups of 5 male and 5 female mice/dose/sacrifice intervals received a single administration of three selected doses of each test agent via oral gavage. Animals were sacrificed at 24, 48 and 72 hours postdosing and bone marrow cells were harvested, processed, spread on to slides, stained and scored; there was no indication whether slides were coded prior to analysis. Appropriate vehicle and positive controls were included in each assay. The incidence of micronucleated polychromatic erythrocytes (MPCE) and the PCE to normochromatic (NCE) ratio were determined by analyzing 1000 PCEs/animal.

All test agents were examined up to the maximum tolerated dose (MTD), based on mortality; the generally comparable MTDs estimate for each test agent were:

- 400 mg/kg 2, 4-D
- 443 mg/kg 2, 4-D DEA
- 397 mg/kg 2, 4-D DMA
- 500 mg/kg 2, 4-D EHE

400 mg/kg 2, 4-D IPE
376 mg/kg 2, 4-D IPA
542 mg/kg 2, 4-D TIPA
375 mg/kg 2, 4-D BEE

Results of the individual micronuclei analysis indicated that neither 2, 4-D nor its seven derivatives were cytotoxic to the bone marrow since all PCE: NCE ratios were generally comparable to the vehicle control values. Similarly, none of the test treatments with 2, 4-D or its seven derivatives induced an aneugenic or clastogenic effect at any dose or sacrifice time in mouse bone marrow. **Accordingly, the test agents were adequately tested and found to be negative in these acceptable assays that fulfill the regulatory requirements for *in vivo* mammalian erythrocyte micronucleus assays.**

2. Assays in Yeast, Plants and Insects

Three publications were available that reported the findings of investigations on genotoxicity and cytotoxicity of 2, 4-D in yeast and human transformed hematopoietic cells (Venkov et al., 2000); in transgenic plants (Filkowski et al., 2003) and in *Drosophila* (Tripathy et al., 1993). Summarized results from these studies are presented in Table 2 and discussed as follows:

Venkov et al. (2000)

In this assay, Venkov et al. (2000) exposed *Saccharomyces cerevisiae* D7*ts1* to 2, 4-D (100%) test concentrations at 4, 8, or 16 mM (~900, 1800 or 3600 µg/mL, respectively) for 1 to 8 hours; cells were recovered and plated for survival and the reversion and mitotic gene conversion rates were calculated. Additionally, 100% pure phenoxyacetic acid, 4-chlorophenoxyacetic acid and 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T) were tested in this yeast assay following a 4-hour treatment. Results show that 2, 4-D at 8 mM induced gene mutations and gene conversions at levels causing ~40% reduction in cell survival. The effect was time and dose dependent with the peak response occurring after 8-hours of treatment with 8 mM. No evidence of genetic activity was seen for phenoxyacetic acid or 2,4, 5-T; 4-chlorophenoxyacetic acid at 16 mM induced mitotic gene conversion but did not increase reversion to isoleucine protrotrophy.

These investigators also examined the cytotoxic effects of 2, 4-D (16, 160 and 1600 µM) and 2, 4, 5-T (16, 160 and 1600 µM) in human transformed hematopoietic cells lines (LAMA-84 cells, derived from chronic myelogenous leukemia cells and U-937 cells, derived from histocytic lymphoma cells). Both test agents were 100% lethal at the high concentration. At 160 µM 2, 4-D, cell survival was 13.4 and 52.5 % for the LAMA-84 and U-937 cell lines, respectively. For the lowest dose of 2, 4-D, survival for both cell lines was ≥ 89%. The data indicate that 2, 4-D was slightly more cytotoxic than 2, 4, 5-T since cell survival at 160 µM 2, 4, 5-T was 41 and 53% for the LAMA-84 and U-937 cell lines, respectively. **Based on these findings, the investigators concluded that 2, 4-D is cytotoxic, mutagenic and recombinogenic in yeast and cytotoxic in transformed human cells.** Data on the cytogenetic analysis of C57b1 male and female mice is discussed under the section on chromosome aberrations and micronucleus induction.

Filkowski et al. (2003)

Filkowski et al. (2003) exposed transgenic seedlings of *Arabidopsis thaliana* to 0, 3, 10, 30, 100, 200 and 300 µg/L 2, 4- D (>98%) for 3 weeks under standard growth conditions; the number treated was not reported. Plants were collected, stained with 5-bromo-4-chloro-3-indolyl glucuronide, incubated for 48 hours and bleached with ethanol. Intrachromosomal homologous recombinations and point mutations were scored as blue sectors on white plants. It was indicated that "1000 'mutation'- and 200 'recombination'-monitoring plants were scored". The experiment was repeated three times. Data were only presented in graphs; thus variability in the data could not be ascertained. The mid-to low-doses of 2, 4-D (3, 10 and 30 µg/L) but not the high doses (100 and 300 µg/L) induced significant ($p<0.05$) increases in A → G point mutations; no explanation was provided for the lack of an effect at the high doses. Results also show that significant ($p<0.05$) increases in the induction of homologous recombinations (suggestive of DNA double strand breaks) occurred at all tested concentrations of 2, 4-D; a clear dose response was, however, not evident. **The authors stated, "…the increase in the recombination frequency at low concentrations of 2, 4-D correlated with the increase in the levels of A → G point mutations".**

Tripathy et al. (1993)

In a *Drosophila melanogaster* wing spot test and sex-linked recessive lethal assay, Tripathy et al. (1993), exposed second- and third- instar *mwh* + / + fl^3 transheterozygous larvae to 2.5, 5.0, 10.0 mM 2, 4-D (98%) prepared in 3% ethanol + 1% Tween 80 and incorporated into the feeding medium for 48 and 72 hours (i.e., "the rest of their larval life"); the high dose represents 50% of the $LD_{50.}$ Following exposure, 40 wings/treatment or control group of "eclosed" flies (i.e., flies that have wings that are highly folded and compact; within an hour, each wing expands) were examined for the type and number of spots. Duplicate experiments were performed; the data were pooled because there were no statistically significant differences among experiments.

In the sex-linked recessive lethal assay, Oregon-R larvae were fed diets containing comparable doses of 2, 4-D as described for 3 days; male flies were mated to 3 *Basc* virgin females for 3 days to obtain the F_1 *Basc*/Y siblings (Brood 1). Brood 1-males and females were mated (1:1) to produce Brood 2 (Brood 2 represents the sampling of germ cells that were primarily late or middle spermatids). X chromosomes from 60-65 males/test group (~1230-1330 X chromosomes/test group) and 150 pooled vehicle controls (3130 X chromosomes) were scored; the X-linked recessive lethals were calculated. Duplicate experiments were performed; the data were pooled because there were no statistically significant differences among experiments.

Results indicated that after 48 hours of exposure to 5.0 and 10.0 mM 2, 4-D, a significant ($p<0.05$) and dose-related increase in the number of spots/per wing (small single and large single wing spots) as well as the total spots was induced. Following 72 hours of exposure, significant increases were obtained only at the high dose for small single and large single and twin wing spots and total spots. The low dose (2.5 mM) was not active. **Based on these data, it was concluded that 2, 4-D induced somatic cell genotoxic damage in the primordial wing cells of *Drosophila*.**

Significant ($p<0.05$) and dose-related increases in the lethal frequencies were calculated at 5.0 and 10.0 mM 2, 4-D (48-hour exposure) and at all doses (72-hour exposure). Accordingly, the **authors concluded that in addition to somatic cell damage, 2, 4-D is also genotoxic in the male germinal cells of *Drosophila***.

3. Cytogenetic and Micronucleus Assays in Human Lymphocytes

Four assays were available on cytogenetic evaluations of structural chromosome aberrations or micronucleus induction in human lymphocytes. Findings from these studies are summarized in Table 2 and discussed below:

Figg et al., 2000

Figg et al. (2000) found no evidence of micronuclei (MN) induction in the peripheral lymphocytes of 12 applicators spraying only 2, 4-D during a 6-month period. In this study, 12 applicators were compared to a control group consisting of 9 controls (matched for gender, age, alcohol and tobacco uses, general health and geographical location). Blood and urine samples were collected prior to spraying and at every other week after a typical day of 2,4-D application. The concentration of 2, 4-D in the collected urinary samples was determined. Lymphocyte cultures were grown for 44 hours, and Cytochalasin B was added to prevent complete cytokinesis. At 72 hours, lymphocytes were separated; slides were prepared, stained with antikinetochore antibody and coated with fluorescent anti-goat human IgG, counterstained with

the DNA dye, 4', 6-diamidino-2-phenylindole, refrigerated for 1 week, coded and scored. At least 1000 binucleated lymphocytes were scored for micronuclei; cell division kinetics was calculated for the count of 400 cells per sample (duplicate cultures). The replicative index (RI) was determined by calculating the percentage of cells with one, two, three or more nuclei per individual. Hematological end points such as hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, red cell count, white blood cell count and leukocyte differential examinations were also conducted. The 2, 4-D concentration 'grand mean among all applicators after spraying 2, 4-D was 240 ± 100 (±SE) ppb. The authors stated, "Urinary concentrations of 2, 4-D increased as hours spent spraying the herbicide increased." Consequently, the 12 individuals were grouped according to hours spent spraying 2, 4-D according to the following urinary concentrations: <20 ppb (4 subjects), 20-40 ppb (3 subjects) or > 40 ppb (5 subjects). Results of the lymphocyte analysis revealed that there was no significant increase in the incidence of micronuclei in the exposed workers. However, the replicative index (RI) for lymphocytes was significantly ($p<0.05$) higher in applicators compared to non-applicants and in applicators after spraying compared to before spraying ($p<0.016$). It was stated that the increase in RI suggests "stimulated cell growth that could contribute to carcinogenesis." **Nevertheless, the authors concluded that their findings did not support a "genotoxic pathway" for 2, 4-D at mean urinary levels of 12 to 1285 ppb.**

Holland et al., 2002

In the *in vitro* micronucleus assays of Holland et al. (2002), two experiments were conducted with either 2 or 5 volunteers (total of 3 females and 2 males, nonsmokers, 26- 45 years). Note: one of the subjects from the first test participated in the second test. Whole blood cultures as well as isolated lymphocyte assays were examined in the first test; the second test only used isolated lymphocytes. Collection of blood samples, culture preparation, treatment conditions and scoring of binucleated lymphocytes was conducted as described above in Figg et al. (2000). The only procedural exceptions were that lymphocytes smears were not stained with antikinetochore antibody or coated with anti-goat human IgG and MIs were determined from the count of 1000 cells/culture. In the first test, samples of whole blood and isolated lymphocytes were exposed *in vitro* to pure (98%) 2, 4-D at concentrations of 0.001-1.0 mM and analyzed for micronucleus induction. In the second test, only isolated lymphocytes were exposed to both pure 2, 4-D or commercial 2, 4-D (9.4%2, 4-D but adjusted to 98% pure 2, 4-D) at concentrations of 0.005 and 0.3 mM.

Cultures exposed to concentrations ranging from 0.001- 1.0 mM of pure 2, 4-D (98%) for 4 hours showed only "a minimal increase in MN…at doses approaching cytotoxic levels" in either whole blood or isolated lymphocytes. Similar findings were obtained for the commercial product at 0.3 mM 2, 4-D in isolated lymphocytes. However, the study authors conceded that these increases were within the "lower range of normal MN for healthy adults". RIs were significantly ($p<0.001$) decreased (~19%) at 0.3 mM of either pure or commercial 2, 4-D, regardless of the solvent (ethanol or culture medium) used. These data suggest a cytotoxic effect. Although Holland et al. (2002) claimed that 0.005 mM of commercial grade 2, 4-D induced an increase (nonsignificant) in the RI of isolated lymphocytes, an examination of the data in Table 1 of this paper revealed that the pooled average RI for both commercial 2,-4 –D groups (10 samples) was 1.65 vs. 1.62 for the pooled solvent control groups. This "increase" cannot be considered evidence of a proliferative response. Similarly, MIs were unaffected by treatment with 0.005 or 0.3 mM 2, 4-D (commercial or pure). Thus, the lack of a meaningful increased RI at this low dose is supported by data showing that MIs were unchanged. **The overall conclusions are that 2, 4 –D, regardless of the formulation, did not induce micronuclei in human lymphocytes**

up to levels approaching cytotoxicity.

Zeljezic and Garaj-Vrhovac, (2004)

Zeljezic and Garaj-Vrhovac (2004) exposed human lymphocytes, harvested from two young, healthy non-smoking volunteers, to Deherban A®, a commercial formulation of 2, 4-D adjusted to contain 0.4 or 4 µg/mL 2, 4-D in the absence and the presence of S9. The S9 fraction was derived from the livers of male Sprague-Dawley rats treated with intraperitoneal (ip) injections of Deherban A® adjusted to contain 500 mg/mL 2, 4-D for 3 consecutive days. Lymphocytes were treated for 24 hours, recovered, processed, stained with Giemsa and scored (1000 metaphases/concentration) for chromosome aberrations. Similar cultures were treated for 24 hours with 0.4 or 4 µg/mL 2, 4-D +/-S9; cytochalasin B was added and cells were harvested, processed, and stained as previously described for the *in vitro* micronucleus assay. Cells were scored (1000 binucleated lymphocytes/concentration) for micronuclei and nuclear buds. Appropriate solvent and positive control cultures were included. The article did not indicate whether slides were coded to eliminate bias prior to scoring.

Both concentrations of 2, 4-D induced statistically significant ($p<0.05$) increases in the percentage of cells with aberrant chromosome aberrations (primarily chromatid and chromosome breaks) in lymphocytes from both donors. The number of micronuclei and nuclear buds at both nonactivated and S9-activated test material concentrations was also significantly increased ($p<0.05$) at 0.4 and 4 µg/mL +/-S9 in cells also harvested from two different donors. The cytogenetic, micronuclei and nuclear bud responses were uniformly and significantly ($p<0.05$) higher in cells treated in the presence of S9 at both test levels. **Hence, it can be concluded that S9 activation was not required to demonstrate the clastogenic effect induced by 2, 4-D in this study.**

Venkov et al. (2000)

As part of the analysis of 2, 4-D and other substituted phenoxyacetic acids in yeast and cytotoxicity assays in human transformed hematopoietic cells (see Section, Assays in Yeast, Plants and Insects), Venkov et al. (2000) also investigate 100% pure 2, 4-D and 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T) for the induction of structural chromosome aberrations following single ip administrations of both agents at 3.5 and 4.0 mg/kg, respectively. Accordingly, bone marrow cells were harvested from groups of four male and four female mice at 12 and 24 hours postdosing. The appropriate vehicle (0.9% NaCl) and positive controls were included. At least 100 metaphases per animal were scored for aberrant structural morphology and the MIs were determined from the count of 1000 cells per animal. It was not stated whether slides were coded prior to analysis. 2, 4-D at 3.5 mg/kg induced significant ($p<0.0.5$) increases in the frequency of cells with aberrations at both sacrifice times (3.4 and 5.5% vs. 1.5 % for the vehicle control at 12 and 24 hours, respectively); the predominant type of aberration induced by 2,4-D was chromatid breaks, suggesting cytotoxicity, and exchanges suggesting clastogenicity. 2, 4-D also reduced the MIs by 69% at 12 hours and 35% at 24 hours. By contrast, 2, 4, 5-T was neither cytotoxic nor clastogenic in bone marrow cells harvested 12 postexposure to 4.0 mg/kg. **Based on the findings from this investigation, the authors concluded that 2, 4-D was clastogenic in mouse bone marrow cells when administered via ip injection.**

4. DNA Damage Assays

Five studies were found in the open literature investigating the potential of 2,4-D to induce DNA damage; these include two studies on sister chromatid exchange (SCE) assays in chick embryos

performed by Arias (2003 and 2007), SCE and comet assays in CHO cells by Gonzales et al, (2005), SCE induction in human whole blood and leukocytes (Soloneski et al., 2007) and SCE induction in mouse bone marrow and spermatogonia (Madrigal-Bujaidar et al., 2001). Findings from these publications are also summarized in Table 2 and discussed below:

Arias 2003, 2007

In the first study, concentrations of 0, 0.5, 1, 2, and 4 mg/embryo of either the pure grade of 2,4-D (99%) or the commercial formulation, Agroxone containing 37% 2,4-D were injected into the air cell of White Leghorn chicken eggs (9-12/dose). Treatment lasted for 3 days; eggs were automatically rotated at 37.8ºC and BrdU (150 µg/embryo) was injected into the air cell. On Day 4, embryos were removed following a 2-hour exposure to colchicine (0.05%). Chromosome preparations were made from harvested embryos and slides were stained using the fluorescent-plus-Giemsa technique; the report did not indicate whether slides were coded. A total of 30-40 second-division metaphases were scored for SCE in the first five pairs of macrochromosomes. The frequency of first (M1), second (M2) and third (M3) division metaphases from 200 cells were scored for mitotic delay and MIs were determined from the count of 1000 cells. No tabular presentation of the data was available. Results, shown in graphs, indicated a significant ($p<0.05$) increase in SCEs for the high concentration of the commercial formulation (~1.43 SCEs/ cell vs. ~1.22 SCEs/cell for the solvent control, acetone) and a nonsignificant increase (~1.41 SCEs/ cell vs. ~1.22 SCEs/cell for acetone) for pure 2,4-D. Although the dose response was concentration related for both test materials, the increase at the high dose was only 1.2-fold higher, compared to the solvent control and 1.2-fold compared to the average spontaneous background of 1.15 SCEs/cell. Additionally, data were presented as the mean and standard error of the mean, which suggests wide variation in the data. The investigator also stated that cell cycle delay was observed at the high concentration (2 and 4 mg/embryo) and that the MI was significantly ($p<0.01$) reduced by both test material at the high dose (4 and 8% reductions for the commercial and pure 2, 4-D, respectively). **Based on these considerations, the values for SCE induction, cell cycling and MIs provide questionable evidence of a positive response.**

For the second study, Arias (2007) used the same purity of the test materials at similar concentrations and the same protocol with the exception of including a 7- and 10-day extended exposure time and increasing the sample size to 35 embryos/concentration. Results were similar to the previous study showing "significant ($p<0.05$)" increases in SCEs/cell at 4.0 mg/embryo (1.1-fold increase) at 4, 7 and 10 days for the commercial product and at 4.0 mg/embryo (1.1 fold-increase) at 7 and 10 days for pure 2,4-D. Similarly, slight but significant ($p<0.01$) effects were reported on cell cycle delay and the MI (≈23.3 for the commercial product and ≈23.1 for the pure substance at the high dose vs. 24 for acetone), which was not associated with prolonged exposure. **Although these effects were statistically significant, the biological relevance is unclear.**

<u>Gonzalez et al., 2005</u>

Gonzalez et al. (2005) evaluated 2,4-D (purity not reported) and commercial grade 2,4-D DMA (containing 60.2% 2,4-D as the active ingredient) for the ability to cause DNA damage in CHO cells using the SCE and Comet tests. Concentration ranges of 2- 10 µg/mL were used in both assays in the absence of S9 activation; treatment times for the SCE assay were 24 and 36 hours and 90 minutes and 36 hours for the comet test. For both the SCE assay and the Comet test,

duplicate cultures were prepared for each dose; three independent experiments were conducted; and cultures treated with the solvent (acetone) were included. The percentage of cells in M1, M2 and M3 division in 200 metaphases/sample, the proliferative rate and the MIs/2000 cells were determined. There were no significant differences for cell-cycle progression or alterations in the rate of cell proliferation. By contrast, 2, 4-D at 24 hours caused 25 and 35% reductions in the MI at 6 and 10 µg/mL; respectively, and 42 and 46% reductions at 6 and 10 µg/mL, respectively (36 hours). For 2, 4-D DMA, reduction of ~38 % (24 hours) and ~28% (36 hours) were recorded for both 6 and 10 µg/mL. SCE data were presented in graphs and showed significant ($p<0.01$) and dose-related increases in SCE/cell at all levels (2, 6 and10 µg/mL) for both test materials, regardless of time. The fold increase at the high dose was 1.5 and 1.8 X for 2,4-D at 24 and 36 hours, respectively and 1.6 and 2.1 X for 2,4-D DMA, at 24 and 36 hours, respectively. Similar evidence of DNA damage was seen in the comet test with significantly ($p<0.01$) increased and dose-related percentages of cells with tail comets, indicative of DNA damage, at 2.0, 6.0 and 10.0 µg/mL in the absence of severe cytotoxicity (≥85% viability) at 90 minutes. At 36 hours, regardless of the test material, all cells had tail comets. **These data provide clear and reproducible evidence of the DNA-damaging activity of 2, 4-D and the DMA derivative**.

Soloneski et al., 2007

In another study of SCE induction, Soloneski et al. (2007) assayed 2, 4-D (purity not reported) and commercial grade 2, 4-D DMA (purity not listed) at levels of 10 to 100 µg/mL in human whole blood (WBC) and plasma leukocyte cultures (PLC). Treatment time was 72 hours. Duplicate cultures were prepared for each dose or the solvent control, acetone and three independent experiments were conducted. Smears were prepared and stained as previously described and metaphases were scored blind for cell cycle progression, MIs and SCE induction. Results show that both test materials significantly (generally $p<0.05$) affected cell cycle progression in WBCs at doses ≥ 25 µg/mL (note: the 100-µg/mL group was not scored for 2, 4-D) and only at 100 µg/mL (2, 4-D) for PLCs. Mean SCE frequencies were also significantly ($p\leq0.05$) increased at 10, 25 and 50 µg/mL (2, 4-D) and at 25, 50 and 100 µg/mL (2, 4-D DMA) when WBCs were tested. No activity was seen at any dose when PLCs were examined. Significant reductions in the proliferative rates and the MIs were found in WBCs and PLCs after treatment with 50 or 100 µg/mL 2, 4-D, respectively. For 2, 4-D DMA reduced proliferative rates and MIs were noted at the high dose but only in WBC. The discrepancies in the data between WBCs and PLCs were considered by the authors to be related to the detoxificating properties of erythrocytes, which are present in the PLC cultures but not the purified leukocyte cultures. **The authors, citing several investigators, further speculated that the cellular response caused by both test agents in this study could be related to oxidative damage since lipid peroxidation has been reported for 2,4-D.**

Madrigal-Bujaidar et al., (2001)

In the study of Madrigal-Bujaidar et al. (2001), SCE induction in mouse bone marrow and spermatogonia were investigated. Groups of five male mice received oral doses of 0, 50, 100 or 200 mg/kg 2,4-D (98.95%) in alkalinized H_2O (pH 10.5) or the vehicle, followed at 2 hours by an intraperitoneal (ip) injection of BrdU/charcoal and at 21 hours by an ip injection of colchicine. Bone marrow cells were harvested, stained by the fluorescent-plus-Giemsa technique, and scored; it was not reported whether slides were coded prior to scoring. Thirty second-division metaphases/animal were scored for SCE induction; the MIs, cell cycle delay and average generation time were also determined. For the examination of spermatogonial cells, groups of five male mice received the test material and BrdU/charcoal as described; cell division was halted by the subcutaneous injection of colchicine at 51 hours after BrdU administration and testes were harvested 4 hours later. Prepared slides were stained as described and 25 mitosis /animal were scored for SCEs. The presented data show that there were no adverse effects on the MI, cell cycle progression on the average generation time in the bone marrow cells. By contrast, significant ($p<0.05$) and dose-related increases in SCE induction (2-fold or approaching 2-fold increases) at 100 and 200 mg/kg were recorded for both cell types. It is also of note that the authors speculate that the fate of the genotoxic damage on the spermatogonial cells is unclear since "teratogenic and embryotoxic studies on rodents treated with 2,4-D have given conflicting results and the dominant lethal mutation assay on mouse and rats has produced negative data. Thus cell repair, cell lethality or zygote damage remains as three possible fates of the observed genotoxic alterations in spermatogonia." Similar speculations can be raised for the fate of DNA damage in bone marrow cells since chronic and oncogenic studies in rats and mice were negative. Thus, this somatic cell damage may be repaired or result in cell death.

5. Other Mechanisms

Two articles were placed in this category: the malignant cell transformation in Syrian hamster embryo (SHE) cells by Maire at al. (2007) and the two studies on apoptosis (Maire at al., 2007 and Tuschl and Schwab, 2003). The article of Maire at al. (2007) also contains the results of a RT-PCR determination of gene expression analysis in SHE cells as well as a comet assay of DNA damage. These findings are summarized in Table 2 and discussed below:

Maire et al. (2007)

In a series of *in vitro* investigations, Maire et al. (2007) exposed Syrian hamster embryo (SHE) cells to 2, 4-D (98%) at concentrations of 0, 4.5, 11.5, 23 or 34 µM (equivalent to 0, 1, 2.5, 5 or 7.5 µg/mL, respectively) and evaluated the cultures for malignant cell transformation, apoptosis, expression of the *c-myc* gene and gene product, and DNA damage (Comet test). For the cell transformation assay, X-irradiated SHE feeder cells were seeded and grown for 25 hours. Target cells were seeded on top of the feeder cells, allowed a 24-hours period of quiescence and 10 dishes /concentration were treated with the selected test article levels of 2, 4-D for 7 days. At the end of exposure, dishes were washed, fixed and stained. Appropriate solvent (H_2O) and positive controls were included in each assay. One thousand colonies/concentration were counted and examined for morphology; cloning efficiency (CE) and the transformation frequency (TF) were determined.

For apoptosis, cells were also sampled 48 hours after seeding, trypinized, reseeded and treated with of 0, 4.5, 11.5 and 23 µM 2, 4-D for 5 and 24 hours. After treatment, cells were suspended in culture medium deprived of growth factors for 3 hours, harvested and centrifuged. Cell pellets

were stained with fluorescent acridine orange and ethidium bromide, mixed, placed on slides, and scored for the number of apoptotic cells/100 cells/slide. Three slides/concentration were examined.

For the RT-PCR determination of gene expression, cells were again sampled 48 hours after seeding, trypinized, reseeded and treated with of 0, 4.5, 11.5 and 23 µM 2, 4-D for 5 hours. After treatment, total RNA was extracted and complementary DNA (c-DNA) was synthesized from 1µg of total RNA using oligo d (T) as a primer and reverse transcriptase. PCR amplification of the c-DNA was performed with *c-myc, bcl-2* and *bax* primers specific to SHE cells. Preparations were denatured and PCR products were sequenced, cloned and electrophoresed. Signals were analyzed by densitometry and data were expressed as density ratios of the transcript corresponding to the *c-myc* gene. Three different experiments were conducted with different batches of SHE cells. Western blot analysis was conducted in parallel with RT-PCR to assess effects on *c-myc* protein.

For the comet assay, SHE cells were trypsinized 48 hours after seeding, reseeded and treated with 0, 4.5, 11.5, or 23µM 2, 4-D for 5 and 24 hours. At least three experiments were performed and included in each assay were the appropriate solvent and positive control. Cells were recovered and electrophoresed; nuclear DNA was stained with ethidium bromide and scored blind. Cells (75/slide/concentration) were scored randomly and the percentage of DNA in the tail was determined as a measure of DNA damage.

Results indicate that 2, 4-D induced a dose-dependent and significant ($p<0.01$) increases in morphological cell transformation of SHE cells at 11.5 (2.4%) and 23µM (3.8%); relative cloning efficiency at 11.5 and 23µM was 14%. No significant increase in transformed cells was observed at the high dose of 34 µM 2, 4-D. There were also no significant increases in apoptotic cells 5 or 24 hours after exposure to the selected doses of 2,4-D. These data are supported by the lack of an effect on expression of *bcl-2* and *bax* genes, which are associated with regulation of the apoptotic pathways. By contrast, a significant ($p<0.05$) increase in the up-regulation of *c-myc* RNA at 11.5 and 23 µM was reported; the increases represented 2.3-fold at these levels and immunoblotting confirmed the increase in the *c-myc* protein. Thus, 2, 4-D induced over expression of *c-myc* at both the RNA and the protein level; this finding is consistent with the transforming activity of 2, 4-D at comparable doses. Data from the comet test revealed that treatment with 11.5 and 23 µM for 5 or 24 hours also induced a dose-dependent and significant ($p<0.01$) increase in the number of DNA-damaged cells. No dead cells were found at any dose or harvest time.

Overall, the investigators concluded that 2, 4-D induces morphologic cell transformation in SHE cells at doses that that extend into the cytotoxic range and caused overexpression of *c-myc* (at both the mRNA and the protein level) and DNA damage.

<u>Tuschl and Schwab (2003)</u>

By contrast, Tuschl and Schwab (2003) found that exposure of the human hepatoma cell line, HepG2 to 2, 4-D (purity no stated) in 1% ethanol induces apoptosis but only at high doses (4, 8 or 16 mM; equivalent to 0.9, 1.8 or 3.6 mg/mL) that induced severe effects on cell cycling, increased necrotic cells and increased breakdown of mitochondrial membranes. The data in this article were only presented in graphic form. It is of note, however, that these doses are ≈ 1000X

higher than the doses reported by Maire et al. (2007). **The relevance of the findings is unclear because of severe cytotoxicity at all levels.**

Table 2. Summarized Results of Recent Genetic Toxicology Studies on 2, 4- Dicholor-phenoxyacetic acid (2, 4-D)

ENDPOINT	CONCENTRATION RANGE	RESULT	REFERENCE
ASSAYS in YEAST, PLANTS and INSECTS			
Saccharomyces cerevisiae D7 *ts1* gene mutations & gene conversions Human transformed hematopoietic LAMA-84 & U-937 cell lines	0, 4, 8, 16 mM 2, 4-D (100%) for 1-8 hrs 0, 16, 160 & 1600 µM 2, 4-D (100%) for 48 hrs	**Pos.**; Time & dose-dependent increases in gene mutations & gene conversions 100% lethal at 1600 µM; 13.4% survival (LAMA-84) & 52.5 % (U-937) at 160 µM & ≥89% survival (both cell lines) at 16 µM	Venkov et al., 2000
Transgenic *Arabidopsis thaliana* point mutations & homologous recombination	0, 3,10, 30, 100, 200, 300 µg/mL for 3 weeks	**Equivocal;** significant increases ($p<0.05$) in A → G point mutations at 3, 10, & 30 µg/mL but not at 100-300 µg/mL; no explanation provided. **Pos.**; significant increases ($p<0.05$) in induction of homologous recombinations at all concentrations but not clearly dose-related.	Filkowski et al., 2003
Drosophila melanogaster wing spot test (somatic cell gene mutations) Sex-linked recessive lethal test (germinal cell chromo-some damage)	0, 2.5, 5.0, 10.0 mM 2, 4-D (98%) larval feeding 48 and 72 hrs (wing spot test) 3 days (X-linked recessive lethal)	**Pos**.; 5.0 & 10.0 mM induced significant & dose-related increases ($p<0.05$) in the number of small and large single spots & frequency of lethal X chromosomes.	Tripathy et al., 1993
MICRONUCLEUS (Mn[a]) AND STRUCTURAL CHROMOSOME INDUCTION			
Mn in lymphocytes of 12 2, 4-D applicators sampled every other week for 12 wks	Urinary range: 12-1285 ppb	**Neg.** No significant increases in Mn of lymphocytes harvested from 2, 4-D workers compared to matched controls but significant increases in RIs for applicators vs. controls.	Figg et al., 2000
Mn in whole blood & isolated lymphocytes from 5 volunteers (*in vitro*)	2, 4-D (98%): 0.001- 1.0 mM; Commercial 2, 4-D (adjusted to contain pure 2, 4D): 0.005- 0.3 mM	**Neg.** Minimal increases in Mn for whole blood & isolated lymphocytes at 0.1 mM (98% 2,4-D) & 0.3mM commercial 2,4-D **but** "within the normal range for healthy adults". Nonsignificant increases RIs at 0.005 but not 0.3 mM; at 0.3 mM RI were decreased ≈19% (both formulations); no effects on MIs at any dose.	Holland et al., 2002
Mn & structural chromosome aberrations in isolated	Deherban A®, adjusted to contain 0.4 and 4µg/mL 2, 4-D +/-S9. S9 derived from the liver of male rats	**Pos.** Significant ($p<0.05$) increases in chromosome aberrations (chromatid & chromosome breaks), micronuclei & nuclear buds at 0.4 and 4 µg/mL +/-S9.	Zeljezic and Garaj-Vrhovac 2004

ENDPOINT	CONCENTRATION RANGE	RESULT	REFERENCE
lymphocytes from 2 volunteers (*in vitro*)	induced with 500 mg/kg 2, 4-D as Deherban A® for 3 days	The response was ≈20% higher in the presence of S9.	
Structural chromosome aberrations in mouse bone marrow (M&F)	0, 3.5 mg/kg (single ip administration); 12 & 24 hrs. harvest times	**Pos** Significant (p<0.05) increases in chromosome aberrations (chromatid breaks & exchanges)	Venkov et al., 2000
DNA DAMAGE			
SCEs in chick embryos	0, 0.5, 1, 2, 4 mg/embryo 99% 2, 4-D & 0, 0.5, 1, 2, 4 mg/embryo 37% 2, 4-D as Agroxone; sampled 4 days post-treatment	**Neg.** Reported to induce a dose-related increase in SCEs at 0.5-4 mg/embryo; significant increase (p<0.05) at 4 mg/embryo (commercial product only) **but** the increases were only 1.1or 1.2X higher than the solvent control or the spontaneous background rate. Also significantly reduced **(4-8%)** MIs at the high dose for both products	Arias 2003
SCEs in chick embryos	0, 0.5, 1, 2, 4 mg/embryo 99% 2, 4-D & 0, 0.5, 1, 2, 4 mg/embryo 37% 2, 4-D as Agroxone sampled 4, 7 & 10 days post-treatment	**Neg.** Results similar to above study (i.e., significant increase (p<0.05) in SCEs at 4 mg/embryo (commercial product) at 4, 7, & 10 days & at 4 mg/embryo (pure 2,4-D) at 7 & 10 days **but** only 1.1X for both products.	Arias 2007
SCE & Comet test CHO cells	2- 10 µg/mL 2, 4-D (unknown purity) 2- 10 µg/mL 2, 4-D DMA (60.2% 2, 4-D); treatment times: 24 & 36 hrs (SCE) & 90 min. & 36 hrs (Comet test) –S9 only.	**Pos.** No significant effects on cell cycling time or RI; reductions in MIs: 25-35% at 6 and 10 µg/mL at 24 hrs & 42-46% at 6 and 10 µg/mL at 36 hrs (2, 4-D) ≈38% (24 hrs) & ≈28% (36 hrs) at 6 and 10 µg/mL (2, 4-D DMA). **Significant (p<0.01) & dose-related increases in SCE** at 2, 6 and 10 µg/mL (both products) regardless of time. **Comet assay**: **Significant (p<0.01) & dose-related increases in** cells with tail comets at 2, 6 and 10 µg/mL (both products) at 90 min. in the absence of severe cytotoxicity (≥85% viability) & all cells, regardless of product or dose had 100% of cells with tail comets at 36 hours.	Gonzalez et al., 2005
SCE human whole blood (WB) & plasma leukocytes (PL)	10 – 100 µg/mL 2, 4-D (unknown purity) 10- 100 µg/mL 2, 4-D DMA (unknown purity); treatment time 72 hrs.	**Pos.** Significant (p<0.05) reduction in cell cycling in WB at ≥ 25 µg/mL (both products) & in PL at 100 µg/mL (2, 4-D only). Significant (p<0.05) reduction in MIs & proliferative rates at ≥ 50 or 100 µg/mLin WB or PL, respectively (2, 4-D) at 100 µg/mL only in WB (2, 4-D DMA). **SCEs significantly increased** at 10, 25 & 50 µg/mL only in PL (2, 4-D) & 25, 50 & 100 µg/mL only in PL (2, 4-D DMA).	Soloneski et al., 2007
SCE mouse bone marrow & mouse spermatogonia	0, 50, 100 or 200 mg/kg 98.95% 2, 4-D in alkalinized H_2O (pH 10.5); oral gavage	**Pos.** No effects on MI, cell cycling or average generation time. **Significant & dose–related increases in SCEs** (both cell types) at 100 & 200 mg/kg.	Madrigal-Bujaidar et al., 2001

ENDPOINT	CONCENTRATION RANGE	RESULT	REFERENCE
	administration; 5 males/group		
OTHER MECHANISMS			
Cell transformation in SHE cells	0, 4.5, 11.5, 23 or 34 µM ≈0, 1, 2.5, 5 or 7.5µg/mL 2, 4-D (98%) 7 days	**Pos.** Dose-dependent & significant (p<0.01) increases in morphological cell transformation at 11.5 & 23 µM; cloning efficiency 14% at both doses.	Maire et al., 2007
Apoptosis in SHE cells	0, 4.5, 11.5, 23 µM 2, 4-D (98%) 5 & 24 hrs	**Neg.** No significant increase at any dose or time.	
RT-PCR gene expression (*c-myc* gene) in SHE cells	0, 4.5, 11.5, 23 µM 2, 4-D (98%) 5 hrs	**Pos.** Significant (p<0.05 up-regulation of *c-myc* gene expression at 11.5 & 23 µM; confirmed at protein level by Western immunoblotting	
Comet test in SHE cells	0, 4.5, 11.5, 23 µM 2, 4-D (98%) 5 & 24 hrs	**Pos.** Dose-dependent & significant (p<0.01) increases in the number of DNA damaged cells at 11.5 & 23 µM (both time points). No dead cells were found at any dose or time	
Apoptosis in human hepatoma cells (HepG2)	0, 4, 8 or 16 mM ≈0, 0.9, 1.8 or 3.6 mg/mL 2, 4-D (unknown purity) treated 4- 48 hrs.	**No conclusions for apoptosis;** severe cytotoxicity at all levels	Tuschl and Schwab (2003)

[a] Abbreviations:

Mn = Micronucleus SCE = Sister Chromatid Exchange
RI = Replicative index CHO = Chinese Hamster Ovary cells
MI = Mitotic index SHE = Syrian Hamster Embryo cells

6. Conclusions

Based on the analysis of the new studies in the open literature it can be concluded that neither 2, 4-D nor its derivatives are mutagenic in bacteria (*Salmonella typhimurium*); however, there is convincing evidence of gene mutations in yeast (*Saccharomyces cerevisiae* D7), plants (*Arabidopsis thaliana*) and possibly insects (*Drosophila melanogaster*). No assays were located for *in vitro* mammalian cell gene mutation studies. 2, 4-D and its derivatives were negative in mouse bone marrow micronucleus assays up to the MTD and did not cause UDS in primary rat hepatocytes. Similarly, 2, 4-D was not genotoxic in the *in vivo* rat hepatocyte UDS assay. Three derivatives of 2, 4-D (2, 4-D IPA, 2, 4-D TIPA and 2, 4-D BEE) were not mutagenic or clastogenic in CHO cells or rat lymphocytes. However, 2, 4-D was not tested in these assay systems. Nevertheless, four earlier *in vitro* chromosome aberration studies of 2, 4-D showed conflicting results for chromosome aberrations (see Table 3). Contrary to the negative *in vivo* findings, 2, 4-D induced structural chromosome aberrations, micronuclei and nuclear buds in human lymphocytes and was clastogenic in mouse bone marrow when delivered by ip injection. There are several studies showing DNA damage, as measured by SCE induction (3 studies) and positive comet assays (2 studies) in CHO cells, SHE cells or human leukocytes. Additionally, 2, 4-D, prepared in alkalinized H_2O and delivered by oral gavage gave positive results in the *in vivo* mouse bone marrow and mouse spermatogonia SCE assay. Finally, 2, 4-D induced malignant cell transformation (an *in vitro* test of carcinogenesis) and overexpression of the *c-myc* at both the mRNA and protein level. The induction in *c-myc* expression is indicative of cell proliferation and is associated with cell transformation, *in vitro*.

7. Revisit of Existing Genetic Toxicology Data on 2, 4-D

Data included in the fourth carcinogenicity peer review of 2, 4- D are summarized in Table 3. These data support the conclusion that 2, 4-D is not mutagenic in bacteria. There is also confirmation of sex-linked recessive lethal induction in *Drosophila* larvae. As previously mentioned, results from the *in vitro* cytogenetic assays were conflicting (negative without S9 but positive with S9 in CHO cells and human lymphocytes; negative in embryonic bovine and peripheral lymphocytes and in lymphocytes from 2, 4- D workers). Two rat bone marrow cytogenetic assays, using ip injection as the exposure route provided only equivocal results. The one mouse bone marrow micronucleus assay (MRID Nos. 41409804, 41870101) is the same assay that was published by Charles et al. (1999b). Similarly, the findings from the *in vitro* UDS assay with 2, 4-D was also previously presented (Charles et al., 1999a). A similar clouded picture emerged for *in vitro* DNA damage; four of five assays were negative (2 SCE assays in rat lymphocytes and human lymphocytes from 2,4- D workers; 1 UDS assay in primary rat hepatocytes; and 1 DNA damage assay in human fibroblasts). The findings from the *in vitro* UDS assay with 2, 4-D were previously presented (Charles et al., 1999a). The remaining SCE assay, which was in CHO cells and was part of the chromosome aberration test performed by Galloway et al. (1987), gave results that conflicted with the cytogenetic data (i.e., positive without S9 but negative with S9).

8. Overall Conclusions

There is a rather extensive Genetic Toxicology database on 2, 4-D and most of the assays required to fulfill the FIFRA guidelines for pesticides are available, negative and acceptable, with the exception of no gene mutation in mammalian cells assays. However, the only conclusions that can be reached with assurance (i.e., confirmed or unambiguous results) is that 2, 4-D induces DNA damage both *in vitro* and *in vivo* and gene mutations and/or chromosome mutations in yeast, plants and insects. These data should nevertheless, be interpreted with caution for two reasons:

a. As stated earlier, DNA damage alone, whether *in vitro* or *in vivo,* cannot be interpreted as a causal event leading to mutagenicity or other adverse outcomes in rodents or humans because the ability of cells or whole animals to repair damage to DNA cannot be overlooked.

b. Confirmed or clear evidence of mutagenicity comes from test systems (i.e., yeasts, plants and insects) that generally are not given the same consideration in a weight-of-the-evidence mutagenicity evaluation of a pesticide. Thus, the relevance to human health cannot be ascertained.

No recommendations for further testing are given at this time. It is doubtful, considering the uncertainty in the Genetic Toxicology database, that additional testing will resolve the issue of whether 2, 4-D is a mutagen.

9. References

Bus, J.S. (2009). Personal communication: E-mail: 1999 Article on 2, 4-D. To Nancy McCarroll, OPP/HED/TEB From: Jim Bus, Dow Chemical, dated 10/01/2009.

US Environmental Protection Agency (USEPA), 1991. Pesticide assessment guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals, Series 84, Mutagenicity, Addendum 9. Washington, DC: Office of Pesticide Programs, USEPA, EPA Publication No. 540/09-91-122.

Table 3. Summary of Mutagenicity Studies with 2, 4-D Acid Presented to the Fourth Cancer Peer Review Committee [#]

Test system	Test object	Concentration	Results	Reference
Ames Test	*Salmonella typhimurium* TA 98, TA 100, TA 1535, TA 1537, TA 1538	100 - 10000 µg per plate with S9; 66.7 - 6670 µg per plate without S9	Negative	Lawlor, 1990 MRID No. 41409801.
Ames Test	*Salmonella typhimurium* TA98, TA100, TA 1535, TA1537, TA1538	0 - 1000 g/plate	Negative	Rashid *et. al.,* 1984
Ames Test	*Salmonella typhimurium*	0 to 3333 µg/plate	Negative	Soler-Neidzieler, 1988
Ames Test	*Salmonella typhimurium* TA 97, TA 98, TA 100, TA 1535, TA 1537, TA 1538	0 - 1000 g/plate (Although, weakly positive in TA97a at 250 µg/plate with S9)	Negative	Kappas, 1988
Ames Test	*Salmonella typhimurium* TA 1538 and TA 1535	0 - 2000 µg/plate	Negative	Rashid and Mumma, 1986
Ames Test	*Salmonella typhimurium* TA 98, TA 100, TA 1535, TA 1538	up to 5000 µg /plate	Negative	Simmon *et al.,* 1977
E. Coli	K12, WP2	0 - 2000 g/plate	Negative	Rashid and Mumma, 1986
E. Coli	PQ 37	0 - 200 g/plate	Negative	Mersch-Sundermann *et al.,* 1989
SLRL Mutation	*Drosophila melanogaster* larvae	0.l - 10,000 ppm	Positive	Kale *et al.,* 1995
SLRL Mutation	*Drosophila melanogaster* Adults	1000 - 10,000 ppm (feeding) 10,000 ppm (injection)	Negative	Zimmering *et al.,* 1985
Hamster-HGPRT	V79 fibroblasts	10 - 100 µg/ml	Inconclusive	Pavlica *et al.,* 1991
Chromosomal Aberrations	CHO Cells	500 - 920 µg/ml	Negative without S9[a]	Galloway *et al.,* 1987
Chromosomal Aberrations	Embryonic bovine kidney and peripheral lymphocytes	1 - 1000 ppm	Negative	Bongso and Basrur, 1973
Chromosomal Aberrations	*In vitro* Human lymphocytes	0.125-0.35 mM 0.125-1.250 mM	Negative without S9 Positive without S9[b]	Mustonen *et al., 1986*
Chromosomal Aberrations	Human lymphocytes (occupational exposure)	(0.03 - 0.04 mg/m^3)[c]	Negative	Mustonen *et al., 1986*

Test system	Test object	Concentration	Results	Reference
Chromosomal Aberrations	***In vivo*** **Charles River Rat (bone marrow)**	**0 - 350 mg/kg i.p./4 or 24 hr; 3 replicates.**	**Equivocal[d]**	**Turkula and Jalal, 1987**
Chromosomal Aberrations	***In vivo*** **Wistar Rat (bone marrow)**	**0, 17.5, 35 or 70 mg/kg/day/ i.p/2 times**	**Equivocal[e]**	**Adhikari and Grover, 1988**
SCE	**Rat lymphocyte**	**100 mg/kg**	**Negative**	**Mustonen *et al.*, 1989**
SCE	**CHO cells**	**50 - 299 μg/ml**	**Positive without S9**	**Galloway *et al.*, 1987**
SCE	**CHO cells**	**500 - 4200 μg/ml**	**Negative with S9**	**Galloway *et al.*, 1987**
SCE	**Human lymphocyte (occupational exposure)**	**Not reported**	**Negative**	**Linnainmaa, 1983**
Micronucleus	**ICR mouse (bone marrow)**	**40 - 400 ~~μg/kg~~ mg/kg [$]**	**Negative**	**Ivett, 1990; MRID No. 41409804, 41870101**
Unscheduled DNA synthesis	**Fischer 344 primary rat hepatocytes**	**~~0.969 - 2890 μg/ml~~ 2.43 – 96.9 μg/ml; cytotoxic at ≥96.9 μg/ml [$]**	**Negative**	**Cifone, 1990; MRID No. 41409807**
DNA damage	**Human Fibroblasts (PM2 DNA)**	**0 - 100 nmol/l**	**Negative**	**Clausen *et al.*, (1990)**

a = Positive with S9 at 10.5 hrs after treatment, but not at 19 hrs.
b = Attributed to "unidentified clastogens"(i.e., contaminants) by investigators.
c = Present in urine at 0.09 -1.14 m/L (non smokers); 0.11-1.56 (smokers).
d = Positive ≤ 75 mg/kg but only in one replicate
e = Positive at top two doses but results were similar to DMSO solvent control

[#] Extracted from the Carcinogenicity Peer Review (4th) of 2, 4-Dichlorophenoxyacetic acid (2, 4-D); TXR #: 0050017, Jan. 29, 1997 (Cover Memo); July 17, 1996 (Actual document).

[$] When these data were revisited, it was noted that the dosage unit (μg/kg) or concentration range (μg/ml) was incorrect.

Bin: NC

(Study) **Citation**: Oakes, D.J. and Pollak, J.K. (2000). The *in vitro* Evaluation of the Toxicities of Three Related Herbicide Formulations Containing Ester Derivatives of 2, 4, 5-T and 2, 4-D Using Sub-Mitochondrial Particles. Toxicology 151: 1-9.
[Human Studies Considerations Apply: No]
Principal Endpoint: [**Bin: NC**]
Methods Summary: not applicable
Results Summary: sub-mitochrodrial particle test is useful as an initial screening assay.
CONCLUSION: essential to establish the toxicities of complete formulations, rather than just evaluating the toxicities of the active components.
COMMENT/HED**:** This is an *in vitro* study (sub-mitochrodrial particle test), which cannot be extrapolated directly to the complex *in vivo* situation. Such assays cannot account for pharmacokinetic and pharmacodynamic changes that occur to xenobiotics in whole animals. Studies in whole animals are more relevant, and there are numerous studies on 2, 4-D that have identified effect levels and no effect levels. Apoptosis is considered a vital component of various processes including normal cell turnover, proper

development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Apoptosis plays a crucial role in developing and maintaining health by eliminating old cells, unnecessary cells, and unhealthy cells. There is no indication of how the doses that resulted in cytotoxicity and induction of apoptosis in mitochondria relate to the dose levels attained in cells in the intact animal.
CITED BY: Beyond Pesticides cited this study as showing 2, 4-D to be "cytotoxic and induces apoptosis *via* direct effect on mitochondrial membranes". However, no review of this paper was provided by Beyond Pesticides, and the cited paper references other studies that demonstrated the effects on mitochondria that BP noted.

Appendix C:
Exposure Data Summaries

Title: Biomonitoring of 2,4-dichlorophenoxyacetic acid exposure and dose in farm families
Journal Citation: Envir. Health Perspectives, V115, No. 3, March 2007 (pps 370-376)
Author(s): Alexander, B.H., Mandel, J.S., Baker, B.A., Burns, C.J., Bartels, M.J., Acquavella,J.F., Gustin, C.
Study Type: PB – Population Based Monitoring
Methods Summary: Farm families were recruited from licensed applicators in Minnesota and South Carolina. Families were recruited from licensed applicators in Minnesota and South Carolina. There were 43 applicators, 34 spouse, and 22 children. None of them had mixed or applied 2,4-D in previous week. The families were instructed to follow their normal routines as related to pesticide application. The farmer and spouse were asked to complete an enrollment questionnaire before and following the study application. Daily 24-hr urine samples were collected one day before through 3 days after an application of 2,4-D, characterizing exposure profiles. Eligibility for the study required that a) the family lived on a farm, b) the family consisted of the farmer, a spouse, and at least one child 4-17 years of age, c) the farmer was planning to personally apply 2,4-D to at least 10 acres of cropland as part of the normal operation, and d) the family members were willing to collect all of their urine for 5 days. Eligible family members collected all urine during five 24-hr intervals, 1 day before through 3 days after an application of 2,4-D. Exposure profiles were characterized with 24-hr urine 2,4-D concentrations, which then were related to potential predictors of exposure. Systemic dose was estimated using the urine collections from the application day through the third day after application.
Results Summary: Median urine 2,4-D concentrations at baseline and day after application were 2.1 and 73.1 μg/L for applicators, below the limit of detection, and 1.2 μg/L for spouses, and 1.5 and 2.9 μg/L for children. The younger children (4–11 years of age) had higher median post-application concentrations than the older children (≥ 12 years of age) (6.5 vs. 1.9 μg/L). The geometric mean systemic doses (micrograms per kilogram body weight) were 2.46 (applicators), 0.8 (spouses), 0.22 (all children), 0.32 (children 4–11 years of age), and 0.12 (children ≥ 12 years of age). Exposure to the spouses and children was primarily determined by direct contact with the application process and the number of acres treated. Multivariate models identified glove use, repairing equipment, and number of acres treated as predictors of exposure in the applicators.
Conclusions: Investigators observed considerable heterogeneity of 2,4-D exposure among farm family members, primarily attributable to level of contact with the application process. Awareness of this variability and the actual magnitude of exposures are important for developing exposure and risk characterizations in 2,4-D–exposed agricultural populations. The FFES is a comprehensive exposure study, but is not without limitations. Overall, the compliance with the urine collection protocol was good, but a few of the 24-hr urine collections were incomplete. Although the 2,4-D concentrations may be well represented in these incomplete samples, the systemic dose will be underestimated. The applications in this study were intended to represent the real-world scenarios that a farm family would encounter year in and year out; however, the study was limited to a single application. The applicators were asked to follow their usual practices to capture real-world exposure scenarios, but it is possible that some altered practices because the field observer was present. Changes in equipment or agricultural practices in the past or future may alter the actual exposure to the family. This study was not able to address how much the exposure will vary over one or more seasons and whether the peak exposures are representative of exposures for that individual or the distribution of expected exposure from applying this chemical.
Comments: This study is part of the industry FFES (Farm Family Exposure Study) conducted in South Carolina and Minnesota using similar methods to the Ag Health Study. The use of the results of this study in the Task Force Comments on the Petition appears reasonable. No environmental media sampling within the subject homes was reported and the results are consistent with other studies of this nature (i.e., the highest exposures are associated with applications).

Title: Predictors of herbicide exposure in farm applicators.
Journal Citation: Int. Arch Occup. Environ. Health (2002) 75: 406-414
Author(s): Arbuckle, T.E., Burnett, R., Cole, D., Teschke, K., Dosemeci, M., Bancej, C., Zhang, J.
Study Type: PB – Population Based Monitoring
Methods Summary: A subset if the participants in a retrospective cohort study of Ontario farm families volunteered for a pesticide exposure assessment study. Immediately prior to and after handling phenoxy herbicides (including 2,4-D) for the first time during the season, 126 applicators provided pre-exposure spot urine samples and a subsequent 24 hour urine sample. They also completed a questionnaire on pesticide use and handling practices for the 1st day of application.
Results Summary: Assuming that the presence of 2,4-D in the urine was a measure of true exposure and that questionnaire indications of 2,4-D use were the exposure classification subject to error, then the questionnaire's prediction of exposure had a sensitivity of 56.7% and specificity of 86.4%. The comparable values for MCPA were sensitivity and specificity of 91.6% and 67.4%, respectively. In multi-variate models, the variables pesticide formulation, protective clothing/gear, application equipment, handling practice, and personal hygiene were significant predictors of urinary herbicide levels in the first 24 hours after application (or spraying) had been initiated (adjusted R^2 = 44% for MCPA and 39% for 2,4-D). Investigators also indicated the only protective factor identified in the 2,4-D multivariate model was the wearing of rubber gloves during mixing or loading of the herbicide.
Conclusions: The investigators concluded that although similar domains of factors were associated with exposure in both models, the specific factors identified and the signs of the coefficients were sufficiently different between the final models for each herbicide that additional investigations appear to be warranted to determine the sources of the differences and assess the validity of the models and their ability to be generalized.
Comments: The investigation in this paper was conducted as part of the Ontario farm family retrospective cohort study. In this study, urinary concentrations for applicators only were reported for 2,4-D and MCPA. No exposure results were reported for spouses or children. A total of 126 families participated in the exposure monitoring component of this study. In Table 2 results were presented for applicators who reported application of 2,4-D (N=43) and MCPA (N=89). Comparatively, as would be expected, those who reported applications had much higher urinary concentrations of each chemical compared to those who did not report application involvement. The arithmetic means for 2,4-D were 27.6 and 2.6 µg/L for applicators and those not involved in application, respectively. The maximum reported 2,4-D concentrations were 410.0 and 66.0 µg/L for applicators and those not involved in application, respectively. The use of the results of this study in the Task Force Comments on the Petition appears reasonable with regard to the efficacy of protective glove use. No environmental media sampling within the subject homes was reported.

Title: Increased lymphocyte replicative index following 2,4-dichlorophenoxyacetic acid herbicide exposure.
Journal Citation: Cancer Causes and Control 11: 373-380 (2000)
Author(s):. Figgs LW, Holland NT, Rothmann N, Zahm SH, et al.
Study Type: EPI/PB – Epidemiology & Population Based Monitoring
Methods Summary: The objective of this study was to evaluate peripheral blood lymphocyte proliferation (replicative index: RI) and micronuclei frequency (MF) among 2,4-D herbicide applicators. Twelve applicators (one 17 years old) spraying only 2,4-D provided a blood and urine specimen upon enrollment, several urine samples during the spraying season, and a blood specimen at the end of the study. Nine controls also provided blood and urine specimens. Standard methods were used to quantify impacts and 2,4-D residues. Applicator RI and MF were compared before and after spraying and with controls.
Results Summary:. Applicators contributed 45 urine specimens with concentrations ranging from 1.0 to 1700 µg 2,4-D/g creatinine/L urine that logarithmically increased as spray time increased. Applicator RI increased after spraying (p=0.016), independent of tobacco and alcohol use, and demonstrated a weak dose-response with increasing urinary 2,4-D levels (p=0.15). Among 2,4-D applicators pre-exposure complete blood counts and lymphocyte immunophenotypes were not significantly different from post-exposure measurements.
Conclusions: Urinary 2,4-D concentration, an exposure biomarker, may be associated with lymphocyte replicative index, a cell proliferation biomarker. Investigators also indicated the results of this study do not support a genotoxic pathway.
Comments: In the NRDC petition this study is cited in a manner that reflects the conclusions drawn by the investigators except it was not explicitly stated by the investigators that there is an increased risk of chronic toxicity due to the apparent exceedance of clearance mechanisms. The 2,4-D task force also cited this study correctly but included characterization elements which were not raised by NRDC including indicating the authors described the study as preliminary, a pilot investigation, and the first to show a relationship between RI and urine levels. The task force also cited the investigator indication that the link between 2,4-D function and lymphocyte replicative index is unclear and that the mechanism, our reports and other recent investigations do not support a genotoxic pathway.

Title: Review of 2,4-dichlorophenoxyacetic acid (2,4-D) Epidemiology and Toxicology
Journal Citation: Critical Reviews in Toxicology 32(4): 233-257 (2002)
Author(s): Garabrant, D.H. and Philbert, M.A.
Study Type: EPI – Epidemiology [various tox]
Methods Summary: A literature review meta-analysis was completed using a variety of animal data and epidemiological evidence to determine if 2,4-D or its salts or esters damage DNA under physiologic conditions or induce oncogenic or carcinogenic effects.
Results Summary: Detailed review of the genotoxic effects of 2,4-D, its salts, and esters showed that 2,4-D does not have any genotoxic/mutagenic potential. It was also found that 2,4-D does not form reactive electrophile metabolites which are associated with carcinogenicity. Human observation studies found an association between exposure to 2,4-D and neurologic effects, including persistent peripheral polyneuropathy, demyelination and ganglion degeneration in CNS, reduced nerve conduction velocities, and myotonia. However, the doses associated with these effects are unknown. There is no convincing evidence in the literature that 2,4-D is associated with human reproductive toxicity. Case-control and cohort epidemiology studies provide scant evidence that supports the conclusion that exposure to 2,4-D is associated with soft tissue sarcoma (STS), non-Hodgkin's lymphoma (NHL), or Hodgkin's disease (HD).
Conclusions: Investigators concluded overall the available evidence from epidemiologic studies is not adequate to conclude that any form of cancer is causally associated with 2,4-D exposure. There is no human evidence of adverse reproductive outcomes related to 2,4-D. The available data from animal studies of acute, subchronic, and chronic exposure to 2,4-D, its salts, and esters show an unequivocal lack of systemic toxicity at doses that do not exceed renal clearance mechanisms. There is no evidence that 2,4-D in any of its forms activates or transforms the immune system in animals at any dose. At high doses, 2,4-D damages the liver and kidney and irritates mucous membranes. Although myotonia and alterations in gait and behavioral indices are observed after overwhelming doses of 2,4-D in the microgram/kg/day range. It is unlikely that 2,4-D has any neurotoxic potential at doses below those required to induce systemic toxicity.
Comments: This study was conducted for the *2,4-D Industry Task Force II on Research Data* to provide an update of the evidence on human toxicity and cancer risks related to 2,4-D on research completed between 1995 and 2001. The use of the results of this study in the Task Force Comments on the Petition appears reasonable. No environmental media sampling or direct exposure monitoring from the considered studies were reported.

Title: Birth Defects, Season of Conception, and Sex of Children Born to Pesticide Applicators Living in the Red River Valley of Minnesota, USA

Journal Citation: *Environ Health Perspect* 110(suppl 3):441–449 (2002).

Author(s): Vincent F. Garry, Mary E. Harkins, Leanna L. Erickson, Leslie K. Long-Simpson, Seth E. Holland, and Barbara L. Burroughs

Study Type: EPI – Epidemiology

Methods Summary: A cross-sectional study of 695 families and 1,532 children conducted during 1997 to 1998 in the Red River Valley of Minnesota was reported with reference to a previous study conducted in 1989-1991. Birth defects, season of conception and the sex of children born to pesticide applicators were evaluated. Associations with varied types of pesticides were considered. All reported anomalies were recorded according to the International Classification of Disease, 9th Revision. Confounding variables such as maternal smoking, drinking, age and chronic diseases (such as diabetes and hypertension), were examined. Where possible, familial genetic history, pregnancy medication use, and non-medicinal drug use (vitamins) was assessed in families with birth defects. Regarding pesticide use, subjects were re-interviewed 6 months later, where possible, by written questionnaire. This was used to document common pesticide use by pesticide class, acreage treated, type of crop, and use of personal protective gear, as well as to validate use of pesticides by class. Where appropriate, regression analysis, two-sided t-tests, and analysis of variance methods were employed. Variables were mother's age, smoking status, alcohol use, and season of conception. Information regarding specific medication used during pregnancy and subjects' diet were not considered in the survey. Residence at a rural site (towns with populations < 3,000) or on a farm during childhood (<18 years of age) was considered in some statistical analysis.

Results Summary: In this current study, in the first year of life the birth defect rate was 31.3 births per 1,000, with 83% of the total reported birth defects confirmed by medical records. Inclusion of children identified with birth or developmental disorders within the first 3 years of life and later led to a rate of 47.0 per 1,000 (72 children from 1,532 live births). Conceptions in spring resulted in significantly more children with birth defects than found in any other season (7.6 vs. 3.7%). Twelve families had more than one child with a birth defect (n = 28 children). Forty-two percent of the children from families with recurrent birth defects were conceived in spring, a significantly higher rate than that for any other season. Three families in the kinships defined contributed a first-degree relative other than a sibling with the same or similar birth defect, consistent with a Mendelian inheritance pattern. The remaining nine families did not follow a Mendelian inheritance pattern. The sex ratio of children with birth defects born to applicator families shows a male predominance (1.75 to 1) across specific pesticide class use and exposure categories exclusive of fungicides. In the fungicide exposure category, normal female births significantly exceed male births (1.25 to 1). Similarly, the proportion of male to female children with birth defects is significantly lower (0.57 to 1; p = 0.02). Adverse neurologic and neurobehavioral developmental effects clustered among the children born to applicators of the fumigant phosphine (odds ratio [OR] = 2.48; confidence interval [CI], 1.2–5.1). Use of the herbicide glyphosate yielded an OR of 3.6 (CI, 1.3–9.6) in the neurobehavioral category.

Conclusions: Investigators concluded that the present and previous studies these studies point out that *a*) herbicides applied in the spring may be a factor in the birth defects observed and *b*) fungicides can be a significant factor in the determination of sex of the children of the families of the RRV. Thus, two distinct classes of pesticides seem to have adverse effects on different reproductive outcomes. Biologically based confirmatory studies are needed. The investigators also indicated use of the phosphonamino herbicides (glyphosate, Roundup) was overrepresented in the adverse birth and developmental effect group. No other commonly used pesticide (i.e., 2,4-D) compared by major organ and/or functional system was uniquely associated with specific adverse birth or developmental effects.

Comments: The use of the results of this study in the 2,4-D Task Force Comments on the Petition appears reasonable in that the investigators only identified a possible relationship with the herbicide glyphosate and not with the use of the chlorophenoxy acid herbicides such as 2,4-D. No environmental media sampling or direct exposure monitoring were discussed or reported.

Title: Pesticide Appliers, Biocides, and Birth Defects in Rural Minnesota
Journal Citation: Environmental Health Perspectives V 104, No. 4, April 1996 (394-399)
Author(s): Garry, V.F.; Schreinmachers, D.; Harkins, M.E.; and Griffith, J.
Study Type: EPI – Epidemiology
Methods Summary: This ecologic study evaluated the hypothesis suggested previously by the investigators that the offspring of pesticide applicators might have increased risks of birth anomalies. This was investigated by linking 4,935 births to 34,772 state-licensed, private pesticide applicators in Minnesota occurring between 1989 and 1992 to the Minnesota state birth registry containing 210,723 live births in the same timeframe. SAS software; Chi-square tests were used to estimate the statistical significance of observed differences in the proportion of birth anomalies between pesticide applicators and the general population, and between populations living in three identified crop regions. Odd ratios were developed to estimate risk. The Mantel-Haenszel method was used to analyze crude and age-adjusted data, and to measure overall association. Logistic regression was used to estimate the effects of potential pregnancy risk factors.
Results Summary: The birth defect rate for all birth anomalies was significantly increased in children born to private applicators. Specific birth defect categories, circulatory/respiratory, urogenital, and musculoskeletal/integuemental showed significant increases. For the general population and for applicators, the birth anomaly rate differed by crop-growing region. Western Minnesota, a major wheat, sugar beet, and potato growing region, showed the highest rate of birth anomalies per 1000 live births: 30.0 for private appliers versus 26.9 for the general population of the same region. The lowest rates, 23.7/1000 for private applicators versus 18.3/1000 for the general population, occurred in noncrop regions. The highest frequency of use of chlorophenoxy herbicides and fungicides also occurred in western Minnesota. Births in the general population of western Minnesota showed a significant increase in birth anomalies in the same three birth anomaly categories as applicators and for central nervous system anomalies. This increase was most pronounced for infants conceived in the spring. The seasonal effect did not occur in other regions. The male/female sex ratio for the four birth anomaly categories of interest in high phenoxy herbicide/fungicide use is 2.8 for applicators versus 1.5 for the general population of the same region ($p=0.05$). In minimal use regions, this ratio is 2.1 for applicators versus 1.7 for the general population. Pesticide applicators had significantly more children with an anomaly than non-applicators ($p<0.001$) in the corn/soybean-growing region. The most consistent results were obtained for 2,4-D and Methyl-4-chlorophenoxyacetic acid (MCPA). The frequency of anomalies in high-use areas significantly increased (OR=1.86; 95% CI, 1.69- 2.05) for combined births with central nervous system, circulatory/respiratory, urogenital, and musculoskeletal anomalies (OR = 1.51; 95%CI, 1.40-1.62).
Conclusions: Investigators concluded that the pattern of excess frequency of birth anomalies by pesticide use, season, and alteration of sex ratio suggests exposure-related effects in applicators and the general population of the crop-growing region of western Minnesota. Birth defects increased in 1) offspring born to licensed private applicators, 2) offspring born to the general population residing in high use chlorophenoxy herbicide/fungicide regions, and 3) infants conceived in spring in western Minnesota.
Comments: The use of the results of this study in the NRDC Petition and in the comments from Beyond Pesticides that reflect the specific conclusions described in the study but NRDC and Beyond Pesticides ignored the comments provided in later publications by the same investigator which were cited by the 2,4-D task force comments on the petition which generally indicate lower incidence rates. The 2,4-D task force commented the study of Garry and co-workers (1996) assessing potential associations of herbicide use and birth defect outcomes in an agricultural region of Minnesota was later described by this same research team as "a first effort" that suffered from a "limited reporting timeframe" (Garry et.al., 2002). In the latter report, the 2,4-D task force indicated the authors concluded that a more detailed cross-sectional analysis of this area showed no statistically significant correlations between 2,4-D use and excess adverse birth or neurodevelopmental effects. No environmental media sampling or direct exposure monitoring were discussed or reported. It is also not clear how the trends in the general population of the Minnesota birth registry compare to the general population of the United States nor was any comparison provided pertaining to other high use areas for 2, 4-D. Analysis was done at the group level, rather than individual level. Individuals were assigned an exposure classification that might not represent their true exposure. This study also did not mention if the maternal factors that can cause birth defects (such as maternal infections, vaccinations, drug/medicine used) were

considered.

Title: Biomarker Correlations of Urinary 2,4-D Levels in Foresters: Genomic Instability and Endocrine Disruption

Journal Citation: Environmental Health Perspectives V109/No. 5 (May 2001) pp. 495-500

Author(s): Vincent F. Garry, Robert E. Tarone, Ilan R. Kirsch, Jorge M. Abdallah, Donald P. Lombardi, Leslie K. Long, Barbara L. Burroughs, Dana B. Barr, and James S. Kesner

Study Type: PB/EPI – Population Based Monitoring/Epidemiology

Methods Summary: Forest pesticide applicators were selected for consideration in this case-control study of genomic stability and reproductive hormone levels related to the use of chlorophenoxy herbicides. Aberrations in G-banded chromosomes, reproductive hormone levels, and polymerase chain reaction-based V(D)J rearrangement frequencies were examined. At the end of the peak of the 2,4-D application season, first-voided morning urine and morning blood specimens were obtained from applicators. A second blood specimen was obtained from exposed subjects, only within 6 weeks of the beginning of the following season's application work and used to compare to the earlier data set. For reproductive hormone analysis, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone concentrations (total and free) in serum or plasma obtained from blood specimens donated by study participants were measured. For chromosome studies, chromosome analysis and PCR-based V (D) J trans-rearrangement assay were performed. Subjects were selected to participate based on the following criteria: a) no chronic disease, b) no chronic medication use, c) have applied 2,4-D more than 5 days per year (for the exposed group), d) did not apply pesticides this past year (for the control group). Analysis of variance methods was used to test for changes in hormone level according to 2,4-D application method. Pearson correlation coefficients were used to quantify the relationship between urinary 2,4-D level and changes in hormone level across all application methods. Wilcoxon rank-sum tests were computed to determine if chromosome aberration frequencies varied according to level of herbicide exposure. Poisson regression analyses were used to examine the relationship between aberration frequencies and urinary 2,4-D levels. Pearson correlation coefficients were used to quantify the relationship between urinary 2,4-D level and V (D)J rearrangement frequencies.

Results Summary: Categorized by applicator method, men who used a hand-held, backpack sprayer in their applications showed the highest average level (453.6 ppb) of 2,4-D in urine. Serum luteinizing hormone (LH) values were correlated with urinary 2,4-D levels, but follicle-stimulating hormone and free and total testosterone were not. At the height of the application season; 6/7 backpack sprayers, 3/4 applicators who used multinozzle mechanical (boom) sprayers, 4/8 aerial applicators, and 2/5 skidder-radiarc (closed cab) applicators had two or more V(D)J region rearrangements per microgram of DNA. Only 5 of 15 minimally exposed (control) foresters had two or more rearrangements, and 3 of these 5 subjects demonstrated detectable levels of 2,4-D in the urine. Only 8/24 DNA samples obtained from the exposed group 10 months or more after their last chlorophenoxy use had two rearrangements per microgram of DNA, suggesting that the exposure-related effects observed were reversible and temporary. Although urinary 2,4-D levels were not correlated with chromosome aberration frequency, chromosome aberration frequencies were correlated with the total volume of herbicides applied, including products other than 2,4-D. One issue the investigators attempted to define was whether or not it was not possible to separate the impacts of adjuvant use. The sample size in this study was very small and most applications use adjuvants which aid in the retention and absorption of pesticides on treated plants. Therefore to the extent to which the cumulative chromosome effect observed can be related to adjuvant use is uncertain. Another issue considered by investigators was to elicit whether or not the volume of pesticide used and/or application equipment used. It is clear more significant effects are noted with higher exposure scenarios.

Conclusions: Investigators concluded that herbicide applicators with high urinary levels of 2,4-D (backpack and boom spray applications) exhibited elevated LH levels. They also exhibited altered genomic stability as measured by V(D)J rearrangement frequency, which appears reversible months after peak exposure. However, there is uncertainty associated with these results because although they are highly detailed, the limited sample size warrants cautious interpretation of the data.

Comments: The use of the results of this study in the comments from Beyond Pesticides appears to be reasonable and it reflects the specific conclusions described by the investigators. However, it was used to make the argument that 2,4-D is an endocrine disruptor which is being evaluated separately and the results are pending. Several uncertainties should be noted including no environmental media sampling were reported and he sample size is small as noted by the investigators and should be considered in the interpretation of the results. Biomonitoring results could be used quantitatively if desired to calculate risk.

Title: Factors Affecting the Exposure of Ground-rig Applicators to 2,4-D Dimethylamine Salt

Journal Citation: Arch. Environ. Contam. Toxicol. 15, 677-686 (1986)

Author(s): Grover, R.; Cessna, A.J.; Muir, N.I.; Riedel, D.; Franklin, C.A.; Yoshida, K.

Study Type: PL – Planned Exposure Monitoring

Methods Summary: A total of 30 individual spray operations using 2,4-D dimethylamine salt were monitored. These operations involved farmers carrying out spray operations with tractor-drawn ground rigs, involving handling, transferring, mixing, and applying to wheat. These individual events lasted 55 to 870 minutes and involved 1 to 11 tankloads, and application of 6.7 to 88.3 kg 2,4-D acid equivalent. Between 16 to 194 hectares were treated. Air sampling using a typical personal sampling pump method was used. Dermal exposure was measured using patches and a handwash method. Patches were worn under 2 work clothing and on the exterior. Some applicators wore protective gloves. Most applicators in the study used tractors with no cabs but 13 of 30 had some sort of enclosure. Urine was also collected and analyzed for 2,4-D residues. A total of nine subjects participated in the research and all but one were monitored multiple times with one worker being monitored during 7 spray operations. The investigators used the results of this study to evaluate various factors which may impact exposures (e.g., area or amount sprayed).

Results Summary: Hand exposures accounted for 80 to 90 percent of the potential cumulative exposure. The 2,4-D on the rest of the body ranged from 10 to 20 percent of the potential cumulative exposure. Urinary 2,4-D excretion accounted for 1 to 2 percent of the potential cumulative exposure. The median air concentration was 2.1 $\mu g/m^3$ with a range of <0.01 to 101 $\mu g/m^3$. Dermal (non-hand) dose values had a median of 27.9 µg/kgBW with a minimum of 7.5 µg/kgBW and a maximum of 278 µg/kgBW. Hand dose values had a median of 120 µg/kgBW with a minimum of 0.1 µg/kgBW and a maximum of 552 µg/kgBW. Correlations were evaluated for various exposure factors. It should be noted the investigators considered all results together with no consideration as to whether or not gloves were used to a cab was present or not on each tractor.

Conclusions: Investigators concluded the total calculated amount of 2,4-D deposited on the body (minus the hands) and the total amount excreted in the urine were highly correlated with the number of tank fills, area sprayed, amount sprayed, and duration of operation.

Comments: There is no mention in the study of consideration by an institutional review board. This study was conducted prior to the initiation of the Pesticide Handlers Exposure Database (PHED). This study reflects the monitoring technology and approaches of that timeframe but the results still appear consistent with current thinking in that hands are large contributors to overall exposures especially when mixing/loading activities are evaluated. The use of the results of this study in the comments from the 2,4-D task force appears to be reasonable and it reflects the specific conclusions described by the investigators. No environmental media sampling was completed. Direct exposure monitoring was reported and could be considered for quantitative use. However, it should be noted that PHED is currently used for routine assessments of this type rather than individual studies.

Title: A Case–Control Study of Non-Hodgkin Lymphoma and Exposure to Pesticides
Journal Citation: CANCER March 15, 1999 / Volume 85 / Number 6 (pp. 1353-1360)
Author(s): Hardell, L. and Ericsson, M.
Study Type: EPI – epidemiology
Methods Summary: A population-based case–control study in northern and middle Sweden was performed. Exposure data were ascertained by comprehensive questionnaires, and the questionnaires were supplemented by telephone interviews. In total, 404 cases and 741 controls answered the questionnaire. Univariate and multivariate analyses were performed with SAS. Cases with a histopathologic diagnosis of NHL were identified from the regional cancer registries. The cases were divided into different groups according to histopathology, i.e. B-cell lymphoma aggressive (n= 157), indolent (n= 185), T-cell lymphoma (n=18), and other (n=6) or unspecified types (n=38). Exposure to each herbicide (MCPA, 2,4-D/2,45-T, Glyphosate, etc.) was analyzed separately.
Results Summary: Increased risk for NHL was found for subjects exposed to herbicides (odds ratio [OR], 1.6; 95% confidence interval [CI], 1.0 –2.5) and fungicides (OR, 3.7; 95% CI, 1.1–13.0). Among herbicides, the phenoxyacetic acids dominated (OR, 1.5; 95% CI, 0.9 –2.4); and, when subclassified, one of these, 4-chloro-2-methyl phenoxyacetic acid (MCPA), turned out to be significantly associated with NHL (OR, 2.7; 95% CI, 1.0–6.9). Odds ratios were lower for 2,4-D than for the other phenoxyacid herbicides. 2,4-D and 2,4,5-T exposure slightly increased the risk of NHL (OR= 1.3, CI 0.7-2.3). For several categories of herbicides, it was noted that only exposure during the most recent decades before diagnosis of NHL was associated with an increased risk of NHL. Exposure to impregnating agents and insecticides was, at most, only weakly related to NHL.
Conclusions: Investigators concluded exposure to herbicides in total, including phenoxyacetic acids (i.e., 2,4-D had lowest reported OR values in this chemical class) during the decades before NHL diagnosis resulted in increased risk for NHL. Thus, the risk following exposure was related to the latency period. Fungicides also increased the risk for NHL when combined, but this group consisted of several different agents, and few subjects were exposed to each type of fungicide.
Comments: This study was population based, using the Swedish Cancer Registry to identify the cases. No environmental or personal sampling was completed. The Swedish compulsory reporting system for malignant diseases makes it likely that almost all incident cases in the study area during the 4 years of inclusion were used. To avoid any selection of cases associated with prognosis, both living and deceased cases with NHL were included in this case–control study. To assess exposure in an equal manner for both cases and controls and to minimize recall bias, deceased controls were used for deceased cases. Regarding farmers, the questionnaire data had to be supplemented over the telephone for all subjects due to the detailed, written instructions for the interviews. Thereby, exposure data had a significant uncertainty associated with it (e.g., type of chemical used, years and number of days for exposure, methods of use). It should also be noted that the combination of 2,4-D and 2,4,5-T, which constituted Agent Orange in U.S. warfare in Vietnam, was the most predominantly used herbicide combination in Swedish forestry. In this study, however, the analysis was restricted to exposure during the last two decades preceding the diagnosis. Since 2,4,5-T was banned in Sweden in 1977 no subjects in this study had their first exposure to this substance during the 10-year period preceding NHL diagnosis. Thus, it seems to be difficult to demonstrate any lymphomagenic effect from 2,4,5-T in subjects with lymphoma diagnosis during later years. Finally, improved requirements for personal protective equipment are used in current practices which would be expected to alter resulting OR (Odds Ratio) values. The citation of this study by Beyond Pesticides reflects the conclusions of the investigators but it does not account for the uncertainty associated with the exposure predictors acknowledged by the investigators in this study.

Title: Distributions and Determinants of Pre-emergent Herbicide Exposures Among Custom Applicators
Journal Citation: Ann. Occup. Hyg. Vol 45, No. 3, pp 227-239 (2001)
Author(s): Hines, C.J.; Deddens, J.A.; Tucker, S.P.; and Hornung, R.W.
Study Type: PL – Planned Exposure Monitoring
Methods Summary: Custom applicators intensively apply herbicides to corn and soybean fields each spring. The primary objective of this study was to characterize the exposure distributions of the herbicides alachlor, atrazine, 2,4-D 2-ethylhexyl ester (2,4-D EH), and metolachlor among a group of applicators during the spring pre-emergent spray season. A secondary objective was to evaluate determinants of exposure and to estimate within- and between-worker variance components. Fifteen applicators were sampled using a systematic design that included spray and non-spray days and multiple measurements (five to seven) on each applicator. Air, patch, and handwash samples were collected on 89 applicator-days. Applicator-days were classified into three categories: target herbicide sprayed, non-target herbicide sprayed, and no herbicide sprayed. The median age for applicators was 40 years (range 23-58). The median number of years of experience as an applicator was 8 (range 1-22), the median number of years worked for the current company was 5 (range 1-28). Of the 89 applicator-days, herbicide spraying was done for 30 days; 23 of these days included use of one or more of the target herbicides. Mixed-model regression analysis was used.
Results Summary: For all exposure metrics, adjusted mean herbicide exposures were significantly higher on days when target herbicides were sprayed as compared to non-spray days. For 2,4-D EH only, adjusted mean exposures on non-target herbicide spray days were significantly higher than on non-spray days. For all exposure metrics, the within-worker variability (GSDW 2.1–5.6) was greater than the between-worker variability (GSDB 1.2–2.7).
Conclusions: Investigators concluded wearing gloves significantly reduced adjusted mean hand exposure for all herbicides (4–20 fold) and adjusted mean thigh exposure for three herbicides (8–53 fold) on days the herbicides were sprayed; however, wearing gloves significantly increased adjusted mean atrazine hand and thigh exposures (9 and 7 fold, respectively) on days that non-atrazine herbicides were sprayed. Few of the other covariates were consistent determinants of exposure. Glove use can offer protection but reusing the same glove can be a source of exposure. Therefore, frequent hand washing and wearing new gloves should be encouraged.
Comments: This study evaluated several determinants of worker exposure and also produced distributions of exposure under varied field use circumstances. It did not examine concurrent health effects. The use of the results of this study in the comments from the 2,4-D task force appears to be reasonable they reflect the specific conclusions described by the investigators in that the study illustrated that protective gloves significantly reduce exposures. This has been widely demonstrated in many circumstances given proper glove use. No environmental media sampling was completed. Direct exposure monitoring was also reported and could be considered for quantitative use. However, it should be noted that PHED is currently used for routine assessments of this type rather than individual studies in most circumstances. According to the author, applicators were not selected by a strictly random process. Therefore, these results may not apply to all custom applicators.

Title: Exposure of Forestry Ground Workers To 2,4-D, Picloram, and Dichlorprop
Journal Citation: Environmental Toxicology and Chemistry (Vol. 6) pp. 209-224 (1987)
Author(s): T.L. Lavy; L.A. Norris; J.D. Mattice; and D.B. Marx
Study Type: PL – Planned Exposure Monitoring
Methods Summary: Urine samples from 80 forest workers applying 2,4-D plus dichlorprop or picloram by ground techniques were monitored to determine the dose of chemical absorbed. Four crews of 20 workers each were studied to compare the exposures during application by backpack, injection bar, hypohatchet or a hack and squirt method. Total urine output was analyzed to compare the absorbed dose to each worker under two operational modes. In the first (T1) the workers wore their usual clothing and followed the work habits they ordinarily used on their job. In the second (T2), conducted 6 days after the first, workers were given new leather gloves and boots and asked to take all feasible precautions (e.g., wash hands, bathe, wash clothes, avoid sprayed areas) to prevent herbicide exposure and to wash immediately if they come in contact with the chemical.
Results Summary: For all application methods except backpacks the T-2 treatment decreased the absorbed dose. During both T-1 and T-2 the clothing of backpack sprayers often became saturated with spray, dew and perspiration, and these workers received a higher absorbed dose of 2,4-D (0.04 – 0.24 mg/kg/bw) than did workers in other crews. The absorbed dose of dichlorprop ranged from undetectable to 0.18 mg/kg. Hypohatchet workers received a greater dose than did injection bar or hack-and-squirt workers. The absorbed dose of picloram ranged from undetectable to 0.02 mg/kg/bw. If equal dermal penetration of 2,4-D and picloram is assumed, this represents much less dermal absorption than would have been predicted from the relative amounts of 2,4-D and picloram in Tordon 101-R.
Conclusions: Investigators evaluated the utility of using improved hygiene practices with fresh gloves and boots as a means of reducing exposures. In cases where exposures impinge mainly on the feet and hands the T-2 conditions described by the authors coupled with enhanced hygiene appear to reduce exposures. As noted by the authors, T-2 conditions and enhanced hygiene were not as effective with backpack applicators where much of the exposure occurs around the neck and upper chest/back area and they were noted to have been working in dense vegetation areas which had been previously treated (i.e., 2 to 7 meters high). Investigators also calculated various margins of safety with the lowest one being 272 for the highest exposed backpack applicators in T-2 conditions (based on geometric mean and NOEL of 24 mg/kg/d). It was concluded that having a calculated MOS less than the level of concern was attributable to very low dermal absorption since the backpack applicators had very high levels of contact with the 2,4-D spray solution as evidenced by their wet clothing and observed activities.
Comments: This study evaluated several determinants of worker exposure and also produced distributions of exposure under varied field use circumstances. The use of the results of this study in the comments from the 2,4-D task force appears to be reasonable in that they reflect the specific conclusions described by the investigators. No environmental media sampling was completed. The direct exposure monitoring could be considered for quantitative use. However, PHED is currently used for routine assessments of this type rather than individual studies but some values for the monitored forestry operations are not addressed by PHED.

Title: Study of Reproductive Function In Persons Occupationally Exposed to 2,4-D
Journal Citation: Mutation Research, 262 (1991) pp. 47-50
Author(s): Lerda, D. and Rizzi, R.
Study Type: PB&EPI – Population Based Monitoring & Epidemiology
Methods Summary: In this experimental study, sperm quality of fifty-seven male subjects, thirty two 2, 4-D exposed (farmers, mean ages 38 years) and 25 non-exposed (controls, mean ages 35 years) were assessed. These evaluations were completed in March through July 1989. Farmers working with 2, 4-D but without history of urologic abnormalities, severe testicular injury, and recent illness, which can affect spermatogenesis, were selected. Factors such as socio-economic status, cigarette smoking, drug use, radiation exposure, general health, and sexual history were also considered in both control and exposed groups. The exposure periods were August to September 1988 with effects being determined in March 1989 and possible recovery being evaluated in July 1989. Controls were evaluated in April 1989. Sperm quality was assessed by four parameters: volume, sperm count, motility, and morphology. The samples of sperm in unexposed and exposed groups were coded in order to avoid subjectivity in analysis. All sperm parameters were evaluated 3 times per individual and mean values were calculated. The levels of 2,4-D in urine were also determined. It is not clear when the urine samples were collected relative to exposure events. The locale in which this study was conducted was not clear nor was a description provided regarding the cultural practices used by the test subjects who applied 2,4-D in this study .
Results Summary: The results indicate a mean urinary level of 2,4-D of 9.02 mg/liter in the 32 farmers selected for this study. There were increased percentages of abnormal sperm morphology, mobility, and sperm count in the exposed group than in controls. The Chi-square test showed a significant difference between control and exposed groups. Sperm volume did not show significant differences between the exposed subjects, compared to that of the controls. Following a short recovery period (after 4 months), asthenospermia (poor sperm motility), motility, and necrospermia (dead sperm) disappeared, while teratospermia (defects of sperm morphology) remained.
Conclusions: Investigators concluded that 2,4-D exposure can affect spermatogenesis and male fertility in farmers. Significant levels of asthenospermia, necrospermia and teratospermia were found in exposed workers as compared to unexposed controls. Over time, asthenospermia and necrospermia diminished but the abnormal spermatozoa (teratospermia) continued.
Comments: This study evaluated male reproductive effects in conjunction with the collection of urine from 32 applicators of 2,4-D. The investigators concluded 2,4-D can produce harmful reproductive effects. In the petition NRDC cited the results of this study in a manner reflective of the conclusions of the investigators. Beyond Pesticides also cited the results of this study in a manner reflective of the conclusions of the investigators. No environmental media sampling was completed. The direct exposure monitoring could be considered for quantitative use. However, PHED is currently used for routine assessments of this type rather than individual studies. No explanation of the circumstances of how exposures occurred was included in the discussions in the paper so those aspects of the study could not be considered. Additionally, the locale of this study was not described so it is not clear that the practices evaluated in this study are reflective of current cultural practices in the United States. Finally, requirements for endocrine screening of 2,4-D have just been released by the Agency under its updated strategy and this study should be considered in conjunction with any results from those requirements. Urine and sperm samples should have been taken at the beginning of study to compare with post exposure samples, although many of the subjects had already worked with 2,4-D at that time. An experimental study such as this can provide the evidence of cause and effect, but there is still a chance of unrecognized confounders. For example, this study did not mention in detailed which drugs were considered in drug history. There are several drugs and chemicals which can affect the male reproductive system (e.g., antihypertensives, chemotherapy).

Title: Insect Repellents, Phenoxyherbicide Exposure, and Non-Hodgkin's Lymphoma

Journal Citation: JOEM • Volume 47, Number 8, August 2005 (pp. 806-815)

Author(s): Helen H. McDuffie, PhD; Punam Pahwa, PhD; Diane Robson, BSc; James A. Dosman, MD; Shirley Fincham, PhD; John J. Spinelli, PhD; John R. McLaughlin, PhD

Study Type: EPI – epidemiology

Methods Summary: A hypothetical explanation was tested of contradictory results in studies of phenoxyherbicides and non-Hodgkins lymphoma (NHL), that the exposure of rubber gloves recommended for use by farmers when mixing or applying pesticides simultaneously to 2,4-D (2,4-dichlorophenoxyacetic acid), DEET (N,N-diethyl-m-toluamide), and ultraviolet rays increased their permeability to 2,4-D. A case (NHL n =513)/control (n=1506) study among men was conducted using age; province of residence; exposure to insect repellents containing DEET, phenoxyherbicides, or dicamba; and gloves when handling pesticides. This population-based case–control epidemiologic study was completed using mail questionnaires, modified from previous studies in Kansas and Nebraska, for the entire study group and structured pesticide exposure telephone interviews for those who indicated exposure to 10 hours or more per year of pesticide exposure, and a 15% random sample of the remaining cases and controls. Case subjects were ascertained from provincial cancer registries except in Quebec where hospital ascertainment was used. The control subjects were men aged 19 years or older who were residents in the same province as the appropriate case and were free of the cancers of interest and selected at random within age constraints from the provincial Health Insurance records (four provinces), computerized telephone listings (one province) or voters' lists (one province). Demographic data, detailed lifetime occupation and occupational exposure history, indoor pesticide application, and usage pattern of use of insect repellents on skin or clothing, home/garden use by days per year, average acres sprayed per year, details of occupational hygiene practice (such as use of gloves and protective clothing) data were collected. Descriptive analyses of each variable, including frequencies, ranges, means, standard deviations, and median values for cases and controls were done separately. Conditional logistic regression was used to compute odds ratios (OR), and conditional logistic regression subanalyses of farm residents using the variables related to phenoxyherbicide exposure, insect repellents, use of rubber gloves, and potential association with NHL. No environmental or human exposure monitoring data were collected in this study.

Results Summary: Using conditional logistic regression, the stratum with reported exposure to mecoprop, to DEET and the use of rubber gloves had higher odds ratios (3.86; 95% confidence interval _ 1.57–9.49) compared with strata with other combinations (e.g., 2,4-D with OR = 1.77 and range from 0.90 –3.45). The investigators acknowledged that they did not determine the exact type of rubber gloves used, which may vary in their permeability or whether gloves were specifically used while mixing or applying 2,4-D or other phenoxyherbicides of interest. The temporal relationship between the mixing or application of pesticides and the use of DEET is unknown. Lack of information in each of these instances would tend to bias the results toward the null. They also acknowledged the case– control design which they used has inherent limitations, including recall bias and selection bias.

Conclusions: Investigators concluded the etiologic complexity of NHL was demonstrated. The investigators indicated in future research sample sizes must be large enough to statistically address the issues of exposure to multiple pesticides, either simultaneously or concurrently and other effect modifiers or confounders, which include personal hygiene habits during and after handling pesticides and other agricultural chemicals, personal and family medical history, and cigarette-smoking practices. Biomonitoring of selected herbicides or their metabolites in biological tissues/fluids among large numbers of subjects in conjunction with questionnaires was also recommended as a useful addendum in the identification of those exposed who may be at elevated risk of developing NHL.

Comments: NRDC indicated in the petition that there is a relationship between NHL and phenoxyacid herbicide use, DEET use and the use rubber gloves. NRDC also indicated that this study suggests that herbicides could be penetrating the skin even when gloves are worn and this could be leading to development of NHL in humans. There are several critical uncertainties which have not been addressed by NRDC that were described by the investigators. For example, the types of gloves used by subjects were not ascertained and the timeframe of DEET application relative to 2,4-D use was not described. Exposure that occurs even with glove use is anticipated based on existing monitoring data (e.g., PHED). Investigators also suggested that several exposure related aspects should be further investigated because of the uncertainty.

Title: Effect of the Mosquito Repellent DEET and Longwave Ultraviolet Radiation On Permeation of the Herbicide 2,4-D and the Insecticide DDT in Natural Rubber Gloves
Journal Citation: Am. Ind. Hygiene Assoc. J. 53(7): 436-441 (1992)
Author(s): Moody, R.P.; Nadeau, B.
Study Type: PL – Planned exposure monitoring
Methods Summary: A laboratory based permeation cell test was conducted to determine the effect of the commonly used insecticide DEET on the permeability of 100 percent latex rubber gloves (Becton Dickson N36-124). These gloves could be worn by applicators as part of a chemical protective clothing ensemble. Glove permeation analysis was conducted with an automated in vitro diffusion analysis (AIDA) method employing an in-house, flow through permeation cell design.
Results Summary: Permeation of 14C ring labeled 2,4-D in natural rubber glove material was 2.4±1.81 percent at 48 hours after treatment of the glove with 2,4-D applied with DEET; this was not significantly different (Student's t-test; $p<0.05$) from 3.2±3.46 percent permeation of 2,4-D observed without DEET. Results were similar for DDT in that no differences were noted when DEET was used. Scanning electron microscopy of the natural rubber glove material, however, demonstrated disruption of the surface ultrastructure following a 24 hour treatment with DEET. The AIDA analysis also suggested that exposure of the glove material to longwave ultraviolet radiation enhanced the glove permeability to 2,4-D (6.2±0.73% [+UVA]; 0.3±0.14% [-UVA]).
Conclusions: Investigators concluded that because chemical protective clothing is commonly exposed to solar UVA this study raises concerns about the efficacy and safety of protective equipment in general.
Comments: This study describes the permeability changes in a single glove material after exposure to UVA and also DEET coupled with 2,4-D. The manner in which this study is cited by NRDC is not appropriate because they indicate that the gloves are highly permeable when the glove is exposed to DEET and UVA. The investigators reported that with DEET the permeation rate of the glove material was similar with 2,4-D whether or not DEET was present. The latex material tested did indicate increased permeability to UVA. NRDC indicates it then follows that enhanced exposures would occur. This conclusion is not warranted because it is not clear the application of UVA in this study mimics real world conditions (e.g., intensity of light beam). Also, only one type of latex rubber glove material was evaluated and its composition was not clear (e.g., UV stabilization or not?). Current production methods likely use UVA and UVB stabilizers in the manufacturing process. It is also easy to recommend other types of glove materials which as UVA/UVB impervious. Finally, it should be noted that in PHED exposures under real world conditions with actual glove use are measured and used to develop risk estimates. While chamber permeability tests of glove materials are useful they do not necessarily accurately predict protection factors for gloves which can also be impacted by design and seam sealing methods. The Agency concurs with the comments of the 2,4-D task force on the NRDC use of this study.

Title: Adult and children's exposure to 2,4-D from multiple sources and pathways
Journal Citation: Journal of Exposure Science and Environmental Epidemiology (2008) 18, 486–494
Author(s): Marsha K. Morgan, Linda S. Sheldon, Kent W. Thomas, Peter P. Egeghy, Carry W. Croghan, Paul A. Jones, Jane C. Chuang and Nancy K. Wilson
Study Type: PB/PL – Population Based Monitoring/Planned Exposure Monitoring
Methods Summary: This study investigated the 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide exposures of 135 preschool-aged children and their adult caregivers at 135 homes in North Carolina (NC) and Ohio (OH). Participants were randomly recruited from six NC and six OH counties. Monitoring was performed over a 48-h period at the participants' homes. Environmental samples included soil, outdoor air, indoor air, and carpet dust. Personal samples collected by the adult caregivers concerning themselves and their children consisted of solid food, liquid food, handwipe , and spot urine samples. All samples were analyzed for 2,4-D (free acid form) by gas chromatography/mass spectrometry.
Results Summary: 2,4-D was detected in all types of environmental samples but most often in carpet dust samples, with detection frequencies of 83% and 98% in NC and OH, respectively. The median level of 2,4-D in the carpet dust samples was about three times higher in OH homes compared to NC homes (156 vs. 47.5 ng/g, P<0.0002). For personal samples, 2,4-D was more frequently detected in the handwipe samples from OH participants (>48%) than from NC participants (<9%). Handwipe levels at the 95th percentile were about five times higher for OH children (0.1 ng/cm2) and adults (0.03 ng/cm2) than for the NC children (0.02 ng/cm2) and adults (<0.005 ng/cm2). 2,4-D was detected in more than 85% of the child and adult urine samples in both states. The median urinary 2,4-D concentration was more than twice as high for OH children compared to NC children (1.2 vs. 0.5 ng/ml, P<0.0001); however, the median concentration was identical at 0.7 ng/ml for both NC and OH adults. The intraclass correlation coefficient of reliability for an individual's urinary 2,4-D measurements, estimated from the unadjusted (0.31–0.62) and specific gravity-adjusted (0.37–0.73) values, were somewhat low for each group in this study. The variability in urinary 2,4-D measurements over the 48-h period for both children and adults in NC and OH suggests that several spot samples were needed to adequately assess these participants' exposures to 2,4-D in residential settings.
Conclusions: Investigators concluded results from this study showed that children and their adult caregivers in NC and OH were likely exposed to 2,4-D through several pathways at their homes. In addition, our findings suggest that the OH children might have been exposed to higher levels of 2,4-D through the dermal and nondietary routes of exposure than the NC children and the NC and OH adults.
Comments: This study was designed using the CTEPP (Children's Total Exposure To Persistent Pesticides and Other Persistent Organic Pollutants) multi-media approach. The key difference is was that in this case potential exposures were quantified for children who did not attend daycare and their adult caregiver's exposure was also evaluated. The investigators concluded 2,4-D urinary levels were low compared to established reference doses established by IRIS. This study was cited appropriately by the 2,4-D task force including in its description of possible body burden values that could be experienced in households (i.e., NC and OH families were sampled). Additionally, the reported house dust levels were compared by the 2,4-D task force to previous values defined by Nishioka (1996) cited by NRDC. Additionally, investigators indicated levels did not indicate a health concern compared to IRIS and existing BE values. The New York Departments of Environmental Conservation and Health both indicated that EPA should consider this study in its 2,4-D risk assessment. The values could be considered quantitatively in an assessment but the results would always be compared to those calculated using applicable existing methods (e.g., SOPs For Residential Exposure Assessment). Finally, this study was cited in the NRDC supplement to the petition. It was cited properly and NRDC indicated residues can be tracked into indoor environments. NRDC also indicated the Agency did not account for this exposure pathway. NRDC indicates the data from this study could be used quantitatively for risk assessment purposes. In fact, in its updated SOPs for Residential Exposure Assessment the Agency has proposed a method for completing such assessments. It could be incorporated as appropriate.

Title: Measuring Transport of Lawn-Applied Herbicide Acids from Turf to Home: Correlation of Dislodgeable 2,4-D Turf Residues with Carpet Dust and Carpet Surface Residues
Journal Citation: *Environ. Sci. Technol.*, 1996, *30* (11), pp 3313–3320
Author(s): Marcia G. Nishioka, Hazel M. Burkholder, Marielle C. Brinkman, and Sydney M. Gordon
Study Type: PL – Planned Exposure Monitoring
Methods Summary: Transport of lawn-applied herbicides into the home via walking over treated turf, defined here as track-in, was measured at five different times out to 1 week after application. At five discrete times after herbicide application, study participants walked through a treated turf plot and then over a section of carpet to simulate the transfer that might occur with a homeowner walking across turf (e.g., to a mailbox). Dislodgeable turf residues were collected prior to walking; dislodgeable carpet surface residues and carpet dust were collected after walking. Residues of turf-applied 2,4-D and dicamba were measured in carpet dust and on the carpet surface after track-in. The protocol was approved by the Battelle Human Subjects Review Committee in compliance with government regulations.
Results Summary: Both carpet dust levels and carpet surface dislodgeable residue levels were highly correlated with turf dislodgeable residue levels. Turf dislodgeable residues were 0.1−0.2% of turf application levels. Transfer of herbicides from turf to carpet dust was 3% of the turf dislodgeable residues. Transfer from turf to carpet surface was 0.3% of the turf dislodgeable residues. Herbicide 2,4-D was measured in the carpet dust of suburban homes at the 0.1−5 µg/g levels that are predicted by this efficiency of mass transfer. New tools used to collect these samples included the polyurethane foam roller dislodgeable residue sampler and the high-volume solid surface sampler.
Conclusions: Investigators concluded they expect to find 2,4-D in residential carpet dust up to 1 year after turf application at concentrations of approximately 0.5 µg/g and at carpet loadings of approximately 0.3 µg/m^2. These estimates are in agreement with the range of 2,4-D found in nine residential dust samples: 0.1-5 µg/g and 0.02−1.1 µg/m^2. These 2,4-D levels suggest track-in as a contributing transport mechanism, with the number of children correlated with both carpet loading (r^2 = 0.49; p = 0.04) and with dust concentration (r^2 = 0.35; p = 0.09) as measured in regression analyses. The investigators also indicated in a recently reported study, the urine from matched populations of children in two Arkansas towns was analyzed for 2,4-D and 2,4-D was detected in urine of 18% of the children from the town containing a 2,4-D manufacturing plant *and* was present in the urine of 23% of the children in the town with no manufacturing plant. The similarity of exposure rates and detection levels indicated an exposure source other than the manufacturing plant, though the source was unknown. Given the fact that children's hand-to-mouth activity promotes ingestion of contaminated carpet dust, we might assume that chronic indoor exposure will follow a lawn application and may result in measurable urinary levels. Certainly this assumption needs to be fully investigated by analysis of matched samples of house dust and urine from resident children prior to and after turf application. [Note: No mention was made concerning potential dietary or drinking water sources.]
Comments: This study evaluated 2,4-D residues in both carpet dust and as transferable residues on turf using the PUF roller method in a simulated carpet sampling approach. Both NRDC and Beyond Pesticides cited the conclusions by the investigators that 2,4-D persists indoors for up to one year. This value was not empirically derived through measurements but instead it was concluded by the investigators based on a series of assumptions and sampling which only occurred out to 1 week after an application of 2,4-D. The Agency does not concur with these conclusions by the investigators. The 2,4-D task force correctly indicates that the levels were calculated by the investigators and then compared them to empirically derived dust concentrations by Morgan (2008) which were lower than those calculated by the investigators. The 2,4-D task force did not discuss the temporal considerations associated with the interpretation in this comparison. Also, it should be noted that the Agency does not concur with the conclusions by the investigators because the assumptions do not account for any degradation processes nor is the short sampling timeframe of 1 week discussed as an uncertainty in the conclusions.

Title: Urinary Excretion of 2,4-D In Commercial Lawn Specialists
Journal Citation: Appl. Ind. Hyg. 3:119-121; 1986
Author(s): Yeary, R.A.
Study Type: PL – Planned Exposure Monitoring
Methods Summary: Occupational exposure to the herbicide 2,4-D was measured in 45 commercial lawn care specialists who had been spraying the herbicide on lawns on a daily basis, excluding Sundays, for a period of at least 3 weeks. Exposure was determined by collecting all urine voided during a 24 hour period, and analysis of 2,4-D was made by gas chromatography. The 24 hour urinary excretion of 2,4-D was considered to be a reasonable estimate of the daily absorbed dose. When handling concentrated product workers wore latex gloves, an apron or coveralls, goggles or faceshield, and rubber boots. When spraying dilute mixtures workers wore rubber boots, long pants, and short-sleeved shirts. Glove use was optional.
Results Summary: The urinary concentrations of 2,4-D ranged from non-detectable at a sensitivity of 0.01 ppm to 2.29 ppm with a median of 0.18 ppm. The quantity excreted on a body weight basis was 0.0032 ± 0.0010, 0.0063 ± 0.0018, 0.00035 ± 0.0005, and 0.00138 ± 0.0005 mg/kg respectively at 4 work locations.
Conclusions: Investigators concluded these values are considerably less than the WHO/FAO acceptable daily dietary intake of 0.3 mg/kg for 2,4-D residues in food.
Comments: This study evaluated worker exposure and also produced distributions of exposure in varied field locations. The use of the results of this study in the comments from the 2,4-D task force appears to be reasonable in that they reflect the specific conclusions described by the investigators. However, it should be noted that, as written, the 2,4-D task force summary of the study could be misleading because they indicate exposures did not exceed a level of 6 µg/kg/day which was really the maximum mean value from particular site with the highest average exposures. Exposures on a individual basis actually ranged about 10 times higher that this value as noted in the highest urinary concentration reported of 2.29 ppm. No environmental media sampling was completed. The direct exposure monitoring could be considered for quantitative use. However, PHED is currently used for routine assessments of this type rather than individual studies but some values for the monitored forestry operations are not addressed by PHED.

Appendix D:
Pet Incident Summaries

Title: Canine Exposure To Herbicide Treated Lawns and Urinary Excretion of 2,4-Dichlorophenoxyacetic Acid
Journal Citation: Cancer Epidemiology, Vol. 3, 233-237 (April/May 1994)
Author(s): Paula M. Reynolds, John S. Reif,1 Howard S. Ramsdell, and John D. Tessari
Study Type: Pet Exposure
Methods Summary: A recent study by Hayes *et aL* (Jı NatI. Cancer. Inst., *83:*1 226-i 231 , 1991) found an increased risk of malignant Iymphoma associated with exposure to 2,4-dichlorophenoxyacetic acid (2,4-D) in pet dogs. A study was conducted to determine the extent to which dogs absorb and excrete 2,4-D in urine after contact with treated lawns under natural conditions.
Results Summary: Among 44 dogs potentially exposed to 2,4-D-treated lawns an average of 10.9 days after application, 2,4-D concentrations greater than or equal to 10.0 µg/l were found in 33 dogs (75%) and concentrations of ≥50 µg/l were found in 17 (39%). Among 15 dogs with no known exposure to a 2,4-D-treated lawn in the previous 42 days, 4 (27%) had evidence of 2,4-D in urine, 1 at a concentration of ≥50 µg/l . The odds ratio for the association between exposure to a 2,4-D-treated lawn and the detection of ≥50 µg/l 2,4-D in urine was 8.8 (95% confidence interval, 1 .4-56.2). Dogs exposed to lawns treated within 7 days before urine collection were more than 50 times as likely to have 2,4-D at concentrations ≥50 µg/l than dogs with exposure to a lawn treated more than 1 week previously (odds ratio = 56.0; 95% confidence interval, 10.0-312.2). The highest mean concentration of 2,4-D in urine (21 3 mg/l) was found in dogs sampled within 2 days after application of the herbicide.
Conclusions: Investigators indicated these findings demonstrate that dogs living in and around residences with recent 2,4-D lawn treatment absorb measurable amounts of the herbicide for several days after application and thus may constitute a useful animal model for evaluation of the effects of herbicides on the induction of lymphoid cancer.
Comments: This study was considered in the comments of Beyond Pesticides and was cited appropriately except that the comparison made to previous data could not be verified. It should be noted that the NRDC petition does not make any mention of exposure to pets or via pets.

Title: Case-Control Study of Canine Malignant Lymphoma: Positive Association With Dog Owner's Use of 2,4-Dichlorophenoxyacetic acid herbicides
Journal Citation: J National Cancer Institute 1991; 83:1226-1231
Author(s): Hayes, H.M., Tarone, R.E., Cantor, K.P., Jessen, C.R., McCurnin, D.M., Richardson, R.C.
Study Type: Pet Exposure
Methods Summary: Hospital-based case control study of companion dogs which examined developing canine malignant lymphoma associated with the use of chemicals in and around the home. A self administered owner questionnaire and/or phone interviews were completed for 491 cases, 466 nontumor controls, and 479 tumor controls. Dogs diagnosed between January 1984 and February 1988 were identified from 3 veterinary teaching hospitals (MI, IN, CO). Tumor controls included only animals excluding diseases that could be related to chemical exposures. A total of 588 patients met eligibility criteria for malignant lymphoma and 491 responded (84% participation rate).
Results Summary: Dogs living in households where 2,4-D was applied more frequently had significantly higher odds ratios that control situations (Odds Ratio=1.3). Risk of canine malignant lymphoma rose to twofold excess with four or more yearly owner applications of 2,4-D. No difference was observed in the results based whether applications were completed by homeowners or professional applicators. A positive trend ($P<0.02$) was observed for the yearly number of applications. Other possible factors (e.g., flea product use, cigarette use, carpet presence) were evaluated but found not to be a factor. Known susceptible breeds (e.g., boxers, mastiffs) were also addressed. The investigators commented on how recall biases were addressed in the study but they acknowledged a major weakness is a lack of precise exposure data.
Conclusions: The investigators concluded that the study in consistent with results in humans where modest relationships between lymphoma incidence and 2,4-D use is observed. They also indicate the investigation began with an a priori hypothesis that exposure to 2,4-D and canine malignant lymphoma might be observed and that the findings support this supposition. They indicate further investigation is warranted.
Comments: No environmental or personal sampling was completed and the author s acknowledge significant uncertainty pertaining to a lack of exposure data. It is also not clear how germane the results of this study are given the timeframe over which this study was conducted compared to modern use patterns associated with 2,4-D. For example, certain manufacturing contaminants were eliminated from 2,4-D products so it could be plausible that exposures to older types of products could have occurred in the positive cases considered. The citation of this study by Beyond Pesticides reflects the conclusions of the investigators but it does not account for the uncertainty associated with the exposure predictors acknowledged by the investigators in this study. It should also be noted that in the follow-up 1995 paper to this investigators note significant levels of criticisms that are addressed in the subsequent document described below. Please refer to that summary of Hayes *et al* 1995 for additional information.

Title: On the Association Between Canine Malignant Lymphoma and Opportunity for Exposure to 2,4-Dichlorophenoxyacetic Acid
Journal Citation: Environmental Research 70, 119-125 (1995)
Author(s): Hayes, H.M., Tarone, R.E., Cantor, K.P
Study Type: Pet Exposure
Methods Summary: This article was written in response to specific criticisms leveled at the Hayes et al 1991 article described above. Several specific considerations were addressed including how control groups were treated, whether responses were via mail or phone, potential hospital selection bias, difficulties associated with exposure classification including frequency of use, and knowledge related to the specific use of 2,4-D products.
Results Summary: The authors addressed each of the applicable criticisms. The results of Hayes et al 1991 were supported in their analysis. The authors clarified the conclusions to better address uncertainty issues (see below).
Conclusions: Selection and recall bias can never be eliminated and every attempt was made to do so. In this additional analysis the use of a lawn service and its implications on the results was further examined and it does not appear to impact results. The investigators also commented on the magnitude of the associated odds ratios. The investigators also acknowledged that the reported values could be explained by bias or confounding factors. It was also indicated that the results should be interpreted with caution because of low exposures. The investigators also concur with several commenters that the study does not prove that 2,4-D exposure in a home environment is a cause of malignant lymphoma in companion dogs. They again indicate it does warrant further research on the issue.
Comments: The authors addressed several specific criticisms of the 1991 study in an appropriate manner. The citation of this study in the Beyond Pesticides comments to the NRDC petition is correct. However, it is somewhat misleading because they do not reiterate the level of uncertainty voiced by the investigators in their comments about the applicability of the results and the call in both Hayes 1991 and 1995 for additional research to investigate this relatively weak relationship between 2,4-D exposure and canine malignant lymphoma.

Appendix E:
Meta Information

Title: Regenerative 21st Century Farming: A Solution to Global Warming
Journal Citation: http://www.ifoam.org/growing_organic/1_arguments_for_oa/environmental_benefits/pdfs/Rodale_Research_Paper_Regenerative_Agriculture.pdf
Author(s): Dr. Timothy J. LaSalle, CEO; Dr. Paul Hepperly, Director of Research and Fulbright Scholar; Rodale Institute ©
Study Type: Sustainable Agricultural Systems Analysis
Methods Summary: This paper discusses organic alternatives to 2,4-D.
Results Summary: Organic alternatives to 2,4-D are illustrated.
Conclusions: Investigators conclude there are viable organic alternatives to 2,4-D.
Comments: This study was cited properly in the Beyond Pesticides Comment. It does not discuss human health aspects of exposure to 2,4-D but only discusses herbicide use as it relates to conventional large scale farming practices.

Title: Handbook of Successful Ecological Lawn Care
Journal Citation: Edaphic Press 1995, ISBN 9780963605313
Author(s): Paul D. Sachs
Study Type: Sustainable Agricultural Systems Analysis
Methods Summary: This paper discusses organic alternatives to 2,4-D.
Results Summary: Organic alternatives to 2,4-D are illustrated.
Conclusions: Investigators conclude there are viable organic alternatives to 2,4-D.
Comments: This study was cited properly in the Beyond Pesticides Comment. It does not discuss human health aspects of exposure to 2,4-D but only discusses organic alternatives herbicide use in lawncare.

Title: The Herbicide Resistance Action Committee (HRAC). 2005. Classification of Herbicides According to Mode of Action
Journal Citation: http://www.hracglobal.com/Publications/ClassificationofHerbicideModeofAction/tabid/222/Default.aspx
Author(s): Dr. Hubert Menne , Bayer CropScience AG Frankfurt Germany
Study Type: Rating System to categorize herbicide mode of action
Methods Summary: This paper discusses herbicide mode of action
Results Summary: This paper discusses herbicide mode of action
Conclusions: This paper discusses herbicide mode of action
Comments: This study was cited properly in the Beyond Pesticides Comment in that it does describe how herbicide modes of action can be categorized. It does not discuss human health aspects of exposure to 2,4-D or the need to conduct a cumulative human health risk assessment that Beyond Pesticides raises partially based on this information.

[i] NAFTA Dermal Absorption Group Position Paper on Use of In Vitro Dermal Absorption Data in Risk Assessment (2009).