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

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

Date: **June 29, 2015**

SUBJECT: **EDSP Weight of Evidence Conclusions on the Tier 1 Screening Assays for the List 1 Chemicals**

PC Code: See table, Attachment A
Decision No.: NA
Petition No.: NA
Risk Assessment Type: NA
TXR No.: See table, Attachment A
MRID No.: NA

DP Barcode: NA
Registration No.: NA
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EPA has completed its Weight of Evidence (WoE) assessment evaluating results of the Endocrine Screening Program (EDSP) Tier 1 screening assays for the List 1 chemicals. The WoE documents for the 52 chemicals are listed in Attachment A along with the chemical and report identifiers.

Attachment A. EDSP List 1 Chemicals

Chemical Name	PC Code	TXR Number
2,4-D	030001	0057151
Abamectin	122804	0057152
Acephate	103301	0057153
Acetone	044101	0057154
Atrazine	080803	0057155
Benfluralin	084301	0057156
Bifenthrin	128825	0057157
Captan	081301	0057158
Carbaryl	056801	0057159
Carbofuran	090601	0057160
Chlorothalonil	081901	0057161
Chlorpyrifos	059101	0057162
Cyfluthrin	128831	0057163
Cypermethrin	109702	0057164
DCPA	078701	0057165
Diazinon	057801	0057166
Dichlobenil	027401	0057167
Dimethoate	035001	0057168
EPTC	041401	0057169
Esfenvalerate	109303	0057170
Ethoprop	041101	0057171
Fenbutatin-Oxide	104601	0057172
Flutolanil	128975	0057173
Folpet	081601	0057174
Glyphosate	417300	0057175
Imidacloprid	129099	0057176
Iprodione	109801	0057177
Isophorone	847401	0057178
Linuron	035506	0057179
Malathion	057701	0057180
Metalaxyl	113501	0057181
Methomyl	090301	0057182
Metolachlor	108801	0057183
Metribuzin	101101	0057184
MGK-264	057001	0057185
Myclobutanil	128857	0057186
Norflurazon	105801	0057150
o-Phenylphenol	064103	0057146
Oxamyl	103801	0057142
PCNB	056502	0057138
Permethrin	109701	0057149
Phosmet	059201	0057145
Piperonyl Butoxide	067501	0057141
Pronamide	101701	0057137
Propargite	097601	0057148
Propiconazole	122101	0057144
Pyriproxyfen	129032	0057140
Simazine	080807	0057136
Tebuconazole	128997	0057143
Tetrachlorvinphos (TCVP)	083701	0057147
Triadimefon	109901	0057139
Trifluralin	036101	0057135

EDSP: WEIGHT OF EVIDENCE ANALYSIS OF POTENTIAL INTERACTION WITH ESTROGEN, ANDROGEN OR THYROID PATHWAYS

CHEMICAL: 2, 4-DICHLOROPHENOXY ACETIC ACID (2,4-D)

OFFICE OF PESTICIDE PROGRAMS

OFFICE OF SCIENCE COORDINATION AND POLICY

U.S. ENVIRONMENTAL PROTECTION AGENCY

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Abbreviations

Abbreviation	Terminology
A	Androgen (hormonal pathway)
ADME	Absorption, Distribution, Metabolism, Excretion
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AMA	Amphibian Metamorphosis Assay
ARTA	Androgen Receptor Transcriptional Activation
AST	Aspartate Aminotransferase
ANOVA	Analysis of Variance
AOP	Adverse Outcome Pathway
AR	Androgen Receptor
B_{max}	Binding at maximum
BROD	Benzyloxyresorufin-O-dealkylase
BUN	Blood Urea Nitrogen
CAR	Constitutive Androstane Receptor
CFR	Code of Federal Regulations
CG	Cowper's Gland
ChE	Cholinesterase
ChEI	Cholinesterase inhibition
CMC	Carboxymethyl cellulose
CTA	Comparative Thyroid Assay
CV	Coefficient of Variation
CYP	Cytochrome 450
DER	Data Evaluation Record
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DP	Dorsolateral Prostrate
E	Estrogen hormonal pathway
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EDRT	Endocrine Disruptor Review Team
EDSP	Endocrine Disruptor Screening Program
EE	Ethinyl Estradiol
ELISA	Enzyme Linked Immunosorbent Assay
EOGRTS	Extended One-Generation Reproductive Toxicity Study (Rat)
ER	Estrogen Receptor
EROD	Ethoxyresorufin-O-dealkylase (or deethylase)
ERTA	Estrogen Receptor Transcriptional Activation
EtOH	Ethanol
F	Female
F1	First filial generation

Abbreviation	Terminology
F2	Second filial generation
Fcd	Fecundity
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FOB	Field Observation Battery
FQPA	Food Quality Protection Act
Frt	Fertility
FSH	Follicle Stimulating Hormone
FSTRA	Fish Short-Term Reproduction Assay
FT	Flutamide
GD	Gestation Day
GGT	Gamma-glutamyl Transpeptidase
GnRH	Gonadotropin-releasing hormone
GP	Glans Penis
GSI	Gonado-Somatic Index
H	High
HLL	Hind Limb Length
HPG	Hypothalamic-Pituitary-Gonadal Axis
HPLC/MS/MS	High Pressure Liquid Chromatography/Mass Spectroscopy
HPT	Hypothalamic-Pituitary-Thyroidal Axis
I	Inadequate
IC50	Inhibitory Concentration at 50% of response
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative
K_d	Equilibrium Dissociation Constant
K_{ow}	Octanol/Water Partition Coefficient
L	Low dose
LABC	Levator Ani-Bulbocavernosus
LAGDA	Larval Amphibian Growth and Development Assay
LC50	Lethal Concentration in 50% of test organisms
LD	Lactation Day
LH	Luteinizing hormone
LOAEC	Lowest Observed Adverse Effect Concentration
LOAEL	Lowest Observed Adverse Effect Level
LOQ	Limit of Quantitation
M	Male
MDL	Minimum Detection Level
MEOGRT	Medaka Extended One Generation Reproduction Test
MH	Medium high
ML	Medium low
MoA	Mode of Action
MOE	Margin of Exposure
MRID	Master Record Identifier
MROD	Methoxyresorufin-O-dealkylase
MTC	Maximum Tolerated Concentration

Abbreviation	Terminology
MTD	Maximum Tolerated Dose
N	Negative
NE	Not examined/evaluated
NF stage	Nieuwkoop and Faber's Staging Atlas
NOAEC	No Observed Adverse Effect Concentration
NOAEL	No Observed Adverse Effect Level
NS	Not Statistically Significant
NR	Not Reported
OCSPP	Office of Chemical Safety Pollution and Prevention
OECD	Organization for Economic Co-Operation and Development
OPP	Office of Pesticide Programs
ORD	Office of Research and Development
OSCP	Office of Science Coordination and Policy
OSRI	Other Scientifically Relevant Information
P	Positive
P	Parental generation
PC	Positive Control
PC_{10}	Positive Control at 10% of response
PC_{50}	Positive Control at 50% of response
PND	Post-Natal Day
POD	Point of Departure
PPS	Preputial Separation
PROD	Pentaoxyresorufin-O-dealkylase (or depentylase)
PXR	Pregnane X receptor
QC	Quality Control
RBA	Relative Binding Affinity
RBC	Red Blood Cells
RfD	Reference Dose
RPC_{max}	Relative to Positive Control at maximum
SAP	Scientific Advisory Panel
SC	Solvent Control
s.c	Subcutaneous
SDH	Sorbitol dehydrogenase
SDWA	Safe Drinking Water Act
SEP	Standard Evaluation Procedure
SD	Standard Deviation or Sprague-Dawley
SVL	Snout-to-Vent Length
SV	Seminal Vesicles
T	Thyroid (hormonal pathway)
T1WoERC	EDSP Tier 1 Weight of Evidence Review Committee
T3	Triiodothyronine
T4	Thyroxine (tetraiodothyronine)

Abbreviation	Terminology
TP	Testosterone Propionate
TR	Thyroid Receptor
TSH	Thyroid Stimulating Hormone
UDPGT	Uridine Diphosphate Glucuronyltransferase (also known as UGT)
VC	Vehicle Control
VO	Vaginal Opening
VP	Ventral Prostate
VTG	Vitellogenin
WoE	Weight-of-Evidence

Executive Summary

The Endocrine Disruptor Screening Programs (EDSP) Tier 1 assay battery is designed to provide the necessary empirical data to evaluate the potential of chemicals to interact with the estrogen (E), androgen (A) or thyroid (T) signaling pathways. This interaction includes agonism and antagonism at the estrogen and androgen receptors, altered steroidogenesis, as well as hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary thyroid (HPT) perturbations. In addition to the available Tier 1 assay data, other scientifically relevant information (OSRI), including general toxicity data and open literature studies of sufficient quality were considered in this weight of evidence (WoE) assessment.

In determining whether 2, 4 dichlorophenoxy acetic acid (2,4-D) interacts with E, A or T hormone pathways, the number and type of effects induced, the magnitude and pattern of responses observed across studies, taxa and sexes were considered. Additionally, the conditions under which effects occur were considered, in particular, whether or not endocrine-related responses occurred at dose(s) that also resulted in general systemic toxicity or overt toxicity.

On October 22, 2014, the EDSP Tier 1 Assay Weight of Evidence Review Committee (T1WoERC) of the Office of Pesticide Programs (OPP) and the Office of Science Coordination and Policy (OSCP) conducted a weight-of-evidence (WoE) analysis of the potential interaction of 2, 4-Dichlorophenoxy acetic acid (2,4-D) with the E, A or T signaling pathways. The T1WoERC conclusions from the WoE evaluation in this report are presented by pathway (E, A and then T) beginning with the results of the Tier 1 *in vitro* assays followed by *in vivo* mammalian and wildlife results, then the results of the cited OSRI for mammalian and wildlife studies (40 CFR Part 158 and literature).

There was no convincing evidence of potential interaction of 2, 4-D with the estrogen pathway. The Tier 1 or OSRI *in vitro* assays were negative with the exception of a 20% increase in estradiol production in the in the steroidogenesis assay at 100 μM. The requirements for the EDSP Tier 1 *in vivo* mammalian uterotrophic and female pubertal assays were satisfied by the extended one-generation reproduction toxicity test (EOGRT) in rats (equivalent to the EDSP Tier 2 study) considered as OSRI. No estrogen-related effects were observed in this study or in any of the mammalian Part 158 toxicity studies in the absence of overt toxicity. No effects were observed in the Part 158 bobwhite quail reproduction study. In the fish short-term reproduction assay (FSTRA), fecundity was decreased (34%) at a concentration of 96.5 mg a.i./L, and while an increase in the number of female ovaries at stage 2 (compared to stage 3 or 4) was observed for the 2,4-D treatment groups compared to the negative control, the incident rates were not dose-responsive, and there were no other effects observed in this study.

There was no convincing evidence of interaction of 2, 4-D with the androgen pathway. The Tier 1 and OSRI *in vitro* assays were negative. The requirements for the Tier 1 *in vivo* mammalian Hershberger and male pubertal assays were satisfied by the extended one-generation reproduction test (EOGRT) in rats considered as OSRI. There were no androgen-related effects observed in this study. Additionally, there were no treatment-related effects in the FSTRA or avian reproduction studies, and there were no effects on the male reproductive parameters in the Part 158 mammalian studies in the absence of overt toxicity.

In the amphibian metamorphosis assay (AMA), while there was a 15% decrease in HLL (at the high test concentration of 113 mg/L), there were no histological effects on the thyroid or effects on developmental stage. Some thyroid-related effects were seen in the mammalian Part 158 studies. The effects were only seen in the presence of overt toxicity or at doses that exceeded the threshold for renal clearance of 2, 4-D in the rat, resulting in systemic toxicity. In the EOGRT, there were no treatment-related thyroid effects in males or females. Therefore, collectively the data across the Tier 1 and Part 158 data do not demonstrated convincing evidence for potential interaction with the thyroid pathway.

Based on weight of evidence considerations, additional EDSP Tier 2 ecological testing is not recommended. It is noted that the agency already has a mammalian toxicity study (extended one-generation reproduction test, EOGRT) that is equivalent to the EDSP Tier 2 study.

I. Introduction

The Endocrine Disruptor Screening Programs (EDSP) Tier 1 assay battery is designed to provide the necessary empirical data to evaluate the potential of chemicals to interact with the estrogen (E), androgen (A) or thyroid (T) signaling pathways. This interaction includes agonism and antagonism at the estrogen and androgen receptors, altered steroidogenesis, as well as hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary thyroid (HPT) perturbations. In addition to the available Tier 1 data, other scientifically relevant information (OSRI), including general toxicity data and open literature studies of sufficient quality were considered in this weight of evidence (WoE) assessment.

In determining whether 2,4 D interacts with E, A or T hormone pathways, the number and type of effects induced, the magnitude and pattern of responses observed across studies, taxa and sexes were considered. Additionally, the conditions under which effects occur were considered, in particular, whether or not endocrine-related responses occurred at dose(s) that also resulted in general systemic toxicity or overt toxicity.

On October 22, 2014, the EDSP Tier 1 Assay Weight of Evidence Review Committee (T1WoERC) of the Office of Pesticide Programs (OPP) and the Office of Science Coordination and Policy (OSCP) conducted a weight-of-evidence (WOE) analysis of the potential interaction of 2, 4-D with the E, A or T signaling pathways. The T1WoERC conclusions from the WoE evaluation in this report are presented by pathway (E, A and then T) beginning with the results of the Tier 1 *in vitro* assays followed by *in vivo* mammalian and wildlife results, then the results of the cited OSRI for mammalian and wildlife studies (40 CFR Part 158 and literature).

2, 4-D has a water solubility of 569 mg/L at 20°C and a octanol/water partition coefficient (log Kow) of 0.177. It has a vapor pressure of 1.4×10^{-7} mm Hg at 25°C and, therefore, volatilization is not expected to significantly contribute to the dissipation of 2, 4-D in the environment. The log Kow suggests that the potential for bioconcentration in aquatic organisms is low. The degradation of 2,4-D acid appears to be dependent on oxidative microbially-related mineralization in the terrestrial environment and photodegradation in water. Results from laboratory studies indicate rapid to moderately rapid degradation under aerobic soil conditions with half-lives ranging from 1.4 days to 12.4 days and a median half-life of 2.9 days. 2,4-D acid was stable to photodegradation in soil. 2,4-D acid was not stable in aerobic aquatic environments (t1/2=15.0 days) but was moderately persistent to persistent (t1/2=28.5 to 333 days) in anaerobic aquatic laboratory studies.

The available information considered in determining the potential interaction of 2,4-D with the E, A, or T pathways include submitted EDSP Tier 1 assays and/or other scientifically relevant information (OSRI) such as general toxicity studies and other published articles. These data are summarized in Sections III.A through III.C. An analysis of the data submitted to the agency,

using the WoE approach outlined by the Agency (USEPA, 2011), is presented in Section IV. The EDSP Tier 2 study recommendations are presented in Section V.

II. Sources of Scientific Data and Technical Information

A. EDSP Tier 1 Screening Assays

The Tier 1 assays and/or other scientifically relevant information (OSRI) submitted to satisfy the agency's test order are shown below in Table 1. Executive Summaries are presented in Appendix 1.

B. Other Scientifically Relevant Information (OSRI)

In response to the Agency's Test Orders, data believed to be relevant to one or more of the Tier 1 assays were submitted as OSRI by the Test Order recipients and/or the public. This included studies published in the open literature and/or data submitted to support pesticide registration (*e.g.*, Part 158 guideline studies). The Agency's review of the initial OSRI is provided in the Report of the Endocrine Disruptor Review Team on 2, 4-D (USEPA, 2010). Since then, the Agency has also conducted a more recent search of available scientific literature for any additional relevant information. Summaries of the available OSRI are presented in Appendix 2. Additionally, literature/studies considered but not utilized for the WoE analysis are listed in Appendix 3.

III. Weight of Evidence (WoE) Evaluation

The principles, criteria and approach used in the WoE determination on the potential of a substance to interact with endocrine-related processes (*i.e.*, E, A, or T hormonal pathways) were as described in the WoE guidance document (USEPA, 2011) and presented at the 2013 FIFRA Scientific Advisory Panel (SAP) (USEPA, 2013). The weight of evidence process identifies how the individual lines of evidence are assembled and integrated along two concepts (*i.e.*, complementarity and redundancy) within the conceptual framework of an adverse outcome pathway). Broadly, there are four main steps outlined in the guidance which provide the foundation for WoE evaluations. The first step is to evaluate the individual studies for their scientific quality and relevance in evaluating potential endocrine interaction(s). The second step is to integrate the data along different levels of biological organization while examining the extent of complementarity (*i.e.*, the concordance of endpoints within an assay that measures multiple endpoints) and redundancy (*i.e.*, the concordance of endpoints/responses across assays) in the observed responses across these different levels of biological organization. The third step is to characterize the main lines of evidence as well any conclusions. Finally, the last step is to evaluate whether additional testing is needed based on the evidence and conclusions described above.

As mentioned, the first step is to assemble and evaluate the available scientific data. Data for the EDSP Tier 1 WoE evaluation falls into one of two categories: 1) EDSP Tier 1 data, or 2) other scientifically relevant information (OSRI). The EDSP Tier 1 data include a battery of 11 assays consisting of *in vitro* and mammalian and wildlife *in vivo* assays. The Tier 1 assays were designed specifically to evaluate a number of key biological events including potential effects on receptor binding (estrogen and androgen agonist and antagonist), steroidogenesis, and other effects on the HPG and HPT axes. OSRI may include published literature studies as well as studies conducted under USEPA (often referred to as Part 158 data) or OECD guidelines submitted in support of pesticide registrations. Each study is evaluated for scientific quality and relevance for informing interactions with the E, A or T pathway. Additionally, the consistency of the responses in the individual study is evaluated. For the Tier 1 *in vivo* assays, often multiple endpoints are measured in each assay.

Evaluation of the potential confounding effects of overt toxicity in the study, as well as the relative degree of diagnostic utility of a specific endpoint for discerning whether or not the chemical has interacted with the endocrine system, are considered. The collective response of the individual endpoints, as well as the conditions under which they were expressed, are considered when evaluating an overall indication of potential interaction as measured by the study.

The second step in this WoE process is to formulate hypotheses and integrate the available data along different levels of biological organization. Two keys elements in the integration of data, as well as characterizing the extent to which the available data support a hypothesis that a chemical has the potential to interact E, A or T pathways, are the concepts of complementarity and redundancy. These two concepts provide a basis for considering the plausibility, coherence, strength, and consistency of the body of evidence. The current EDSP Tier 1 screening assays are meant to evaluate whether or not a chemical can interact with E, A, and T consisting of different levels of biological organization from a molecular initiating event, such as receptor binding, through potential adverse effects in apical endpoints such as sexual development and fecundity at the whole organism level. The extent of expression of responses at higher levels of biological organization can indirectly provide information on potential compensatory capabilities of an individual organism.

After the data have been assembled and integrated, the third step is to characterize the main lines of evidence along with the conclusions; this characterization involves three components. The first component is whether the data provide relevant, robust and consistent evidence in terms of complementarity and redundancy as well as biological plausibility. Second, is at what level of biological organization were the responses observed and whether organisms exhibit compensatory responses at higher levels of biological organization? Finally, an evaluation of conditions where the responses occur including consideration of whether the responses were observed in the presence of overt or systemic toxicity? The presence of overt and/or systemic

toxicity introduces uncertainty in the ability to distinguish effects specifically related to an endocrine-related effect from a non-endocrine toxic response.

This uncertainty in distinguishing whether the responses were endocrine-related was discussed at the FIFRA SAP meeting that evaluated scientific issues associated with the WoE evaluation of the EDSP Tier 1 screening process. In October 2013, the SAP stated that , *"In summary, the Panel agreed that little, if any, weight should be placed on signs of endocrine disruption in the presence of overt toxicity. All effects in endocrine sensitive tissues should be evaluated in terms of primary interactions with the endocrine system vs. secondary effects related to toxicity in non-endocrine organs or overall disruptions in homeostasis"* (USEPA, 2013).

For these WoE analyses, overt toxicity was generally defined in accordance with EPA's current approach as used by OPP in reviewing 40 CFR Part 158 studies for both human and ecological risk assessments. Specifically, in these analyses, the effects that EPA considered to be potential evidence of overt toxicity included, but were not limited to: mortality; clinical signs such as tremors, ataxia and abnormal swimming (fish and aquatic-phase amphibians); and body weight decreases of ≥10% in mammals. Additionally, other effects including morphological (*e.g*., organ weights/histopathology), biochemical (*e.g*., blood chemistry), and other clinical signs (*e.g*., lethargy) were also considered when evaluating overt toxicity, especially if the effects were extreme. In some instances, one parameter (*i.e*., death or >10% decrease in mammalian body weight) was sufficient to consider a dose/concentration to be overtly toxic. However, in other instances, more than one parameter was needed to determine overt toxicity. For example, in the FSTRA, generally, body weight decreases were considered along with other responses when assessing potential overt toxicity. Additionally, effects which were considered to be signs of systemic toxicity were also captured and these effects were generally considered as less severe forms of toxicity (*e.g.*, changes in organ weights or blood chemistry). The circumstances for which a dose/concentration was considered overtly toxic for a particular study are described in Section IV.A.

In summary, EPA considers multiple lines of evidence in including the observed responses in the Tier 1 assays and OSRI in the context of a chemical's physical/chemical properties and its known modes of action in its overall characterization of a chemical's potential to interact with the E, A or T pathway. Adequately addressing the three main components described above is fundamental to the WoE process and in determining whether additional data are needed. In addition to characterizing the WoE, reviewers also considered: 1) uncertainties and their potential impact to conclusions; 2) discussion of key studies; 3) description of inconsistent or conflicting data; 4) overall strength of evidence supporting a conclusion; and, 5) what, if any, additional data are needed and why. Assessing the need for additional data is based on a case-by-case analysis which took all available toxicity data into account.

The WoE approach involved consideration of data (i.e., lines of evidence) from the EDSP Tier 1 assays and OSRI which are depicted in **Tables 2 - 4**. *These tables contain data that are considered scientifically and biologically relevant with regard to a treatment-related effect which supports a conclusion of whether a substance has the potential to interact with the E, A, or T pathway. Effects that occurred in the presence of overt toxicity are discussed in the text for each respective pathway (E, A or T) but are not reported in the table for E, A or T.*

A. EDSP Tier 1 Screening Assays

The Tier 1 assays submitted in response to the agency's test order for 2, 4-D are shown below in **Table 1**.

Table 1. Tier 1 Assays for 2, 4-D.

Tier 1 Assays	Test Guideline	Test Order Status
ER Binding Assay (Rat uterine cytosol)	OCSPP 890.1250	Requirement Satisfied (MRID 48614303)
ERα Transcriptional Activation Assay (Human cell line HeLa 9903)	OCSPP 890.1300; OECD 455	Requirement Satisfied (MRID 48614304)
AR Binding Assay (Rat prostate cytosol)	OCSPP 890.1150	Requirement Satisfied (MRID 48614301)
Steroidogenesis Assay (Human cell line H295R)	OCSPP 890.1550; OECD 456	Requirement Satisfied (MRID 48614305)
Aromatase Assay (human recombinant microsomes)	OCSPP 890.1200	Requirement Satisfied (MRID 48614302)
Uterotrophic Assay (Rat)	OCSPP 890.1600; OECD 440	Requirement satisfied by the Extended One-Generation Reproduction Toxicity (EOGRT) Study (MRID 47972101)
Hershberger Assay (Rat)	OCSPP 890.1400; OECD 441	Requirement satisfied by EOGRT (MRID 47972101)
Pubertal Female Assay (Rat)	OCSPP 890.1450	Requirement satisfied by EOGRT (MRID 47972101)
Pubertal Male Assay (Rat)	OCSPP 890.1500	Requirement satisfied by EOGRT (MRID 47972101)
Fish Short-term Reproduction Assay	OCSPP 890.1350; OECD 229	Requirement Satisfied (MRID 48317007)
Amphibian Metamorphosis Assay (Frog)	OCSPP 890.1100; OECD 231	Requirement Satisfied MRID 48317002)

B. Effects on Hypothalamic-Pituitary-Gonadal (HPG) Axis

1. Effects on Estrogen Pathway

The potential for 2, 4-D to interact with the estrogen pathway is summarized in **Table 2.** The various targets of the estrogen pathway across the relevant Tier 1 assays are delineated so as to facilitate determination of potential for estrogenic, anti-estrogenicor HPG axis effects. This table also includes HPG-relevant findings from data evaluated as OSRI. *Effects that occurred in the presence of overt toxicity are discussed in the text but are not reported in the table and not considered further in the WOE assessment.*

2, 4-D was negative in the Tier 1 *in vitro* ER binding and ERTA assays. Literature studies considered as OSRI also showed no evidence for potential ER-related binding (Blair *et al*, 2002) or transactivation (Orton *et al*., 2009; Hurst and Sheahan, 2003; Kojima *et al*., 2004; Lemaire *et al*., 2006; and Sun *et al*., 2012). Additionally, Soto *et al*. (1995) reported that 2,4-D did not induce cell proliferation in ER-responsive MCF7 cells.

2, 4-D was negative for aromatase inhibition in the Tier 1 aromatase assay. In the *in vitro* Tier 1 steroidogenesis assay, 2,4-D treatment resulted in a 20% increase ($p \leq 0.05$) in estradiol production in all three runs at the highest concentration tested (10^{-4} M). However, in an OSRI *in vitro* study, Orton *et al*., (2009) reported no 2,4-D-related effects (up to 62.5 µM) in a frog oocyte assay evaluating ovarian steroidogenesis.

The *in vivo* uterotrophic and female pubertal assays were not conducted because these requirements were satisfied by an extended one-generation rat reproductive test (EOGRT) considered as OSRI which is equivalent to the EDSP Tier 2 mammalian study.

In the EORGT, although changes were noted in some estrogen-related endpoints, the effects were non-significant (NS) and /or non-dose-related, thereby there were no treatment-related estrogen effects. For example, increased absolute and relative uterine weights were observed at the adult P1 females (17%) and F1 offspring Set 1a (31 and 32%, respectively) at 600 ppm; however, these responses did not attain statistical significance. Non-statistically significant increases in absolute and relative uterine weights were also seen in the Set 3 F1 offspring at 300 ppm (10% absolute and relative) and 600 ppm (10% absolute, 11 % relative). Increased ovarian weight (↑9%) was observed in the 600 ppm F1 Set 1a females, although statistical significance was not attained. In addition, non-significant decreases (9-10%) in pituitary weight were observed in the F1 Set 1a and 3 female offspring. There were no treatment-related effects on VO, anogenital distance, estrous cyclicity, gonadal histopathology or reproductive indices in the study.

In the other part 158 mammalian studies, the only potential -related effects observed occurred in the presence of overt toxicity. These effects included decreased ovarian weights in the subchronic (42%) and chronic/carcinogenicity (41%; NS) in the rat, decreased pituitary weight in the subchronic rat, and decreased F1b litter size/viability in the rat 2-generation reproduction study.

In the FSTRA, there was a 34% decrease in fecundity at the high treatment level (96.5 mg/L). Plasma VTG concentrations and GSI in females were not affected. While not concentration-dependent, an increase in the number of female ovaries at stage 2 (compared to stage 3 or 4) for the 2,4-D treatment groups compared to the negative control was evident.

There were no effects on growth or reproduction up to 962 mg ae/kg-diet in a Part 158 reproduction study with bobwhite quail and 2,4-D acid. In a Part 158 fathead minnow early life stage study with 2,4-D dimethylamine salt larval length was affected with a NOAEC of 14.2 mg ae/L (17.1 mg a.i./L); survival was also reduced at higher concentrations. In another early life-cycle study with fathead minnow and 2,-4-D ethylhexyl ester, larval survival was affected at 0.22 mg a.i./L (NOAEC=0.0792 mg a.i./L), however, high variability in test concentrations was observed in this study.

Table 2. Estrogenic/Anti-Estrogenic Pathway for 2,4-D

Lines of Evidence Indicating Potential Interaction with the Estrogenic/Anti-Estrogenic Pathway for 2,4-D[1]															
Study Type/ Literature Citation	ER Binding	ER Activation	Steroidogenesis	Sex Steroid Hormone	Uterine Weight	Ovarian Weight/GSI	Ovarian/Gonad Staging and Histopathology	Pituitary Weight	Estrous Cyclicity	Age & Weight at VO	2° Sex Characteristics	Fertility(Frt) /Fecundity (Fcd)	Vitellogenin (VTG)	Systemic Toxicity Observed[2]	Overt Toxicity Observed[3]
EDSP Tier 1															
ER Binding (MRID 48614303)	N														
ERTA (MRID 48614304)		N													
Aromatase (MRID 48614302)			N												
Steroidogenesis (MRID 48614305)			P[4]												
Uterotrophic	Requirement satisfied by OSRI (MRID 47972101); see below.														
Female Pubertal Rat	Requirement satisfied by OSRI (MRID 47972101); see below.														
FSTRA (MRID 48317007)				NE		N	N				N	Fcd: ↓34% (H)	N		N

Table 2. Estrogenic/Anti-Estrogenic Pathway for 2,4-D

Lines of Evidence Indicating Potential Interaction with the Estrogenic/Anti-Estrogenic Pathway for 2,4-D[1]															
Study Type/ Literature Citation	ER Binding	ER Activation	Steroidogenesis	Sex Steroid Hormone	Uterine Weight	Ovarian Weight/GSI	Ovarian/Gonad Staging and Histopathology	Pituitary Weight	Estrous Cyclicity	Age & Weight at VO	2° Sex Characteristics	Fertility(Frt) /Fecundity (Fcd)	Vitellogenin (VTG)	Systemic Toxicity Observed[2]	Overt Toxicity Observed[3]
OSRI															
ER Binding (Blair *et al*., 2000)	N														
Recombinant (hER) yeast-based assay/Frog ovulation assay (Orton *et al*., 2009)		N	N[5]												
Recombinant (hER) yeast-based assay (Hurst and Sheahan, 2003)		N													
ERTA (Kojima, *et al*., 2004)		N													
ERTA (Lemaire *et al*., 2006)		N[6]													
ERTA (Sun *et al*., 2012)		N													
Cell proliferation assay (Soto *et al*., 1995)		N													
Subchronic toxicity (Rat; MRIDs 00101599 and 00102451)					NE	NE	N	NE						N	N
Subchronic toxicity (Rat; MRID 41991501)					NE	N	N	N						X (H)	X (H)

Table 2. Estrogenic/Anti-Estrogenic Pathway for 2,4-D

Lines of Evidence Indicating Potential Interaction with the Estrogenic/Anti-Estrogenic Pathway for 2,4-D[1]															
Study Type/ Literature Citation	ER Binding	ER Activation	Steroidogenesis	Sex Steroid Hormone	Uterine Weight	Ovarian Weight/GSI	Ovarian/Gonad Staging and Histopathology	Pituitary Weight	Estrous Cyclicity	Age & Weight at VO	2° Sex Characteristics	Fertility(Frt) /Fecundity (Fcd)	Vitellogenin (VTG)	Systemic Toxicity Observed[2]	Overt Toxicity Observed[3]
Subchronic toxicity (Mouse; MRID 41991502)					NE	N	N	N						N	N
Subchronic inhalation toxicity (Rat; MRID 47398701)					N	N	N	NE						N	N
Developmental toxicity (Rat; MRID 00130407)					N							N		N	N
Developmental toxicity (Rabbit; MRID 41747601)					N							N		N	X (H)
Extended one-generation reproduction (Rat, MRID 47972101)[7]					N	N	N	N	N	N		N		N	N
Two-generation reproduction (Rat; MRIDs 00150557 and 00163996)					NE	NE	NE	NE	NE			N		X (X)	X (H)
Chronic/ Carcinogenicity (Rat; MRID 43612001)					NE	N	N	NE						X (M,H)	X (M, H)
Carcinogenicity (Mouse; MRIDs 43879801 and 43597201)					NE	NE	N	NE						N	N

Table 2. Estrogenic/Anti-Estrogenic Pathway for 2,4-D

Lines of Evidence Indicating Potential Interaction with the Estrogenic/Anti-Estrogenic Pathway for 2,4-D[1]														
Study Type/ Literature Citation	ER Binding	ER Activation	Steroidogenesis	Sex Steroid Hormone	Uterine Weight	Ovarian Weight/GSI	Ovarian/Gonad Staging and Histopathology	Pituitary Weight	Estrous Cyclicity	Age & Weight at VO	2° Sex Characteristics	Fertility(Frt) /Fecundity (Fcd)	Vitellogenin (VTG)	Systemic Toxicity Observed[2]	Overt Toxicity Observed[3]
Avian reproduction (Quail; MRID 45336401)												N		N	N

1 Key to responses: L=Low treatment, Medium treatment, MH=Medium-high treatment, H=High treatment. Arrows (↓ or ↑) indicate the direction of the response. A shaded cell indicates that is parameter is not routinely evaluated or is not applicable in this assay. Changes in weight are absolute unless otherwise indicated. LW= liver weight

2. The systemic toxicity in the Tier 1 assays are presented in this column (*e.g.* KW= kidney weight). The systemic toxicity for the OSRI is indicated by an X in this column. For details see Section IV. A

3. The overt toxicity in the Tier 1 assays are presented in this column (*e.g.* ↓BW). The overt toxicity for the OSRI is indicated by an X in this column. For details see Section IV. A

4. 1.2-fold increase in estradiol production at 100 µM.

5. Negative in an *in vitro* frog oocyte assay evaluating ovulatory response and ovarian steroidogenesis (conc. range: 0.00625- 62.5 µM)

6. Negative for ERα and ERβ transactivation at the single concentration evaluated (10 µM).

7. The extended one generation reproductive study included P1 and F1 generations, and the F1 generation was sub-divided into 5 Subsets at weaning (PND 21) to examine general systemic and thyroid toxicity (Set 1a), developmental neurotoxicity (Set 1b), developmental immunotoxicity (Sets 2a and 2b) and reproductive and endocrine toxicity (Set 3).

P Positive finding

N Negative finding (*in vitro*)/No effect (*in vivo*)

NE Not examined

2. Effects on Androgen Pathway

The potential for 2,4-D to interact with the androgen pathway is summarized in **Table 3.** The various targets of the androgen pathway across the relevant Tier 1 assays are delineated so as to facilitate determination of potential for androgenic, anti-androgenic or HPG axis effects. This table also includes HPG-relevant findings from data evaluated as OSRI. *Effects that occurred in the presence of overt toxicity are discussed in the text but are not reported in the table and not considered further in the WOE assessment.*

2, 4-D was negative (non-binder) in the Tier 1 *in vitro* AR binding assay and negative in the steroidogenesis assay. In *in vitro* literature studies considered as OSRI, 2,4-D also showed no evidence of competitive binding to the AR (Kim *et al*., 2005) or AR activation (Kojima, *et al*., 2004; Orton *et al*., 2009; Sun *et al*., 2012).

The Tier 1 *in vivo* Hershberger and male pubertal assays were not conducted because the requirements for these two assays were satisfied by an OSRI extended one-generation rat reproductive test (EOGRT) conducted with 2,4-D, which is equivalent to the EDSP Tier 2 mammalian test.

In the EOGRT, although changes were noted in some androgen-related endpoints, the effects were non-significant (NS) and /or non-dose-related, thereby there were no treatment-related androgen effects in the study. High-dose animals had non-statistically significant (NS) decreases in several AST weights [(prostate and epididymides (P1 and F1 Set 1a) and seminal vesicles (P1)]. Serum testosterone concentrations were not determined in the EOGRT, or any other Part 158 guideline study with 2, 4-D. The age at PPS was increased ($p<0.05$) 1.6 days in the high-dose F1 male offspring, but this delay was not considered biologically relevant since it was within the normal range of variability. Testes weights were decreased (NS) in P1 high-dose males, but were decreased ($p<0.05$) in all treated F1 PND 22 males by 14 to 15%, but did not show a dose response. No histopathological lesions were observed in the testes or epididymides. Finally, pituitary weights were decreased in several F1 offspring subsets with no histopathological findings; 14 and 10% decreases (NS) in pituitary weights were observed in the F1 Set 1a and PND 22 males, and a 9% decrease ($p<0.05$) was seen in the F1 Set 3 males at the high dose. However, the values were within the historical control range for the testing facility and were not associated with histopathology in the pituitary gland.

The only other potentially androgen-related effects observed in the mammalian Part 158 toxicity studies occurred in the presence of overt toxicity. These include decreased testes weight and increased relative pituitary weights in the subchronic rat and decreased testes weight and testicular atrophy in the rat chronic toxicity/carcinogenicity study. In the FSTRA or Part 158 avian reproduction study, there were no treatment-related effects observed in males.

Table 3. Androgenic/Anti-Androgenic Pathway for 2,4-D

Lines of Evidence Indicating Potential Interaction with the Androgenic/Anti-Androgenic Pathway for 2,4-D[1]															
Study Type/ Literature Citation	AR Binding	AR Activation	Steroidogenesis	Sex Steroid Hormones	Testes Weight/GSI	Gonad Staging and Histopathology	Epididymides Weight	Epididymides Histopathology	Pituitary Weight	Accessory Sex Organ Weights/ 2° Sex Characteristics	Fertility(Frt) /Fecundity (Fcd)	Age and Weight at PPS	Vitellogenin (VTG)	Systemic Toxicity Observed[2]	Overt Toxicity Observed[3]
EDSP Tier 1															
AR Binding (MRID 48614301)	N														
Steroidogenesis (MRID 48614305)			N												
Hershberger	Requirement satisfied by OSRI (MRID 47972101); see below.														
Male Pubertal Rat	Requirement satisfied by OSRI (MRID 47972101); see below.														
FSTRA (MRID 48317007)				NE	N	N				N	N		N	NE	N

Table 3. Androgenic/Anti-Androgenic Pathway for 2,4-D

Lines of Evidence Indicating Potential Interaction with the Androgenic/Anti-Androgenic Pathway for 2,4-D[1]															
Study Type/ Literature Citation	AR Binding	AR Activation	Steroidogenesis	Sex Steroid Hormones	Testes Weight/GSI	Gonad Staging and Histopathology	Epididymides Weight	Epididymides Histopathology	Pituitary Weight	Accessory Sex Organ Weights/ 2° Sex Characteristics	Fertility(Frt) /Fecundity (Fcd)	Age and Weight at PPS	Vitellogenin (VTG)	Systemic Toxicity Observed[2]	Overt Toxicity Observed[3]
OSRI															
AR binding (Kim *et al*., 2005)	N														
AR transactivation (Kojima *et al*., 2004)		N													
AR transactivation (Orton *et al*., 2009)		N													
AR transactivation (Sun *et al*., 2012)		N													
Subchronic toxicity (Rat; MRIDs 00101599 and 00102451)					N	N	NE	N	NE	NE				N	X
Subchronic toxicity (Rat; MRID 41991501)					N	N	NE	N	N	NE				X (MH, H)	X (MH,H)
Subchronic toxicity (Mouse; MRID 41991502)					NE	N	NE	N	N	NE				N	N
Subchronic inhalation toxicity (Rat; MRID 47398701)					N	N	N	N	NE	NE				N	N
Developmental toxicity (Rat; MRID 00130407)											N			N	N

Table 3. Androgenic/Anti-Androgenic Pathway for 2,4-D

Lines of Evidence Indicating Potential Interaction with the Androgenic/Anti-Androgenic Pathway for 2,4-D[1]															
Study Type/ Literature Citation	AR Binding	AR Activation	Steroidogenesis	Sex Steroid Hormones	Testes Weight/GSI	Gonad Staging and Histopathology	Epididymides Weight	Epididymides Histopathology	Pituitary Weight	Accessory Sex Organ Weights/ 2° Sex Characteristics	Fertility(Frt) /Fecundity (Fcd)	Age and Weight at PPS	Vitellogenin (VTG)	Systemic Toxicity Observed[2]	Overt Toxicity Observed[3]
Developmental toxicity (Rabbit; MRID 41747601)											N			N	X (H)
Extended one-generation reproduction (Rat MRID 47972101)[4]					N	N	N	N	N	N	N	N		X (H; males)	X (H; males)
Two-generation reproduction (Rat; MRIDs 00150557 and 00163996)					NE	NE	NE	NE	NE	NE	N			X (H)	X (H)
Chronic/ Carcinogenicity (Rat; MRID 43612001)					N	N	NE	N	NE	NE				X (H)	X (H)
Carcinogenicity (Mouse; MRIDs 43879801 and 43597201)					NE	N	NE	N	N	NE				N	N
Avian reproduction (Quail; MRID 45336401)											N			N	N

1. Key to responses: L=Low treatment, Medium treatment, MH=Medium-high treatment, H=High treatment. Arrows (↓ or ↑) indicate the direction of the response. A shaded cell indicates that is parameter is not routinely evaluated or is not applicable in this assay. Changes in weight are absolute unless otherwise indicated. Abbreviations for androgen sensitive tissues: Seminal vesicles (SV), Ventral prostate (VP), Dorsal prostate (DP), Levator ani-bulbocavernosus (LABC), Epididymides (E), Kidney weight (KW), Kidney histopathology (KH)
2. The systemic toxicity in the Tier 1 assays are presented in this column (*e.g.* KW= kidney weight). The systemic toxicity for the OSRI is indicated by an X in this column. For details see Section IV. A

3. The overt toxicity in the Tier 1 assays are presented in this column (*e.g.* ↓BW). The overt toxicity for the OSRI is indicated by an X in this column. For details see Section IV. A
4. The extended one generation reproductive study included P1 and F1 generations, and the F1 generation was sub-divided into 5 Subsets at weaning (PND 21) to examine general systemic and thyroid toxicity (Set 1a), developmental neurotoxicity (Set 1b), developmental immunotoxicity (Sets 2a and 2b) and reproductive and endocrine toxicity (Set 3).

P Positive finding
N Negative finding (*in vitro*)/No effect (*in vivo*)
NE Not examined

C. Effects on Hypothalamic-Pituitary-Thyroidal (HPT) Axis

The current EDSP Tier 1 battery does not have a specific *in vitro* assay to detect chemicals with the potential to affect hypothalamic or pituitary regulation of thyroid hormone production, but it does include three *in vivo* assays that provide redundancy and have the potential to detect changes in the HPT axis, *i.e.*, the pubertal female and male (rat) assays, and the AMA (frog).

The potential for 2, 4-D to interact with thyroid regulation are summarized in **Table 4.** The various targets of the thyroid pathway across the relevant Tier 1 assays are delineated so as to facilitate determination of potential for thyroid or HPT axis effects. This table also includes HPT-relevant findings from data evaluated as OSRI. *Effects that occurred in the presence of overt toxicity are discussed in the text but are not reported in the table and not considered further in the WOE assessment.*

In the AMA with 2, 4-D, there were no histological effects on the thyroid, and the only developmental change was a decrease ($p<0.05$) in the Day 21 hind-limb length (HLL) value (15%) at the high test concentration (113 mg/L).

The requirements for the Tier 1 *in vivo* male and female pubertal assays were satisfied by an OSRI rat extended one-generation rat reproductive test (EOGRT). In this study, thyroid parameters were evaluated at multiple life stages. Although changes were noted in some thyroid-related endpoints, the effects were non-significant (NS) and /or non-dose-related, or seen at a dose that resulted in overt toxicity (10% decrease in body weight in male offspring), thereby there were no treatment-related thyroid effects in the EORGT. For example, non-significant decreases were observed in thyroid weights in several F1 offspring sub-groups (male and female), as well as a non-dose-responsive increase (NS) in thyroid weight in P1 gestational day (GD) 17 females. The decreased thyroid weights were present with no concomitant histopathological effects. Decreased follicle size was observed in 3/12 high-dose P1 GD 17 females. Non-significant decreases in serum T_4 concentrations generally were observed in several F1 offspring sub-groups (male and female) and in the P1 GD 17 females, with a few exceptions. Serum T_4 concentrations were decreased ($p<0.05$) in the high-dose male F1 PND 22 offspring at an overtly toxic dose. T4 levels were increased in the mid-dose male, and mid- and high-dose female F1 Set 1a offspring, but the changes were not statistically significant. Serum TSH concentrations were unchanged or increased (NS) in several mid- and high-dose F1 offspring sub-groups (male and female) and in the P1 GD 17 females. Finally, non-significant decreases (9-10%) in pituitary weight were observed in the F1 Set 1a and 3 female offspring. Pituitary weights were decreased in several F1 offspring subsets with no histopathological findings; 14 and 10% decreases (NS) in pituitary weights were observed in the F1 Set 1a and PND 22 males. A 9% decrease ($p<0.05$) in pituitary weight was seen in the F1 Set 3 males at the high dose; however, the values were within the historical control range for the testing facility and were not associated with histopathology in the pituitary gland.

Thyroid effects were noted in several other Part 158 studies conducted with 2,4-D. In a subchronic oral toxicity study in rats, thyroid weights at termination were decreased ($p<0.05$) 40% in males and increased ($p<0.05$; relative weight) 68% in females, with an increased incidence of thyroid hypertrophy observed in the high-dose females. Serum T_4 concentrations were decreased for males and females in the high-mid- and high-dose groups, with a dose response observed in the males, but not the females. However, the observed thyroid-related effects in this study occurred at a dose that resulted in overt toxicity (clinical signs and/or ≥10% decrease in body weight). In a subchronic oral toxicity study with 2,4-D in mice, no effect on thyroid weights or histopathology was observed, but a decrease in serum T_4 concentrations was observed in males and females in the high-mid- and high-dose groups (100 and 300 mg/kg/day, respectively). Finally, thyroid effects were noted in a chronic oral toxicity study with 2,4-D in rats. Thyroid weights were increased ($p<0.05$) in the high-dose (150 mg/kg/day) males, and mid (75 mg/kg/day)- and high-dose females, at 12 and 24 months. Histopathological changes in the thyroid were not noted, except for decreased secretory material in the follicles of high-dose females at the 12-month sacrifice only. Serum T_4 concentrations were decreased (generally $p<0.05$) in all mid- and high-dose males and females. Overt toxicity (>10% body weight loss) was observed in females at the mid and high doses.

In an open literature *in vitro* study, 2,4-D showed no thyroid receptor agonist or antagonist activity at concentrations from 0.003 to 3.0 mg/L in a receptor-related luciferase reporter gene assay in Vero (African green monkey kidney) cells (Sun *et al.*, 2012). In Relyea, 2009, although there were no effects on survival to metamorphosis, mass at metamorphosis, or time to metamorphosis for either leopard frogs or gray tree frogs, it is noted that these endpoints are limited in their ability to inform potential thyroid-related perturbations.

Table 4. Thyroid Pathway for 2,4-D.

Lines of Evidence Indicating Potential Interaction with the Thyroid Pathway for 2,4-D[1]										
Study Type/ Literature Citation	Thyroid Receptor Activation	Thyroid Weight	Thyroid: Gross and Histopathology	Serum T_4 Levels	Serum TSH Levels	Pituitary Weight	Developmental stage (± or asynchronous, HLL)	Growth (BW, SVL)	Systemic Toxicity Observed[2]	Overt Toxicity Observed[3]
EDSP Tier 1 Assays										
Male Pubertal Rat	Requirement satisfied by OSRI (MRID 47972101); see below.									
Female Pubertal Rat	Requirement satisfied by OSRI (MRID 47972101); see below.									
AMA (Frog; MRID 48317002)			N				HLL: ↓15% (Day 21, H)	N		N
OSRI										
Thyroid receptor transactivation (Sun *et al.*, 2012)	N									
Subchronic toxicity (Rat; MRID 41991501)		N	N	N	NE	N			X (MH, H)	X (MH, H)
Subchronic toxicity (Mouse; MRID 41991502)		N	N	↓T_4 (MH, H)	NE	N			X (MH, H)	N
Subchronic inhalation toxicity (Rat; MRID 47398701)		NE	N	NE	NE	NE			N	N
Extended one-generation reproduction (Rat MRID 47972101)[4]		N	N	N	N	N			X[5] (H)	X (males: H)
Two-generation reproduction (Rat; MRIDs 00150557 and 00163996)		NE	NE	NE	NE	NE			N	X (H)

Table 4. Thyroid Pathway for 2,4-D.

Lines of Evidence Indicating Potential Interaction with the Thyroid Pathway for 2,4-D[1]										
Study Type/ Literature Citation	Thyroid Receptor Activation	Thyroid Weight	Thyroid: Gross and Histopathology	Serum T_4 Levels	Serum TSH Levels	Pituitary Weight	Developmental stage (± or asynchronous, HLL)	Growth (BW, SVL)	Systemic Toxicity Observed[2]	Overt Toxicity Observed[3]
Chronic/ Carcinogenicity (Rat; MRID 43612001)		↑18% (males: H)	N	↓32 % (males: M) ↓64 % (males: H)[5]	NE	NE			X (female: M, H; male: H)	X (female; MH, H)
Carcinogenicity (Mouse; MRIDs 43879801 and 43597201)		NE	N	NE	NE	NE			N	N

1. Key to responses: L=Low treatment, Medium treatment, MH=Medium-high treatment, H=High treatment. Arrows (↓ or ↑) indicate the direction of the response. A shaded cell indicates that is parameter is not routinely evaluated or is not applicable in this assay. BW= Body weight; HLL=Hind limb length; SVL=Snout to vent length
2. The systemic toxicity in the Tier 1 assays are presented in this column (*e.g.* KW= kidney weight). The systemic toxicity for the OSRI is indicated by an X in this column. For details see Section IV. A
3. The overt toxicity in the Tier 1 assays are presented in this column (*e.g.* ↓BW). The overt toxicity for the OSRI is indicated by an X in this column. For details see Section IV. A
4. The extended one generation reproductive study included P1 and F1 generations, and the F1 generation was sub-divided into 5 Subsets at weaning (PND 21) to examine general systemic and thyroid toxicity (Set 1a), developmental neurotoxicity (Set 1b), developmental immunotoxicity (Sets 2a and 2b) and reproductive and endocrine toxicity (Set 3).4 Decreased follicle size in 3/12 Parental GD17 females. 2,4-D has been shown to exhibit non-linear toxicokinetics in mammals. Toxicokinetic information was used to select doses for the rat extended one-generation reproduction study. The high doses for both sexes in this study were selected to be at or slightly above the inflection point for non-linear toxicokinetics. Data from the extended one-generation study show that the high dose for males (800 ppm) and females (600 ppm) exceeded the threshold for saturation of renal clearance.
5. T_4 was decreased in both sexes at 6, 12, 18 and 24 months; $p<0.05$. The decrease in females occurred in the presence of overt toxicity. At 24 months, T4 level in males were decreased 32% and 64% at 75 and 150 mg/kg/day, respectively.

N Negative findings

NE Not examined

IV. Committee's Assessment of Weight of Evidence

This section of the document describes the weight of evidence (WoE) determination on the potential of 2, 4-D to interact with endocrine related processes (*i.e.*, E, A, or T hormonal pathways) as well as recommendations regarding Tier 2 testing. The results of the Tier 1 assays are considered, along with other scientifically relevant information (*e.g.,* 40 CFR Part 158 test guidelines and published or publicly available peer-reviewed studies). WoE analysis in the context of the EDSP follows the Agency's guidance (USEPA 2011) and is conducted on a case-by-case basis by first assessing the different lines of evidence (*i.e.*, specific Tier 1 assays and OSRI), then performing an integrated analysis of those lines of evidence.

The WoE evaluation includes considerations of biological plausibility of the findings from the different lines of evidence by examining the consistency, coherence, and interrelationships among the measured endpoints within and across studies. The available findings from standard toxicology studies on the substance may contribute to the WoE evaluation in helping elucidate if effects seen in the Tier 1 assay are related to perturbations of the endocrine system *per se* or alternatively a sequelae of systemic effects. Endocrine modes of action may elicit a number of phenotypic consequences other than those evaluated in the Tier 1 assays.

Endocrine-related findings in the presence of overt toxicity may result in uncertainty as to whether or not the responses are related through an endocrine pathway, therefore non-endocrine toxic responses (including but not limited to mortality or body weight changes) are also considered in this WoE evaluation. The FIFRA SAP that evaluated scientific issues associated with weight of evidence evaluation of the results of the Tier 1 assays stated that *"In summary, the Panel agreed that little, if any, weight should be placed on signs of endocrine disruption in the presence of overt toxicity. All effects in endocrine sensitive tissues should be evaluated in terms of primary interactions with the endocrine system vs. secondary effects related to toxicity in non-endocrine organs or overall disruptions in homeostasis"* (USEPA, 2013).

A. Systemic/Overt Toxicity in the *in vivo* Tier 1 Assays and OSRI Studies

Effects that were considered to be systemic or overt toxicity for the *in vivo* Tier 1 assays and OSRI studies are described below. Generally, one parameter (i.e., death or >10% decrease in mammalian body weight) was sufficient for a dose/concentration to be considered overtly toxic. However, in other instances, more than one parameter was needed to determine overt toxicity. Effects which were considered to be signs of systemic toxicity were generally less severe forms of toxicity (e.g., changes in organ weights or blood chemistry).

1. Tier 1 *in vivo* Assays

The requirements for the Hershberger, male and female pubertal assays, and uterotrophic assays with 2, 4-D were satisfied by OSRI (extended one-generation reproduction test, EOGRT). Overt toxicity was not observed in either the AMA or FSTRA.

2. OSRI

In an extended one-generation reproduction toxicity test in rats, systemic toxicity was noted, presenting primarily as effects on the kidney. P males administered 800 ppm 2, 4-D in the diet had increased absolute and relative (to body) kidney weights, with microscopic findings of degenerative lesions in the proximal convoluted tubules in the outer zone of the medulla. In the F1 generation, all PND 22 unselected treated (100, 300, and 800 ppm) males had decreased body weights (↓9%-10%), and the 800 ppm males had decreased absolute (↓37%) and relative (↓29%) adrenal weights, and absolute kidney (↓15%) and liver (↓18%) weights. In the F1 offspring observed to PND 70, the 800 ppm males had decreased body weight (↓11%-17%) and body weight gains (↓11%-25%) throughout the study period, with the magnitude of the reduction diminishing with time of exposure. Both sexes displayed a slight increase in ALT and an increase in triglyceride levels at the high dose. The 800 ppm males had a 10% decrease in terminal body weights, and decreases in liver (↓16%), pituitary (↓14%), and adrenal glands (↓12%). Increased absolute (↑9%) and relative (↑11%) kidney weights were observed in the 300 and 600 ppm females. An increased incidence of degeneration of the proximal convoluted tubule in the kidney was observed in the 300 and 800 ppm males and in the 600 ppm females. Similar decreases in body weights were noted in pups used for immunotoxicity investigations (PND 67-73).

In a guideline subchronic oral toxicity study in Fisher 344 rats, one 100 mg/kg/day male lost weight on Days 55 and 62, with subsequent weight recovery until Day 91. Decreased mean body weights were observed in the 150 mg/kg/day males and 100 and 150 mg/kg/day females beginning on Day 41, and in the 60 and 100 mg/kg/day males beginning on Day 59, all continuing through study termination. Terminal body weights were decreased in the 150 mg/kg/day animals, with lesser decreases (NS) at 100 mg/kg/day. The primary target organ was the kidney with increases in kidney weights in all males, and 150 mg/kg/day females. A degenerative change was seen in the kidneys of male rats treated with ≥60 mg/kg/day. The degree of degeneration was more pronounced in the two highest dose groups.

In a guideline subchronic oral toxicity study in Fisher 344 rats, clinical signs of toxicity (hunched posture, depressed activity, and few/no feces) occurred mainly in the 300 mg/kg/day females. Decreased ($p<0.05$) body weights were observed in the 100 and 300 mg/kg/day males and females, reaching 7-23% in males and 7-28% in females at Week 13. Decreased overall (Weeks 0-13) body-weight gains were also noted in the 100 and 300 ppm males (↓9-37%) and females (↓11-57%). A corresponding decrease in food consumption was observed in both sexes at these doses. Changes ($p<0.05$) in absolute and/or relative organ weights [including adrenals (↑ males;

↓ females), testes with epididymides (↑), ovaries (↓), pituitary (↑ males; ↓ females), liver (↑ males and females), and thyroids/parathyroids (↑ males and females)] were observed primarily at 300 mg/kg/day in both sexes; many of these changes may be attributable to decreased body weight. Gross findings, mainly in the high-dose group, included small testes and epididymis and opaque eyes (females). Treatment-related histopathological changes were observed primarily at 300 mg/kg/day and included centrilobular hepatocellular hypertrophy, atrophy of the testes, hypertrophy of the zona glomerulosa (adrenal cortex, both sexes) and follicular cells (thyroid, females).

In a guideline subchronic oral toxicity study in B6C3F1 mice, treatment-related effects at 100 mg/kg/day included increased mean absolute and relative kidney weights in females. Treatment-related effects at 300 mg/kg/day included transient decreased ($p<0.05$) food consumption (up to Week 7) and histopathological changes in the liver characterized by nuclear hyperchromatism and decreased glycogen in periportal hepatocytes.

In a guideline subchronic inhalation toxicity study in Sprague-Dawley rats, clinical signs associated with exposure at 1.0 mg/L included excessive salivation, labored breathing and chromodacryorrhea. Decreased body weights were observed in the 1.0 mg/L females from Day 14 to the end of dosing (↓10%) and recovery (↓12%). Body weight gains were also reduced in these females throughout the study and recovery, and was accompanied by a reduction in food intake.

In a guideline developmental toxicity study in pregnant Fischer 344 rats, dams treated at 75 mg/kg/day displayed a decrease (NS) in body-weight gain during GD 6-15 (↓79%). The corrected body-weight gain was comparable among the groups.

In a guideline developmental toxicity study in artificially-inseminated female New Zealand White rabbits, two 90 mg/kg/day does aborted (GDs 21 and 24) and were observed with ataxia, decreased motor activity, loss of righting reflex, extremities that were cold to the touch, and/or dried feces. Body weight gains were decreased at 90 mg/kg/day (↓27%) during the dosing period (GDs 6-19; NS). On GDs 7-8, the 10 and 30 mg/kg/day groups showed no body-weight gain, and the 90 mg/kg/day group displayed a body weight loss. On GDs 15-19, the 90 mg/kg/day group displayed no body weight gains, and a decrease in the corrected body weight gains (↓23%; NS).

In a guideline two-generation reproduction study in Fischer 344 rats, decreased ($p<0.05$) body weights were consistently observed at 80 mg/kg/day in the P males (↓3%) after Week 6 and in the P females (↓4%) by Week 12. The decreased body weights could not be attributed to reduced food consumption since food consumption and food consumption per gram body weight gain were slightly increased. Body weights were also decreased ($p<0.05$) in the P females during gestation of the F_{1a} (↓5-6%) litters on GD 7, 13 and 20, and the F_{1b} litters (↓10%) on GD 20. In the 80 mg/kg/day F_{1a} pups, body weights were decreased ($p<0.05$) by 11-29% during PND 1-28

in the males, and by 15-25% during PND 4-28 in the females. In the 80 mg/kg/day F_{1b} pups, body weights were decreased ($p<0.05$) by 22-42% in the males and 17-38% in the females during PND 1-28. In the 20 mg/kg/day F_{1b} pups, body weights were decreased ($p<0.05$) in the males by 7% and 16% on PND 4 and 28, respectively, and in the females by 13% on PND 28. Additionally at 80 mg/kg/day the following differences (p,0.05) in gestation and survival were observed: (i) gestation duration was increased ($p<0.01$) for the F_{1b} litters (22.5 days vs 21.9 days); (ii) gestation survival index was decreased ($p<0.01$) for the F_{1b} litters (31.7% vs 97.8%); (iii) the number of F_{1b} pups born dead/dying by PND 1 was increased (110 treated vs. 5 controls); (iv) F_{1b} litter size was decreased ($p<0.01$; 5.1 treated vs. 9.5 controls); v) F_{1b} pup viability was lower ($p<0.01$) throughout the weaning period; (vi) there were decreases ($p<0.01$) in F_{1b} pup survival to LD 4 (86.3% treated vs. 100% controls); and (vii) survival to LD 28 was decreased (71.4% treated vs. 100% controls).

In a guideline combined chronic toxicity/carcinogenicity study in Fischer 344 rats, body weights were lower than respective controls in females at 75 mg/kg/day (↓14%) and in males (↓8%) and females (↓26%) at 150 mg/kg/day at termination. Body weight gains were lower than respective controls in females at 75 mg/kg/day (↓24%) and in males (↓17%) and females (↓48%) at 150 mg/kg/day. A corresponding depression in mean food consumption occurred in females at 75 mg/kg/day (↓4%) and in males (↓5%) and females (↓12%) at 150 mg/kg/day. Statistically significant ($p < 0.05$) increases in plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP) and/or cholesterol were seen in females at 75 mg/kg/day and in males and females at 150 mg/kg/day at various time periods. After 12 months of treatment, treatment-related effects included: decreased hematopoiesis of the bone marrow of females at 150 mg/kg/day; altered tinctorial properties in the liver of females at 75 mg/kg/day and both sexes at 150 mg/kg/day; bilateral retinal degeneration of the eyes of females at 150 mg/kg/day; multifocal alveolar histiocytosis in the lungs of females at 75 mg/kg/day and both sexes at 150 mg/kg/day; degeneration of the descending portion of the proximal convoluted tubules of the kidneys in both sexes at 75 mg/kg/day and 150 mg/kg/day; atrophy of the adipose tissue of females at 75 and 150 mg/kg/day; atrophy of the testes in males at 150 mg/kg/day; and decreased secretory material in the thyroid follicles of females at 150 mg/kg/day. After 24 months of treatment, treatment-related non-neoplastic lesions were limited to the eyes, liver, lung, and the mesenteric fat. Eye lesions were characterized as slight to severe bilateral retinal degeneration and lenticular cataracts in both sexes at 150 mg/kg/day. Liver lesions manifested as increases in the size of hepatocytes, often accompanied by altered tinctorial properties that involved all hepatocytes within the hepatic lobule of both sexes at 150 mg/kg/day. Lesions of the respiratory system included subacute to chronic inflammation of the lungs in females at 75 mg/kg/day and both sexes at 150 mg/kg/day. Atrophy of the adipose tissue was increased in both sexes at 150 mg/kg/day. It is interesting to note that lesions seen in the spleen, kidneys, testes and thyroid glands in rats sacrificed at 12 months were not seen in those sacrificed at 24 months.

In a guideline carcinogenicity study in B6C3F1 mice, the 300 mg/kg/day females had slightly lower ($p<0.05$) body weights at the 3- and 6-month intervals (↓4%). Body-weight gains were decreased ($p<0.05$) in the 5 (↓7%), 150 (↓6%), and 300 (↓14%) mg/kg/day females at the 3-month interval, and the 300 mg/kg/day females had decreased ($p<0.05$) body-weight gain at the 6- and 12-month (↓9%) intervals. Microscopically, there was an increased incidence of lesions in the kidneys of 62 and 120 mg/kg/day males and the 120 and 300 mg/kg/day females.

In a guideline avian reproduction toxicity study, northern bobwhite quail (*Colinus virginianus*) fed diets containing concentrations of 0, 160, 400, and 1000 ppm [(mean-measured concentrations of 0 (basal diet), 147, 382, or 962 ppm)] for approximately 21 weeks did not display any signs of systemic or overt toxicity.

B. Estrogen Pathway

There was no convincing evidence of potential interaction of 2, 4-D with the estrogen pathway. The Tier 1 or OSRI *in vitro* assays were negative with the exception of a 20% increase in estradiol production in the in the steroidogenesis assay at 100 μM. The requirements for the Tier 1 *in vivo* mammalian uterotrophic and female pubertal assays were satisfied by the extended one-generation reproduction test in rats (equivalent to EDSP Tier 2 study) considered as OSRI. No estrogen-related effects were observed in this study or in any of the mammalian Part 158 toxicity studies in the absence of overt toxicity. No effects were observed in the Part 158 bobwhite quail reproduction study. In the FSTRA, fecundity was decreased (34%) at a concentration of 96.5 mg a.i./L, and while an increase in the number of female ovaries at stage 2 (compared to stage 3 or 4) was observed for the 2,4-D treatment groups compared to the negative control, the incident rates were not dose-responsive, and there were no other effects observed in this study.

C. Androgen Pathway

There was no convincing evidence of interaction of 2, 4-D with the androgen pathway. The Tier 1 and OSRI *in vitro* assays were negative. The requirements for the Tier 1 *in vivo* mammalian Hershberger and male pubertal assays were satisfied by the extended one-generation reproduction test in rats (equivalent to EDSP Tier 2 study) considered as OSRI. There were no androgen-related effects observed in this study. Additionally, there were no treatment-related effects in the FSTRA or avian reproduction studies, and there were no effects on the male reproductive parameters in the Part 158 mammalian studies in the absence of overt toxicity.

D. Thyroid Pathway

In the AMA, while there was a 15% decrease in HLL (at the high test concentration of 113 mg/L), there were no histological effects on the thyroid or effects on developmental stage. Some thyroid-related effects were seen in the mammalian Part 158 studies. The effects were only seen in the presence of overt toxicity or at doses that exceeded the threshold for renal clearance of 2,

4-D in the rat, resulting in systemic toxicity. The requirements for the Tier 1 *in vivo* mammalian male and femal pubertal assays were satisfied by the extended one-generation reproduction test in rats (equivalent to EDSP Tier 2 study) considered as OSRI. In that study, there were no treatment-related thyroid effects in males or females. Therefore, collectively the data across the Tier 1 assays and Part 158 data do not demonstrated convincing evidence of potential interaction with the thyroid pathway.

E. Conclusions

The conclusion of the WoE evaluation is that 2, 4-D demonstrates no convincing evidence of potential interaction with the estrogen, androgen or thyroid pathways.

V. EDSP Tier 2 Testing Recommendations

Based on weight of evidence considerations, additional EDSP Tier 2 ecological testing is not recommended. It is noted that the agency already has a mammalian toxicity study (extended one-generation reproduction) that is equivalent to the EDSP Tier 2 study.

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APPENDIX 1. EDSP Tier 1 Screening Assays

Amphibian Metamorphosis Assay; (OCSPP 890.1100)

The 21-day assay (MRID 48317002) of 2,4-D (purity 98.6%) on amphibian metamorphosis of South African Clawed Frog (Xenopus laevis) was conducted under flow-through conditions. Amphibian larvae at Nieuwkoop-Faber (NF) stage 51 (80/control and treatment group; 20/replicate) were exposed to a negative control and test chemical nominal concentrations of 0.4, 4.0, 40.0, and 100.0 mg a.i./L. Mean measured concentrations were <0.120 (<LOQ, negative control), 0.273, 3.24, 38.0, and 113 mg a.i./L. The test system was maintained at 21.9 to 22.7 °C and a pH of 7.0 to 7.8.

Only one incidence of tadpole mortality occurred in the mid-low treatment group; the cause of death was unknown. No clinical signs of toxicity were noted.

2,4-D did not affect Day 7 normalized (for snout-vent length) hind-limb length (HLL). However, there was a statistically significant ($p<0.05$) decrease of 15% in Day 21 normalized HLL at the highest treatment level compared with the negative control. There was no significant effect on median NF developmental stage, snout-vent length (SVL), or body weight at Day 7 or Day 21. Asynchronous development was not observed. There were no effects on thyroid gland histopathology. Late stage (>NF stage 60) tadpoles were observed in the negative control and in all treatment levels; consistent with the guideline recommendations, these tadpoles were excluded from analyses of growth and normalized HLL.

Androgen Receptor (AR) Binding Assay; (OCSPP 890.1150)

In an androgen receptor (AR) binding assay (MRID 48614301), ventral prostate cytosol from Sprague Dawley rats was used as the source of AR to conduct a competitive binding experiment to measure the binding of a single concentration of [^{3}H]-R1881 (1 nM) in the presence of increasing concentrations (10^{-11} to 10^{-4} M) of 2,4-D (98.5% purity). The test guideline recommends testing up to 10^{-3} M; however, the sponsor selected 10^{-4} M as the highest concentration based on *in vivo* toxicokinetic analyses in the rat; concentrations higher than 10^{-4} M were not relevant for testing in this assay as they are substantially above the inflection point for linear toxicokinetics (see Appendix B of study report). Ethanol was used as a solvent at a final concentration of <3%. A total of three independent runs were performed, and the assay included dexamethasone as a weak positive control, and R1881 as the ligand reference standard.

Saturation binding experiments were conducted to demonstrate that the AR in the rat prostate cytosol was present at adequate levels and functioning with appropriate affinity for the radiolabeled ligand. The saturation binding experiment resulted in a maximum binding capacity (B_{max}) of 3.245 fmol/100 µg protein and the dissociation constant (K_d) was 0.4641 nM. Although these values were slightly below the range of values from the validation studies, the results were highly reproducible and all other performance criteria and the competitive binding assays indicated acceptable performance of the assay. The Scatchard plot indicated a linear response across the concentrations of ligand added. Nonspecific binding as a percent of total binding was less than 20% across the entire concentration range in the saturation binding assays (range 6.2-19.8%, with the exception of the high concentration (10 nM) in one assay, which was 24.6%).

There were no appreciable alterations in R1881 AR binding activity at 2,4-D concentrations ranging from 10^{-11} to 10^{-4} M in the competitive binding experiments, therefore, the log IC_{50} and relative binding affinity (RBA) for 2,4-D could not be calculated. The log IC_{50} values for R-1881 alone and the positive control, dexamethasone, were −9.0 and −4.4 M, respectively. Compared to R1881, the RBA for dexamethasone was 0.0027 %. In all instances, R1881 and the positive control met the QC performance criteria established in the test guideline.

Based on the results from the three runs, 2,4-D is classified as a Non-binder in the Androgen Receptor Binding Assay.

Aromatase Assay; (OCSPP 890.1200)

In an *in vitro* aromatase (CYP 19) assay (MRID 48614302), 2,4-D (98.5% a.i., lot#: 2006 2433 8006-USA) in ethanol (1%) was incubated with human recombinant aromatase and tritiated androstenedione (1-β [^{3}H(N)]-androst-4-ene-3,17-dione; [^{3}H]ASDN) for 15 minutes at 37 °C to assess the potential of 2,4-D to inhibit aromatase activity. 2,4-D was tested at logarithmic concentrations from 10^{-10} M to 10^{-4} M in three independent runs. The test guideline recommends testing up to 10^{-3} M; however, the sponsor selected 10^{-4} M as the highest concentration based on *in vivo* toxicokinetic analyses in the rat. The sponsor considered concentrations higher than 10^{-4} M to be not relevant for testing in this assay as they are substantially above the inflection point for linear toxicokinetics (See Appendix B of the AR binding assay study report, MRID 48614301).

Aromatase activity was determined by measuring the amount of tritiated water produced at the end of a 15-minute incubation for each concentration of chemical. Tritiated water was quantified using liquid scintillation counting (LSC). Three independent runs were conducted and each run included a full activity control, a background activity control, a positive control series (10^{-10} to 10^{-5} M) using a known inhibitor (4-hydroxyandrostenedione; 4-OH ASDN), and the test chemical series (10^{-10} to 10^{-4} M) with 3 repetitions per concentration.

Aromatase activity in the full activity controls ranged from 0.131 to 0.244 nmol·mg-protein^{-1}·min^{-1} for the three test runs, with a mean and standard deviation of 0.186±0.036 nmol·mg-protein^{-1}·min^{-1}. Activity in the background controls ranged from 7.31 to 11.51% of the full activity controls. The responses of full activity controls were outside of the 90 to 110% range in the 2nd and 3rd replicates in the Run 1 (86.6 and 113.8%, respectively).

Results for the positive control were generally within the recommended ranges for the top of the curve, bottom curve, Hill slope, log IC_{50}, and coefficient of variation for replicates of each concentration within runs, with the exception that the bottom of the curve in Runs 2 and 3 (-8.2 and -7.2, respectively) exceeded the acceptable range (-5 to +6). Also, the coefficients of variations (%CVs) for replicates of each concentration of 2,4-D within a run were generally within the 15% guideline, with the exception that the overall %CV for the highest two concentrations (-45.2 and 415.9%) exceeded the acceptable limit. For 4-OH ASDN, the estimated log IC_{50} averaged −7.23 M and the slope was −0.92.

For 2,4-D, aromatase activity averaged 0.195±0.045 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration (10^{-10} M) and 0.168±0.025 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration (10^{-4} M). The data for 2,4-D were modeled for Run 1, and the goodness of fit (R^2) value was 0.83; however, the 2,4-D data from Runs 2 and 3 could not be modeled. The average dose-response curve indicated that the aromatase activity of the test material at concentrations ranging from 10^{-10} M to 10^{-4} M was essentially equivalent to the activity observed in the full activity controls. At 10^{-4} M, aromatase activity was approximately 91%. Since the average lowest portion of the activity response curve was greater than 75% activity, 2,4-D is classified as a non-inhibitor of aromatase activity up to the highest concentration tested (10^{-4} M). High CVs were observed for 4-OH ASDN at the two highest concentrations and at a single concentration for 2,4-D. Individual values were occasionally outside of the performance criteria ranges (with the mean value within range).

Based on the data from the average response curve, 2,4-D is classified as a Non-inhibitor of aromatase activity in this assay.

Estrogen Receptor (ER) Binding Assay; (OCSPP 890.1250)

In an estrogen receptor (ER) binding assay (MRID 48614303) for 2,4-D (98.5%, Lot# 2006 2433 8006-USA) , uterine cytosol from Sprague Dawley rats was used as the source of ER to conduct saturation binding and competitive binding experiments in this assay. The competitive binding experiment was conducted to measure the binding of a single concentration of [^{3}H]-17β-estradiol (1 nM) in the presence of logarithmic increasing concentrations of 2,4-D from 10^{-11} to 10^{-4} M, rather than 10^{-10} to 10^{-3} recommended in the test guideline. The justification for lowering the

top test concentration was based on toxicokinetic data in the rat; concentrations higher than 10^{-4} M were not relevant for testing in this assay as they are substantially above the inflection point for linear toxicokinetics. Ethanol was used as a solvent at a final concentration of <3%. The assay included 19-norethindrone as a weak positive control, octyltriethoxysilane as a negative control, and 17β-estradiol as the natural ligand reference material, and three independent runs were performed on separate days.

Summary data pertaining to the saturation binding experiment were reported separately in the study profile submitted by the test order recipient. The K_d for [^{3}H]-17β-estradiol was 0.1032 nM and the B_{max} (nM) was 0.07097 for the prepared rat uterine cytosol used in these experiments. The K_d for the run was within the expected range of 0.03 to 1.5 nM, and the B_{max} was within the expected range of 10-150 fmol/100 μg protein. The data produced a linear Scatchard plot.

In the competitive binding experiment, no precipitation was observed at any concentration tested. The mean specific binding in the presence of 2,4-D was >95% at 2,4-D concentrations of $\leq 10^{-4}$ M in all three runs. The estimated mean log IC_{50} and RBA was not calculated for 2,4-D as the percent binding inhibition did not reach 50% for any run.

The estimated mean log IC_{50} for the natural ligand, 17β-estradiol, and the weak positive control (19-norethindrone) was −9.0 and −5.5 M, respectively. The mean RBA was 0.034% for 19-norethindrone. All performance criteria were met for 17β-estradiol, 19-norethindrone and octyltriethoxysilane.

2,4-D was tested over a concentration range that fully defined the top of the curve. The mean specific radioligand binding in the presence of 2,4-D was >95% at 2,4-D concentrations of $\leq 10^{-4}$ M. Based on the results from the three runs, 2,4-D is classified as Not Interactive in the Estrogen Receptor Binding Assay.

ERα Transcriptional Activation (ERTA) Assay; (OCSPP 890.1300)

In an estrogen receptor transcriptional activation assay (MRID 48614304), hERα-HeLa-9903 cells cultured *in vitro* were exposed to 2,4-D (98.5% a.i., Lot #2006 2433 8006 USA) at logarithmically increasing concentrations from 10^{-10} to 10^{-4} M in DMSO (0.1%) for 24 hours. A total of four separate runs were performed. Each run was performed using 96-well plates and each 2,4-D concentration was tested in triplicate (3 wells/plate). Cells were exposed to the test agent for approximately 24 hours to induce reporter (luciferase) gene products. Luciferase expression in response to activation of the estrogen receptor was measured upon addition of a luciferase substrate and detection with a luminometer with acceptable sensitivity.

2,4-D was tested up 10^{-4} M based on solubility, cytotoxicity and *in vivo* toxicokinetic analysis. There were deviations from expected performance criteria for all of the four reference chemicals,

but these deviations do not affect the interpretation of this study. The RPC_{Max} was <0% for the first run, 8.8% for the second run, 5.3% for the third run, and 7.0% for the fourth run; the associated PC_{Max} was 10^{-4} M for runs 1-3 and 10^{-5} M for run 4. Because the $RPC_{Max} < PC_{10}$ in all assay runs, 2,4-D was considered negative for estrogen receptor transcriptional activation in this test system.

Fish Short-Term Reproduction Assay (FSTRA); (OCSPP 890.1350)

The 21-day short-term reproduction assay (MRID 48317007) of 2,4-D with fathead minnow (*Pimephales promelas*) was conducted under flow-through conditions. Adult fish (20 spawning groups; 2 males and 4 females in each group; 4 groups/treatment; *ca*. 6 months old) were exposed to 2,4-D (98.6% purity) at nominal concentrations of 0 (negative control), 0.400, 4.00, 40.0, and 100 mg a.i./L concentrations with corresponding mean-measured concentrations of <0.10 (<LOQ, negative control), 0.245, 3.14, 34.0, and 96.5 mg a.i./L, respectively. The test system was maintained at 24.5 to 25.2°C and a pH of 7.02 to 7.76.

The single mortality observed during the assay occurred in a female in the high treatment group. There were no significant differences for male or female body weight or length relative the negative control. At test termination, observations of secondary sex characteristics were observed in the negative control and treated groups; no treatment-related effects were reported. Clinical signs included the loss of an eye, ascites, and scoliosis (bent tail) which were observed in single fish in the negative control or treatment groups.

Spawning occurred in the negative control at least every 4 days in 3 of the 4 replicates, and mean fecundity was 29.8 eggs/female/day/replicate; fertility in the negative control was 96.9%. Fecundity was significantly decreased (Jonckheere-Terpstra; $p<0.05$) by 34% in the high treatment group with a non-significant ($p>0.05$) concentration-dependent trend of decreased fecundity in the lower treatment groups compared to the negative control. There were no significant differences for fertility between the 2,4-D treatments and the negative control.

There were no significant differences ($p>0.05$) between the 2,4-D treatment groups and the negative control for male or female gonado-somatic index (GSI) or plasma vitellogenin (VTG). There was also no significant difference ($p>0.05$) for male nuptial tubercle scores; no tubercles were observed for females. No apparent treatment-related histopathological effects were observed in males and females. Although not concentration-dependent, an increase in the number of female ovaries that were observed as Stage 2 (compared to Stage 3 or 4) was reported for the 2,4-D treatments compared to the negative control. Plasma sex steroid concentrations were not reported.

Hershberger Assay; (OCSPP 890.1400)

The requirement for a Tier 1 Hershberger assay is satisfied by OSRI: an extended dietary one-generation reproductive toxicity study (MRID 47972101, 2010). The results from this study are presented with the OSRI in Appendix 2.

Female Pubertal Assay; (OCSPP 890.1450)

The requirement for a Tier 1 female pubertal assay is satisfied by OSRI: an extended dietary one-generation reproductive toxicity study (MRID 47972101, 2010). The results from this study are presented with the OSRI in Appendix 2.

Male Pubertal Assay; (OCSPP 890.1500)

The requirement for a Tier 1 male pubertal assay is satisfied by OSRI: an extended dietary one-generation reproductive toxicity study (MRID 47972101, 2010). The results from this study are presented with the OSRI in Appendix 2.

Steroidogenesis Assay; (OCSPP 890.1550)

In a steroidogenesis assay (MRID 48614305), H295R cells cultured *in vitro* in 24-well plates were incubated with 2,4-D (98.5% purity, Lot # 2006 2433 8006-USA) at log concentrations of 10^{-10} to 10^{-4} M for 48 hours in triplicate in three independent experiments. Dimethyl sulfoxide (DMSO) was used as the vehicle, at a final concentration in the assay of 0.1%.

Testosterone and estradiol levels were measured using LC-APPI-MS/MS. A Quality Control (QC) plate was run concurrently with each independent run of a test chemical plate to demonstrate that the assay responded properly to positive control agents at two concentration levels. The positive controls included a known inhibitor (prochloraz) and a known inducer (forskolin) of estradiol and testosterone production.

Guideline acceptability recommendations and requirements were generally met, including lack of cytotoxicity, adequate production of testosterone and estradiol, acceptable reproducibility (low %CV), and appropriate induction and inhibition with positive controls, with two exceptions. The required concentration for estradiol production (40 pg/mL) was not met in any run for blank or solvent control, with values ranging from 26.7 to 38.3 pg/mL, but the basal concentrations were greater than 2.5-fold of the minimum detection level (10 pg/mL) as required. Secondly, all three

runs of 1 μM prochloraz only reduced estradiol to 0.6-fold that of the solvent control (instead of 0.5-fold).

2,4-D had no effect on testosterone production at concentrations up to 10^{-4} M, and no effect on estradiol production at concentrations up to 10^{-5} M. At the highest concentration tested (10^{-4} M), 2,4-D increased ($p \leq 0.05$) estradiol levels by 20% (1.2-fold) in all three runs relative to the DMSO-treated cells.

Based on hormone responses in each of the independent runs, 2,4-D treatment resulted in statistically significant and reproducible increases in estradiol production. 2,4-D treatment did not result in statistically significant and reproducible alterations in testosterone production.

Uterotrophic Assay; (OCSPP 890.1600)

The requirement for an uterotrophic assay is satisfied by OSRI: an extended dietary one-generation reproductive toxicity study (MRID 47972101, 2010). The results from this study are presented with the OSRI in Appendix 2.

APPENDIX 2. Other Scientifically Relevant Information (OSRI)

Published literature and Part 158 studies considered relevant for determining the potential interaction of 2,4-D with the endocrine system are summarized below. Given the breath of toxicity data available, the only Part 158 studies discussed in this appendix are those that fulfill current guideline requirements and are directly relevant to assessing potential endocrine effects (i.e. reproductive and developmental studies, subchronic and chronic toxicity studies, special mechanistic studies, etc.). In addition, recent re-evaluations of 2,4-D by the Agency (USEPA, 2012) were considered in summarizing the available information.

ER Binding

In a non-guideline published study, **Blair *et al.*, 2000** screened a large, structurally diverse group of chemicals for estrogen receptor (ER) affinity using a standardized ER competitive-binding assay. The assay was tested using multiple chemicals including the EDSP List 1 chemicals 2,4-D, atrazine, carbaryl, metolachlor and simazine; only data for the List 1 chemicals are presented herein.

Uterine cytosol from ovariectomized Sprague-Dawley rats was used as the ER source for the competitive-binding assay. Test compounds were first screened at high concentrations to determine whether the compounds competed with [^{3}H]-estradiol (E2) for the ER. Compounds that demonstrated affinity for the ER were further assayed with a wide range of concentrations to determine the IC_{50} and relative binding affinity (RBA) values of the compounds.

Adult (~245 days old; retired breeders), non-pregnant female Sprague-Dawley rats (NCTR:SDN, an outbred albino rat stock originating from CRL:CD [SD]BR rats) were ovariectomized (mean n = 14 rats/cytosol batch) 10 days prior to receptor preparation. After sacrifice by CO_2 asphyxiation, the uteri were excised, trimmed of excess fat and mesentery, weighed and placed in ice-cold TEDG buffer that included 10 mM Tris, 1.5 mM EDTA, 10 mM dithiothreitol, and 10% glycerol (pH 7.4). The pooled uteri were homogenized at 4°C in fresh, ice-cold TEDG buffer (1 g tissue/10 mL buffer). The resulting homogenate was centrifuged at 105,000 × *g* for 60 min at 4°C, separated into 3-mL portions, and stored at –70°C until used in the competition assays.

The test compounds 2,4-D (99% purity), atrazine (98%), carbaryl (99%), metolachlor (98.7%), and simazine (99%) were obtained from Supelco. The preparation of the test compounds including the solvent used was not reported in the article.

In the competitive binding assay, 1×10^{-9} M [^{3}H]-E2 (10 µL) was incubated with 10 µL of increasing concentrations of the test compound, 50 µL of uterine cytosol preparation, and 230 µL of 50 mM Tris buffer in duplicate tubes, and incubated at 4°C for 20 hours. After incubation,

750 µL of a cold 60% hydroxylapatite (HAP) slurry in 50 mM Tris buffer was added to separate the bound ligand from the free ligand, and the tubes were incubated in an ice water bath for 20 minutes, and then centrifuged at 4 °C. The resulting HAP pellet was resuspended in 2.0 mL of cold 50 mM Tris buffer and centrifuged again. After three washes, the supernatant was discarded and 2.0 mL of cold ethanol was added to extract the radiolabeled E2 from the HAP. Tubes were incubated on ice for 15 min, centrifuged at 4°C, and the resulting supernatant was decanted into vials containing 10 mL of scintillation cocktail. Radioactivity was measured with a liquid scintillation analyzer.

Each assay also included a "zero" tube (no competitor added; representing the total binding of [^{3}H]-E2; average ~15,000 dpm) and E2 standard curve (1×10^{-7}, 1×10^{-8}, 1×10^{-9}, 3.33×10^{-10}, 1×10^{-10}, and 3.33×10^{-11} M concentrations) for quality control. The 1×10^{-7} M E2 tube contained a 100-fold molar excess of radioinert E2 (compared to [^{3}H]-E2), and represented non-specific binding (NSB; average ~800 dpm). Radioactivity counts (dpm) of the NSB tubes were subtracted from all tubes prior to calculation of percent bound [^{3}H]-E2. Data for each test compound and the E2 standard curve were plotted as percent bound [^{3}H]-E2 versus molar concentration, and the IC_{50} (50% inhibition of [^{3}H]-E2 binding) for each test compound was determined. The RBA for the test compound was calculated by dividing the IC_{50} of E2 by the IC_{50} of the competitor and was expressed as a percentage (E2=100).

Compounds with a log RBA of >0 were considered strong binders by the study authors, moderate binders had a log RBA between 0 and –2, and weak binders had a log RBA of <–2. As expected, all of the tested steroidal estrogens, synthetic estrogens, and anti-estrogens demonstrated an affinity for the ER under the conditions of the assay. 17β-Estradiol had a mean IC_{50} of 8.99×10^{-10} M.

RBAs for the EDSP List 1 chemicals 2,4-D, atrazine, carbaryl, metolachlor, and simazine could not be calculated as the EC_{50} estimates were $>1.00 \times 10^{-4}$ (2,4-D, atrazine, carbaryl, and metolachlor) or $>3.33 \times 10^{-5}$ (simazine). Therefore, these compounds were not considered binders of the ER.

ER and AR Activation

In a published, non-guideline study, **Kojima *et al.*, (2004)** screened and evaluated 200 pesticides (including 2,4-D), covering nine chemical classes, for their potential to interact with two human estrogen receptor subtypes (hERα and hERβ) and one human androgen receptor (hAR) in transactivation assays with Chinese hamster ovary cells (CHO-K1 cells).

The human estrogen receptor (hERα) and androgen receptor (hAR) expression vectors (pcDNAERα and pZeoSV2AR, respectively) were constructed as described previously (Kojima et al, 2003). The hERβ expression vector was constructed by cloning the hERβ cDNA with reverse transcriptase-polymerase chain reaction from human placental RNA. The sequence was verified and inserted into the mammalian expression vector pcDNA3.1Zeo(-) creating pcDNAERβ. The construction of the estrogen or androgen responsive elements containing reporter plasmid (pGL3-tkERE and pIND-ARE, respectively) was described previously (Kojima et al, 2003). pRL-SV40 containing the *Renilla* luciferase gene was used as an internal control for transfection efficiency.

For detection of hERα or hERβ activity, CHO cells were transfected with pcDNAERα or pcDNAERβ, 50 ng pGL3-tkERE, and pRL-SV40. For detection of hAR activity, cells were transfected with pZeoSV2AR, pIND-ARE and pRL-SV40. After a 3 h transfection period cells were dosed with various concentrations of test compounds (95-100% purity) or with 0.1% DMSO (vehicle control) in complete medium. For measurement of antagonistic activity to hERα, hERβ and hAR, either 10^{-11} or 10^{-10} M 17β-estradiol (E_2, >97% purity) or 10^{-10} M 5α-dihydrotestosterone (DHT, 95% purity) was added to the cell cultures along with the test compound, respectively. Cells were incubated for 24 hours and luciferase activity was measured with a luminometer and was normalized based on the activity of cotransfected pRL-SV40. At least three independent experiments were conducted for each compound.

Agonistic activities were evaluated by relative activity expressed as 20% relative effective concentration (REC_{20}), the concentration of the compound showing 20% of the activity of 10^{-10} M E_2, 10^{-9} M E_2, or 10^{-9} M DHT for ERα, ERβ, and AR, respectively. Antagonistic activities were evaluated by relative activity expressed as 20% relative inhibitory concentration (RIC_{20}), the concentration of the compound showing 20% inhibition of the activity of 10^{-10} M E_2, 10^{-9} M E_2, or 10^{-9} M DHT for ERα, ERβ, and AR, respectively. The range of concentrations tested was $\approx 10^{-8}$ to 10^{-5} M; all pesticides were tested at concentrations $\leq 10^{-5}$ M to avoid cytotoxicity. When the activity of the test compound was higher than the REC_{20} the pesticide was judged to be positive for activity. When the activity of the test compound was higher than the RIC_{20} the pesticide was judged to be positive for inhibitory activity. The statistical significance of differences was evaluated with a two-tailed Student's *t*-test with a nominal level of significance of $p < 0.05$.

E_2 demonstrated dose-dependent transactivation of ERα and ERβ which indicated that both receptors can be activated at very low hormone concentrations. The maximal ERα activity was achieved at 10^{-10} M E_2 or more, exhibiting approximately 10-fold that of the control solvent. The maximal ERβ activity induced was 8.5-fold that of the solvent control at 10^{-9} M E_2 or more. Thus, E_2 was more potent for ERα than for ERβ. From the dose–response curves, REC_{20} values of E_2 for ERα and ERβ were determined to be 2.5×10^{-12} M and 5.3×10^{-12} M, respectively. 5α-DHT caused dose-dependent transactivation of AR and was detectable from 10^{-11} M DHT and

plateaued at 10^{-9} M DHT. The maximum induction was 21-fold that of the control solvent. The REC_{20} value of DHT for AR was 3.1×10^{-11} M. 2,4-D was negative for both ER (α and β) and AR agonist and antagonist activity in this test system.

ER Activation

In a published, non-guideline study, **Lemaire *et al.* (2006)** evaluated 49 pesticides (including 2,4-D; purity 95-100%) covering nine chemical classes, for ERα and ERβ activation or inhibition in stable reporter cell lines, HELN ERα and ERβ (HeLa cells stably transfected with an ERE-driven luciferase plasmid).

For the ERα and ERβ competitive-binding assay, ERα and ERβ cells were seeded at a density of 1.5×10^5 cells per well in 24-well tissue culture plates. Cells were labeled with 0.1 nM [^{3}H]-E_2 ([^{3}H]-17β-estradiol, 41.3 Ci/mmol specific activity) in the presence or absence of increasing concentrations of cold E_2 (as control) or each of the tested compounds in 400 µl of DMEM supplemented with 6% DCC-FBS.

The effect of pesticides on cell viability was assessed with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) toxicity assay. Treatment with 2,4-D, as well as all other pesticides, did not result in cytotoxicity in the study.

To evaluate the estrogenic potential of pesticides, HELN ERα and HELN ERβ were used to identify the active compounds and discriminate between ERα and ERβ activities. To determine ERα and ERβ agonistic activity of pesticides, the relative transactivation activity on HELN ER cells was computed as the ratio of luciferase reporter gene induction values of each compound at a concentration of 10 µM to the luciferase reporter gene induction value of E_2 at 10 nM. Antagonistic effects of pesticides in the ERα and ERβ transactivation assay were determined over a range of five concentrations from 10^{-7} to 10^{-5}.

To confirm the ER antagonist effects in the transactivation assays reflected an inhibition of E2 binding to ERα and/or ERβ, whole cell competitive binding assays were conducted with the HELN ERα and ERβ cells. The binding affinity for ER of anti-estrogenic pesticides was assessed with 0.1 nM [^{3}H]-E_2 as a tracer. As expected, non-radiolabelled E_2 displaced [^{3}H]-E_2 by 50% at a concentration of 0.12 nM for ERα and 0.18 nM for ERβ, and ICI 164,384 inhibited [^{3}H]-E_2 binding by 50% at the concentration of 2.5±0.5 nM for ERα and 3.3±1.0 nM for ERβ.

2,4-D was reported negative with a percent activity in ERα and ERβ of 8.4 ± 1.4% and 10.2 ± 2.1%, respectively, which was not statistically different from the solvent DMSO (9.3 ± 1.3% and 10.8 ± 1.6%, respectively).

ER Activation

In a published, non-guideline study, **Hurst and Sheahan, 2003)** screened 26 pesticide compounds (including 2,4-D) and storm water from two agricultural catchments for estrogenic activity using an *in vitro* recombinant yeast-based reporter assay. The assay screens for the binding and activation of human estrogen receptor (hER-α) by measuring estrogenic activity relative to 17β-estradiol (E2).

The pesticide standards used in the study were obtained from Dr. Ehrenstorfer (GmbH); purities ranged 95-99.5%. The yeast assay uses a genetically modified yeast strain in which hER-α has been integrated into the yeast genome, together with expression plasmids carrying estrogen-responsive elements (EREs) which control the expression of the reporter gene *Lac-Z.* In the presence of estrogens or chemicals with estrogenic activity, the yeast synthesizes β-galactosidase which is secreted into the assay medium. The β-galactosidase breaks down the chromogenic substrate chlorophenol red β-galactopyranoside (CPRG) into a product that is quantified by absorbance.

For the initial screening, yeast cells were combined in microtitration plates with pesticide standards dissolved in ethanol at 12 different concentrations (not reported). A dilution series of E2, as the positive control and estrogenic standard, and an ethanol solvent blank were assayed along with the pesticide standards. An estrogenic response was defined as an increase by the pesticide standard of at least 0.1 optical density (OD) units color (at 540 nm) in comparison with the solvent control. The estrogenic potency was reported as the potency relative to E2, where E2 has a potency value of 1; the lowest observed effect concentration (LOEC) for an estrogenic response was also determined.

In this test system, 2,4-D did not demonstrate an estrogenic response up to cytotoxic concentrations. A toxic response (inhibition of yeast growth) was observed at 50 mg/L.

ER Activation/Proliferation

In a published non-guideline study, **Soto et al, 1995**, using the E-SCREEN assay assessed the potential estrogenicity of environmental chemicals using the proliferative effect of estrogen and estrogenic chemical on and estrogen-sensitive target cell line. This quantitative *in vitro* assay compares the cell number achieved by similar inocula of human breast cancer estrogen-sensitive (MCF-7) cells in the absence of estrogens (negative control), and in the presence of 17β-estradiol (E_2, positive control) and a range of concentrations of chemicals suspected to be estrogenic. The assay was developed based on the premise that a human serum-borne molecule specifically inhibits the proliferation of human estrogen sensitive cells and estrogens or estrogen-like chemicals induce cell proliferation by blocking this inhibitory effect. The aim of the work was

to: 1) validate the E-SCREEN Assay, 2) screen a variety of chemicals, 3) examine whether the effects of different chemicals act cumulatively and 4) assess the reliability of the assay for use as a screening tool.

Proliferation rates are measured by comparing the cell yield achieved by similar inocula harvested simultaneously during the late exponential phase of proliferation. The proliferative effect is measured as the ratio between the highest cell yield obtained with the test chemical and the hormone free control. In this experimental design, MCF-7 cell yields were measured after 6 days of exposure and differences between treatment and positive (E2) control were apparent after 4 days. Estrogenic activity was assessed by 1) relative proliferative potency (RPP) which is the ratio between the minimal concentration of estradiol needed for maximum cell yield and the minimum dose of the test compound needed to achieve a similar effect, and 2) measuring the relative proliferative effect (RPE) which is 100 times the ratio between the highest cell yield obtained with the chemical and with E2l. The RPE allows one to quantitatively compare the agonist effect relative to E2. Results were expressed as mean ± SE. Proliferation yield experiments were conducted in duplicate wells with a minimum of 5 replicated. Differences between groups were assessed by analysis of variance and the a posteriori Shaffe's test. A p value ≤ 0.05 was regarded as significant. 2,4-D was tested over a concentration range of 10^{-9} to 10^{-3} M and found to have no activity in the E-SCREEN Assay.

ER, AR and TR Transactivation

In a published, non-guideline study, **Sun, *et al.* (2012)** developed receptor-related luciferase reporter gene assays to evaluate and compare estrogen receptor (ER), androgen receptor (AR) and thyroid hormone receptor (TR) activities of target chemicals. The assay was tested using 2,4-dichlorophenoxyacetic acid (2,4-D), chlorpyrifos, di-2-ethylhexyl-phthalate, and bisphenol A; only data for 2,4-D are discussed herein.

All the assays utilized Vero (African green monkey kidney) cells which do not contain the endogenous receptors. For the ER Reporter Gene Assay, the cells were transfected with pERE-TATA-Luc, rERα/pCl, and phRL-tk (used as internal control to assess transfection efficiency and cytotoxicity of test chemicals). For the AR Reporter Gene Assay, the cells were transfected with pMMTV-Luc, AR/pcDNA3.1, and phRL-tk, and for the TR Reporter Gene Assay, the cells were transfected with pUAS-tk-luc, pGal4-L-TR, and phRL-tk.

Agonistic activity was reported as the 20% relative effective concentration (REC_{20}), the concentration at which the tested chemical showed 20% of the maximum activity of the positive control (E_2, testosterone, or T_3). Antagonistic activity was reported as the 20% relative inhibitory concentration (RIC_{20}), the concentration at which the tested chemical induced a 20% reduction of activity in E_2, testosterone, or T_3. The REC_{20} and RIC_{20} were calculated using nonlinear regression analysis with a sigmoid dose-response (variable slope) curve. Datasets were tested for homogeneity of variance and normality; all datasets met these criteria. Data were

then analyzed by one-way ANOVA followed by Duncan's multiple comparisons test when appropriate. Statistical significance was determined at $p<0.05$. Hormone agonistic treatments were compared to the vehicle (DMSO) control group and antagonistic treatments were compared to the positive control (E_2, testosterone, or T_3) group.

The cytotoxicity of the test chemicals was determined prior to conducting the receptor assays. The test chemicals did not affect the viability or proliferation of the Vero cells with or without E_2, testosterone, or T_3. All three positive controls exhibited appropriate concentration-dependent responses in the respective assays. Based on the concentration-response curves, the EC_{50} values were 1.78×10^{-6} mg/L for E_2, 3.32×10^{-4} mg/L for testosterone, and 2.09×10^{-3} mg/L for T_3.

2,4-D showed no ER, AR or TR agonist or antagonist activity at 0.003-3.0 mg/L in this test system. The authors, however, suggested that 2,4-D may have acted as a synergist at 3.0 mg/L in the antagonist assay, significantly ($p<0.01$) enhancing the effects of testosterone.

AR Activation

In a published, non-guideline study, **Kim *et al*., 2005**, characterized the androgenic activity of 2,4-D (>98% pure) and its metabolite 2,4-dichlorophenol (DCP) with 5α-dihydrotestosterone (DHT) using an *in vitro* mammalian system with prostate cancer cell lines. Additionally, the molecular mechanisms of phenoxy compound action related AR activation were investigated.

Two human prostate cancer cell lines were used in the study: AR expressed 22Rv1 (derived from a relapsed tumor) and androgen-independent PC3 (androgen-insensitive cell line used for validation). African green monkey kidney cells (COS-1) were used for the *in vitro* AR-binding assay.

In the cell proliferation assay, the 22Rv1 cells were seeded in 96-well plates (3×10^3 cells/well) and DHT was added to the experimental medium (phenol red-free RPMI 1640 containing 10% dextran charcoal-stripped FBS) 24 hours later. The bioassay was terminated on the fourth day and the cells were fixed and stained with sulforhodamine-B (SRB). Briefly, the cells were treated with 10% trichloroacetic acid and incubated at 4°C for 1 hour, washed with phosphate-buffered saline (PBS), and the proteins stained with 0.4% SRB at room temperature for 1 hour. The wells were washed with acetic acid, air dried, and the bound dye solubilized with 10 mM Tris base (pH 10.5). The amount of SRB product in each well was determined with a plate reading spectrophotometer at 570 nm; the assays were performed in triplicate for each cell line.

In the transactivation assays, the 22Rv1 and PC3 cells (1.2×10^5) were seeded in 24-well plates and transfected with mouse mammary tumor virus luciferase vector (MMTV-Luc) and pCMV-hAR expression vector or with MMTV-Luc alone using ExGene 500. After transfection (24 hours), DHT and the test chemical were added at specified concentrations with the experimental

medium. After another 24-hour incubation, the cells were lysed and the extracts were assayed for luciferase activity with a dual-luciferase reporter system and liquid scintillation counter luminometer. Luciferase activity was normalized for transfection efficiency using pRL-TK as the internal control. Cell cytotoxicity was determined by using the colorimetric MTS cell cytotoxicity assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay).

For the competitive steroid binding assays, whole-cell binding assays were performed. Briefly, COS-1 cells were transfected with pCMV-hAR in the presence of lipofectamine reagent. The cells were placed in serum-free, phenol red-free medium and incubated with [^{3}H]5α-DHT for 2 hours at 37°C in the presence and absence of increasing concentrations of the test compounds. Nonspecific binding of [^{3}H]5α-DHT was determined by adding a 100-fold molar excess of unlabeled 5α-DHT. Cells were washed in PBS, harvested in buffer containing 2% SDS, 10% glycerol, and 10 mM Tris (pH 6.8), and the radioactivity was determined with a scintillation counter.

For fluorescence imaging, the PC3 cells were cultured and the GFP-AR expression vector was transiently transfected with ExGene500 reagent. After 30 hours, the transfected cells were mixed with fresh medium containing 10% dextran charcoal-stripped FBS, and treated for 30 min with the vehicle (ethanol), 10 nM DHT, or 10 nM DHT in combination with each of the test chemicals also at 10 nM. Next, the cells were fixed with 10% formaldehyde, rinsed with PBS, dried, and fluorescence was analyzed with a confocal laser system connected to a fluorescence microscope. For the evaluation of GFP-AR localization, the relative nuclear and cytoplasmic fluorescence in >100 cells per condition from three experiments was determined and scored according to a 5-grade nuclear localization score: exclusive nuclear fluorescence (4); nuclear fluorescence exceeding cytoplasmic fluorescence (3); equivalent nuclear and cytoplasmic fluorescence (2); cytoplasmic fluorescence exceeding nuclear fluorescence (1); and exclusive cytoplasmic fluorescence (0).

The study authors concluded that 2,4-D and DCP in combination with DHT have androgenic activity in cell proliferation and androgen-induced transactivation, possibly through promotion of AR translocation to the nucleus. In the *in vitro* assay systems, while 2,4-D or DCP alone did not show androgenic activity, 2,4-D or DCP with DHT exhibited synergistic androgenic activities. Co-treatment of 2,4-D or DCP with DHT stimulated cell proliferation by 1.6-fold, compared to DHT alone. In transient transfection assays, androgen-induced transactivation was also increased to a maximum of 32-fold or 1.28-fold by co-treatment with 2,4-D or DCP with DHT, respectively. However, 2,4-D and DCP had no effect on either mRNA or protein levels of the AR. In a competitive AR binding assay, 2,4-D and DCP inhibited androgen binding to the AR (up to 50% at concentrations of approximately 0.5 mM for both compounds). The nuclear translocation of green fluorescent protein-AR fusion protein in the presence of DHT was further promoted with the addition of 2,4-D and DCP. The authors therefore concluded that the results

indicate that 2,4-D and DCP enhancement of DHT-induced AR transcriptional activity may be attributable, at least in part, to the promotion of AR nuclear translocation.

ER/AR Activation and Ovarian Steroidogenesis

In a published study, **Orton et al. (2009)**, investigated eleven pesticides (including 2,4-D (>97% pure)) for AR/ER related activity in a recombinant yeast screen. The same chemicals were also evaluated for effects on the ovulatory response and ovarian steroidogenesis in *Xenopus* oocytes. In the yeast (anti-) estrogen screen (YES) and yeast (anti-) androgen screen (YAS), activation of transfected human estrogen or androgen receptor results in a color change in the media as measured by absorbance at 540 nm. Turbidity was monitored to check for any cytotoxic effects. It was reported that 2,4-D did not elicit (anti-) estrogenic or (anti-) androgenic activity in this test system (no data presented).

In the ovulation assay, ovaries from sexually mature *Xenopus laevis* were removed, cultured in Modified Barth's Medium, and cut into 10 oocyte fragments. The oocytes were incubated with 6.25, 12.5, 25, and 50 IU of human chorionic gonadotropin (hCG) or control media for 20 h. The media was sampled and hormone analysis was performed by RIA. After incubation, a submaximal concentration of hCG (~60% of maximum ovulatory response) was chosen for co-incubation with pesticides at concentrations of 6.25 and 62.5 µM. 2,4-D had no significant effect on the ovulatory response or steroid hormone production in this ovulation assay (no data presented).

90-Day (Subchronic) Oral Toxicity in Rodents (Rat)

A 13-week subchronic oral toxicity study (MRIDs 00101599 and 00102451, 1981) with 2,4-D (97.73% purity; Lot/Batch # V602 Tank) in Fischer 344 rats was conducted on 15 animals/sex/group administered at target doses of 0, 15, 60, 100 or 150 mg/kg/day. Statistical significance is presumed to be α=0.05, but is illegible in the available copy of the DER.

All rats survived until scheduled termination. Although body weights and food consumption were recorded throughout the study, achieved doses were not reported. No overt toxicity was observed, but one male in the high-mid-dose group (100 mg/kg/day) lost weight on two days mid-study (Days 55 and 62), with subsequent weight recovery until Day 91. Decreased mean body weights were observed beginning on Day 41 in the high-dose males, and high-mid- and high-dose females, and continuing through study termination. Decreased mean body weight also was observed beginning on Day 59 in the low mid- and high-mid-dose males, and continuing through study termination. Body weight gains were decreased (NS) in the high-dose animals, and food consumption was occasionally decreased in the high-dose animals throughout the

study. Terminal body weights were decreased in the high-dose animals, with lesser decreases (NS) in the high-mid-dose group.

Concentrations of T_4 were increased in the high-mid-dose males, and the low and low-mid-dose females, but decreased in the high-mid- and high-dose females. The primary target organ was the kidney (see below) with concomitant increases in kidney weights in all males, and females in the high-dose group. Liver weights were affected in the high-dose animals, but these changes were secondary to decreased terminal body weights.

Histopathology was conducted on 10 animals/sex/group. A degenerative change was seen in the kidneys of all male rats treated with ≥60 mg/kg/day (low-mid dose) and in 1/10 male rats in the low-dose group. The degree of degeneration was more pronounced in the two highest dose groups. The original pathology report of this study stated that there was an increased epithelial cytoplasmic homogeneity of the convoluted tubules in the kidneys of male rats treated with 2,4-D at ≥60 mg/kg. The interpretation of this finding was that there were decreased amounts of protein absorption droplets, which are primarily α2u-globulins which are sex-dependent urinary proteins of hepatic origin. Decreased amounts of protein droplets could represent changes in the nutritional status of the rats, a metabolic change in the liver cells where α2u-globulins are produced, or a direct effect on the kidneys.

The dose of 60 mg/kg/day exceeded the maximum-tolerated dose as evidenced by cellular alterations in the kidneys of both sexes. The 15 mg/kg/day dose was considered the NOEL for female rats; in male rats this dose produced no effects except for a slight increase in relative kidney weight.

90-Day (Subchronic) Oral Toxicity in Rodents (Rat)

In a subchronic oral toxicity study (MRID 41991501, 1991), 10 Fischer 344 rats/sex/group were administered 2,4-D (96.1% purity; Lot/Batch #909) in the diet for 13 weeks at target doses of 0, 1, 15, 100, and 300 mg/kg/day. Body weights and food consumption were recorded throughout the study. Average daily compound intake during the study was 0.93, 13.98, 93.93, and 278.39 mg/kg/day for males and 0.96, 14.39, 96.16, and 293.42 mg/kg/day for females.

There were no treatment-related deaths. Clinical signs of toxicity occurred mainly in the high-dose females, and demonstrated as hunched posture, depressed activity, and few/no feces (included four high-dose males at Week 1). There was a dose-related increase in the incidence of pale/opaque eyes in both sexes. Decreased ($p<0.05$) body weights were observed during the study in the high-dose males and females, with the magnitude of the deficit increasing with time (males ↓15%/23% and females ↓21%/28% at Weeks 6/13, respectively). Decreased body-weight gains (Weeks 0-6: males ↓28%/females ↓50%; Overall: males ↓37%/females ↓57%) were observed in the high-dose animals. At the high mid-dose, decreased body weight (↓7% at Week 13; both sexes) and body-weight gain (Overall: males ↓9%/females ↓11%) were observed.

A corresponding decrease in food consumption was observed in both sexes at the two highest doses.

Decreased ($p<0.05$) T_3 and T_4 concentrations were observed at the high-mid- and high-doses at both intervals, except for T_3 concentrations in the high-mid-dose males (Weeks 6 and 13) and in the high-dose females at Week 6 (NS). Changes ($p<0.05$) in absolute and/or relative organ weights [including adrenals (↑ males; ↓ females), testes with epididymides (↑), ovaries (↓), pituitary (↑ males; ↓ females), liver (↑ males and females), and thyroids/parathyroids (↑ males and females)] were observed primarily at the high-dose in both sexes, and many of these changes may be attributable to decreased body weight. Gross findings, mainly in the high-dose group, included small testes and epididymis and opaque eyes (females). Treatment-related histopathological changes were observed primarily in the high-dose animals and included centrilobular hepatocellular hypertrophy, atrophy of the testes, hypertrophy of the zona glomerulosa (adrenal cortex, both sexes)' and follicular cells (thyroid, females). Many of the lesions correlated well with the alterations observed in hematology and clinical chemistry parameters and/or organ-weight data of the high-dose animals.

The LOAEL is 100 mg/kg/day based on decreased body weight/body-weight gain, alterations in some hematology and clinical chemistry [decreased T_3 (females) and T_4 (both sexes)] parameters, and cataract formation in females. The NOAEL is 15 mg/kg/day.

90-Day (Subchronic) Oral Toxicity in Rodents (Mouse)

In a 90-day oral toxicity study (MRID 41991502, 1991), male and female B6C3F1 mice were fed diets containing 2,4-D (96.1% purity; Lot/Batch #909) at target doses of 0, 1, 15, 100, or 300 mg/kg/day for 13 weeks. Body weights and food consumption were recorded throughout the study. The average daily compound intake during the study was 0.98, 14.71, 98.2, and 292.61 mg/kg/day for males and 0.99, 14.84, 98.85, and 295.88 mg/kg/day for females.

There were no adverse treatment-related effects on survival, body weight, body weight gain, food consumption, and gross pathology up to the high intermediate dose (100 mg/kg/day). Treatment-related effects at the high-mid dose included decreased glucose ($p<0.05$; females) and T_4 concentrations (statistical analysis confounded; males), and increases in mean absolute and relative kidney weights (females). Treatment-related effects at 300 mg/kg/day included transient decreased ($p<0.05$) food consumption (up to Week 7); decreased glucose ($p<0.05$; females) and T_4 concentrations (statistical analysis confounded; males); and histopathological changes in the liver characterized by nuclear hyperchromatism and decreased glycogen in periportal hepatocytes. Under the conditions of this study, a NOEL of 15 mg/kg/day and a LOEL of 100 mg/kg/day is established for the 90-day oral toxicity of 2,4-D to male and female mice. The LOEL is based on decreased glucose and T_4 concentrations, and increased mean and relative kidney weights, observed at 100 mg/kg/day.

28-Day (Subchronic) Inhalation Toxicity in Rodents (Rat)

In a subchronic inhalation toxicity study (MRID 47398701, 2008), 2,4-D *(*99% purity, Lot/Batch #2006 24833 8006-USA) was administered to 10 or 20 SD CD® rats/sex/concentration by dynamic (nose only) exposure at concentrations of 0, 0.05, 0.10, 0.30 or 1.00 mg/L for 6 hours per day, 5 days/week for 28 days (for a total of at least 20 exposures). Body weights and food consumption were recorded throughout the study.

After nose-only inhalation exposure, 2,4-D was associated with portal-of-entry effects that consisted of squamous metaplasia and epithelial hyperplasia with increased mixed inflammatory cells within the larynx. The incidence and severity of the effects were increased in a dose-related manner, and the effects persisted after the 4-week recovery period, although the incidence and severity were reduced. Clinical signs associated with exposure at the high dose included excessive salivation (Day 13 and subsequently), labored breathing (Day 13 and subsequently), and chromodacryorrhea (Day 12 and intermittently thereafter). A slight decrease in body weight was observed in the high-dose females by Day 14 and continued throughout the remainder of the dosing (↓10%) and the recovery periods (↓12%). Body-weight gain was reduced in the high-dose females throughout the study and recovery period, and was accompanied by a reduction in food intake. Alkaline phosphatase concentrations were increased ($p<0.05$ and 0.01, respectively) in the mid-high and high-dose females, and aspartate aminotransferase concentrations were increased ($p<0.01$) in the high-dose females, but no correlating microscopic pathology findings in the liver were observed. Lung weights were unaffected by treatment. High-dose females displayed slight reductions in spleen ($p<0.05$) and thymus (NS) weights; organ weights were comparable among the male groups.

The systemic toxicity LOAEL is 1.0 mg/L/day, based on increased alkaline phosphatase and aspartate aminotransferase concentrations, and decreased spleen weights, in females. The NOAEL is 0.30 mg/L/day. The LOAEL for portal-of-entry effects (squamous metaplasia and epithelial hyperplasia with increased mixed inflammatory cells within the larynx; not totally resolved following a 4-week recovery period) is 0.05 mg/L, the lowest dose tested. A NOAEL for portal-of-entry effects was not determined.

Prenatal Development in Rodents (Rat)

In a developmental toxicity study (MRID 00130407, 1983), pregnant Fischer 344 rats (35/group) were administered 2,4-D (97.5% purity; Lot/Batch #3/82) by gavage at doses of 0 (corn oil), 8, 25, and 75 mg/kg/day from gestation day (GD) 6 through 15. All surviving animals were euthanized on GD 20.

There were no treatment-related deaths. Clinical signs were comparable among the groups. Body weights were comparable among the groups throughout the study, but dams at the high-dose displayed a decrease (NS) in body-weight gain during the dosing period (79% of control for

GD 6-15; 57% of control for GD 6-10). The corrected body-weight gain was comparable among the groups.

There was a slight decrease in pregnancy rate with increasing dose, but the numbers of corpora lutea, implantations, and live fetuses were comparable among the groups, and there were no dead fetuses. The number of resorptions, as well as pre- and post-implantation losses, were not adversely affected by treatment. The number of the dams with 100% resorptions was similar (0 to 2) among study groups. Fetal body weight and crown-rump length were comparable among the groups, as was the sex ratio.

There were no treatment-related differences in the incidence of fetal external or visceral malformations. Although none of the increases attained statistical significance, they were attributed to treatment since some of the findings were also observed in the F1b pups of dams fed 2,4-D at 80 mg/kg/day (actual dose 75 mg/kg/day) in a 2-generation reproduction study (MRIDs 00150557 and 00163996) in the same strain of rat. Additionally, skeletal findings [2nd wavy ribs, lumbar ribs] and missing sternebrae were observed in another developmental toxicity study in the SD rat (Schwetz *et al.*, 1971) at a comparable 2,4-D dose of 87.5 mg/kg/day.

The maternal toxicity NOAEL is 25 mg/kg/day, and the maternal toxicity LOAEL is 75 mg/kg/day, based on decreased body-weight gain. The NOAEL for developmental toxicity is 25 mg/kg/day, and the developmental toxicity LOAEL is 75 mg/kg/day, based on an increased incidence of skeletal abnormalities.

Prenatal Development in Non-Rodents (Rabbit)

In a developmental toxicity study (MRID 41747601, 1990), artificially-inseminated female New Zealand White rabbits (20/group) were administered 2,4-D (96.1% purity; Lot/Batch #909) at doses of 0 (aqueous 0.5% methylcellulose), 10, 30, and 90 mg/kg/day from GDs 6 through 18. All surviving does were euthanized on GD 29.

There were no treatment-related deaths. Two high-dose does aborted (GDs 21 and 24). Treatment-related clinical signs of toxicity were observed in the does that aborted; ataxia was observed in both does, and decreased motor activity, loss of righting reflex, extremities that were cold to the touch, and dried feces were observed in the doe that aborted on GD 21. Body weights were comparable among the groups throughout the study, but body-weight gain was decreased at the high-dose (↓27%) during the dosing period (GDs 6-19; NS). On GDs 7-8, the low- and mid-dose groups showed no body-weight gain, and the high-dose group displayed a negative body-weight gain. On GDs 15-19, the high-dose group displayed no body-weight gain, and corrected body-weight gain was decreased at the high-dose (↓23%; NS).

Pregnancy rates were comparable among the groups; similar numbers of corpora lutea, implantations, and live fetuses were observed among the groups with no dead fetuses. One

control doe had 100% resorptions, and the number of resorption, and pre- and post-implantation losses, were comparable among the groups. Gravid uterine weights also were comparable among the groups.

No differences were observed in mean fetal body weight due to treatment. At the high-dose, there was an increase ($p<0.05$) in the percent of live male fetuses (71.2%) compared to control (52.8%) and the other dose groups (low: 54.4%; mid: 59.4%). There were no apparent differences in the incidence of external, visceral, or skeletal variations, anomalies, retardations, or malformations among the groups.

The maternal toxicity NOAEL is 30 mg/kg/day, and the maternal toxicity LOAEL is 90 mg/kg/day based on abortions, decreased body-weight gain, and clinical signs of toxicity. The developmental toxicity NOAEL is 30 mg/kg/day, based on abortions at the developmental toxicity LOAEL of 90 mg/kg/day.

Extended One-Generation Reproduction in Rodents (Rat)

In an extended dietary one-generation reproductive toxicity study (MRID 47972101), 2,4-dichloro phenoxyacetic acid (2,4-D; 97.85%-98.6% a.i.; lot # 2006 2433 8006-USA) was administered to 27 Crl:CD(SD) young adult rats/sex/dose via the diet at dose levels of 0, 100, 300, or 600 (females)/800 (males) ppm [equivalent to 0, ≈5, 15, or 30 (females)/40 (males) mg/kg bw/day] for approximately four weeks prior to mating and continuing through mating (up to 2 weeks), gestation, and lactation. P1 males were exposed for a minimum of 11 weeks including 7 weeks from the initiation of the mating phase. P1 females were exposed until lactation day 22 (LD22). A satellite group of P1 females (12/dose) were subject to the same exposures as the P1 females on the main study (exposure for 4 weeks during the pre-mating, up to 2 weeks during the mating period and during gestation until termination on gestation day 17 (GD 17). Satellite males were not exposed to dietary 2, 4-D except during co-housing with satellite females during the mating period.

Doses were selected based on a combined analysis of toxicity and toxicokinetic (TK) data collected from the 2,4-D range-finding/TK study (MRID 47417901) and titration studies (MRID 47417902). The high dose was set at or slightly above the threshold for nonlinear kinetics. The range-finding study confirmed a gender-based difference in the renal clearance of 2,4-D in adult rats, and different high-dose levels were selected for the sexes. The male high dose was 40 mg/kg/day (dietary concentration of 800 ppm), slightly higher than the inflection point for nonlinear TK in male pups from PND 35 to adulthood. The female high dose was 30 mg/kg/day (600 ppm), which was clearly higher than the inflection point in female pups and adults throughout the entire (range-finding) study.

P1 Generation: A comprehensive evaluation of P1 male and P1 female reproductive system was conducted, including an evaluation of gonadal function, the estrous cycle, sperm parameters,

mating performance, conception, gestation, parturition and lactation, as well as survival, growth and development of the offspring. Selected systemic toxicity parameters were also evaluated in the P1 males and P1 females.

Satellite GD 17 Females: A satellite group of P1 females (12/dose) was included for assessments of selected systemic toxicity parameters, clinical chemistry/hematology, thyroid hormone levels, thyroid weights, plasma 2, 4-D levels, histopathology, and selected reproductive parameters during gestation (corpora lutea and implantation numbers).

F1 Generation: F1 offspring were evaluated for potential effects on the nervous system, immune system, reproductive and endocrine systems, thyroid function, and other systemic toxicity parameters. 2, 4-D plasma levels were also assessed in the F1 offspring. In-life parameters in all F1 offspring included clinical observations, body weights, feed consumption, anogenital distance, nipple retention and puberty onset. Selected F1 offspring were divided into three different groups (Sets 1, 2, and 3) at weaning (postnatal day 21; PND 21). Each set of F1 offspring was maintained on the test diet until PND 60 (Set 1b F1 offspring), ≈PND 70 (Sets 1a and 2a F1 offspring), or ≈PND 90-139 (Sets 2b and 3 F1 offspring).

Set 1a (10/sex/dose): assessment of general systemic and thyroid toxicity, which included clinical chemistry/hematology parameters, thyroid hormone assessment, and urinalysis (males only). Post-mortem evaluations in Set 1a (PND70) included gross pathology, organ weights and histopathology on a wide range of tissues, including thyroids.

Set 1b (10/sex/dose): developmental neurotoxicity (DNT) assessment, which included functional observational battery (FOB), motor activity and acoustic startle response (ASR). On PND 60, Set 1b animals were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry. A special stain (Luxol Fast Blue) was used to evaluate brain myelination.

Set 2a (10sex/dose): assessment of potential developmental immunotoxicity (DIT): examination of humoral immune function using the sheep red blood cell (SRBC) antibody-forming cell (AFC) assay on PND 70-74.

Set 2b (10/sex/dose): assessment of potential developmental immunotoxicity (DIT): examination of innate cellular immunity using the natural killer cell (NK) assay on PND 87-93.

Set 3 (23-27/sex/dose): assessment of reproductive/endocrine toxicity, which included estrous cycle evaluation and post-mortem evaluations that focused on reproductive organs, sperm assessment, and ovarian follicle counts on PND 139. TK analyses were conducted on Set 3 males and females on PND 63 and 84 to determine plasma 2, 4-D levels.

In addition, selected pups culled on PND 4 were used to assess thyroid hormone levels. Additional data were gathered from F1 offspring not assigned to Sets 1-3. On PND 22, unselected weanlings were either perfused for examination of neuropathology (12/sex/dose) or euthanized for assessment of systemic toxicity, which included thyroid hormone assessment, organ weights, and post-mortem examinations (gross pathology and histopathology) in 10/sex/dose.

Reproductive and selected data from the F1 generation were used to assess whether a second generation would be produced. None of the criteria were met (Table 1), and a second generation was not assessed in this study.

P1 Adult Rats: There were no treatment-related deaths or clinical signs of toxicity in either sex of P1 adults. Body weights and body-weight gains were comparable among the groups during the pre-mating and mating phases (both sexes) and during gestation and the latter part of lactation (dams). Prior to dietary adjustment of 2, 4-D concentration during the second week of lactation, the 600 ppm dams displayed a decrease in body weight (LD 7; ↓5%) and body-weight gain (LD 1-4; ↓64%), which is consistent with reduced food intake during the first week of lactation. The reduction in food intake can be attributed to the increase in the actual dose (≈65 mg/kg/day) above the targeted level (30 mg/kg/day) during this time. After dietary adjustment, food intake for the 600 ppm dams was above control levels.

There were no apparent treatment-related effects on hematology, differential white blood cell counts, and prothrombin time, and clinical chemistry and urinalysis parameters were comparable among the groups (both sexes). P1 males displayed increased kidney weights (absolute and relative) at 800 ppm, which were accompanied by histopathological findings (degenerative lesion in the proximal convoluted tubules in the outer zone of the medulla) and are consistent with previous findings that the kidney is a target organ. There were no treatment-related findings in the P1 female kidney. Decreased reproductive and accessory sex gland weights were observed at 300 ppm and/or 800 ppm. These changes, however, are related to the concurrent control being outside of the laboratory historical control range. P1 females at 600 ppm displayed increased uterine weights (↑17%, both absolute and relative), although statistical significance was not attained. There were no alterations in estrous cycle pattern in the 600 ppm P1 females compared to the control, and no significant difference in mean estrous cycle length in P1 females at any dose level compared to the control. There were no significant, treatment-related effects on sperm motility or progressive motility, no differences in testicular spermatid and epididymal sperm counts, and no differences in the proportion of abnormal sperm. Male and female mating, conception, fertility, and gestation indices were comparable among the groups, and post-implantation loss was comparable among the groups. Both the time to mating and gestation length were comparable among the groups.

GD 17 Satellite Females: All P1 satellite females survived to scheduled sacrifice, and body weights were comparable among the groups. Hematology and clinical chemistry parameters were comparable among the groups. Reproductive indices and the numbers of corpora lutea and implantations were comparable among the groups. There was a slight increase in resorptions at 600 ppm (0.9 vs 1.5), although there was wide variability (standard deviations exceed the means). There was a slight increase in post-implantation loss at 600 ppm (9.2 vs 5.5). It should be noted that this observation was not corroborated since post-implantation losses in the P1 adults of the definitive study were comparable amongst all dose groups. Both the 100 ppm and 600 ppm females displayed an increase in thyroid weight (↑9%), but there was no dose-response. There were no statistically significant, treatment-related differences in serum T3, T4, or TSH in the GD 17 satellite females. Although the 600 ppm GD 17satellite females displayed the predicted pattern of thyroid hormone changes (↓ T3 and ↓ T4 with ↑TSH levels) that suggest 2, 4-D exposure may adversely affect thyroid function at doses above the renal saturation clearance, the thyroid effects noted below renal saturation are not considered sufficiently robust to be adverse.

F1 Offspring: There were no treatment-related effects on the numbers of live or dead F1 pups born/litter or on pup survival or sex ratio. Slightly lower body weights were observed in the 600 ppm pups during early lactation, which coincided with the dams decreased food intake LD 1-4 and LD 4-7). Pup body weight (600 ppm) remained lower in the 600 ppm pups (↓6%) during PND 14-21. There was no significant, treatment-related difference in absolute or relative anogenital distance in either sex and no differences in nipple/areolae retention between control and high-dose groups in either sex. F1 males at 800 ppm displayed a 1.6 days delay in preputial separation (well within normal variability), which was accompanied by a very slight reduction in body weight compared to the control (↓2.1 grams; 99% of control). The age at vaginal opening was comparable among the groups of F1 females.

F1 Offspring Thyroid Assessments: PND 4 - There were no statistically-significant differences in serum T3, T4, or TSH in PND 4 culled pups. T4 was reduced to a similar extent in both sexes at the 300 ppm (↓14%-15%) and 600 ppm/800 ppm (↓12%-14%) dose levels, and female PND 4 pups showed an increase in TSH (↑19%) at 600 ppm. F1 PND 22 Weanlings - F1 PND 22 males displayed a statistically-significant reduction (↓28%) in T4 at 800 ppm, and F1 PND 22 females displayed a non-statistically significant reduction (↓20%) in T4 at 600 ppm. T3 was reduced in the males at 300 ppm (↓19%) and 800 ppm (↓13%), but there was no dose response. F1 PND 62-64 - Both sexes displayed increased TSH at 300 ppm (↑26%) and at 800 ppm (males ↑23%))/600 ppm (females ↑24%)), although the increase in males was not dose-related and none of the differences in thyroid hormone levels were statistically significant. T4 was decreased at 800 ppm in males (↓13%). Though these findings suggest that 2, 4-D exposure may adversely affect thyroid function at doses above the renal saturation clearance, the thyroid effects noted below renal saturation are not considered sufficiently robust to be adverse.

F1 Unselected Offspring (PND 22 weanlings): There were no effects on survival of the unselected weanlings used for systemic toxicity (non-perfused). All treated males displayed a decrease in body weight (↓9%-10%) compared to the control males. Decreased adrenal weights were observed in males at 800 ppm (absolute ↓37% and relative ↓29%). The decreases in kidney (↓15%), liver (↓18%), testes (↓15%), and thyroid (↓14%) weights observed in males at 800 ppm were slightly greater than the body-weight deficit of 10%. Organ weights were comparable among the groups of females. There were no significant differences in perfused absolute brain weights, cerebral lengths and widths or cerebellar lengths and widths in perfused F1 PND 22 weanlings of either sex. There were no neuropathological observations attributed to treatment in the perfused F1 PND 22 weanlings, and no treatment-related changes in myelin in either males at 800 ppm or females at 600 ppm.

F1 Offspring Set 1a (PND 70): All Set 1a pups survived to scheduled sacrifice. Males at 800 ppm displayed decreased body weight (↓11%-17%) and body-weight gains (↓11%-25%) throughout the study period, with the magnitude of the reduction lessening with time of exposure. Females displayed comparable body weight/gain among the groups. Platelet counts were reduced in the 800 ppm males but not in the females at any dose level. Both sexes displayed a slight increase in ALT (↑18%/25%) and an increase in triglyceride (↑31%/43%) levels. Although some of the decreases in organ weights observed in the 800 ppm males may be attributed to the 10% decrease in body weight at termination, the decreases in liver (↓16%), pituitary (↓14%), and adrenal glands (↓12%) might be related to treatment. Increased uterine weights (↑31% absolute and ↑32% relative) were observed at 600 ppm. Although statistical significance was not attained, the finding is considered treatment-related since a similar increase was observed at 600 ppm in the P1 and Set 3 F1 females. Increased ovarian weight (↑9%) was observed in the 600 ppm F1 Set 1a females, although statistical significance was not attained. Increased kidney weights (↑9% absolute and ↑11% relative) were observed in the females at 300 ppm and 600 ppm, although there was no dose-response and kidney weights were comparable among the male groups. Decreased thymus weights (↓12% absolute and ↓10% relative) were observed in females at 600 ppm and in Set 3 females at 600 ppm (↓14% absolute and ↓13% relative). An increased incidence of degeneration of the proximal convoluted tubule in the kidney was observed in males at 300 ppm and 800 ppm and in females at 600 ppm. Regarding the terminal stage of estrous, 2 of 10 females at 300 ppm and 3 of 10 females at 600 ppm displayed proestrus, whereas none of the 10 females in the control and 100 ppm groups displayed proestrus.

F1 Offspring Set 1b (PND 54-56): There were no significant differences in body weight/gain in either sex. There was an increase in the level of urination in all treated male groups compared to the control group, but there was no dose response. Developmental neurotoxicity results were not applicable to EDSP evaluation.

F1 Offspring Set 3 (PND 90 or 139): Reproductive Toxicity: There were no treatment-related deaths or clinical signs of toxicity. Terminal body weights were comparable among the groups (both sexes). No significant differences were observed in mean estrous cycle length at any dose level compared to the control. There were no significant, treatment-related effects on the numbers of small follicles, growing follicles, or total follicles. There were no significant, treatment-related effects on sperm motility or progressive motility, no differences in testicular spermatid and epididymal sperm counts, and no differences in the proportion of abnormal sperm between the control and 800 ppm males. Absolute (↓9%) and relative (↓8%) pituitary gland weights were significantly lower in the 800 ppm males and absolute (↓9%) and relative (↓10%) pituitary gland weights were non-significantly lower in the 600 ppm females. There was no associated histopathology in the pituitary glands. Uterine weights were increased at 300 ppm (↑10% absolute and ↑10% relative) and 600 ppm (↑10% absolute and ↑11% relative) compared to the controls. Thymus weights were decreased (↓14% absolute and ↓13% relative) in females at 600 ppm, although statistical significance was not attained. No histopathological changes were observed in the pituitary or thymus in either sex. A degenerative lesion was observed in the kidney (proximal convoluted tubule) in both sexes at 300 ppm and at 600 ppm/800 ppm. Ovarian follicle counts were comparable between the control and 600 ppm females (PND 139).

The parental systemic LOAEL is 800 ppm (45.3 mg/kg bw/day in males), based on nephrotoxicity manifested as increased kidney weights, and degenerative lesions in the proximal convoluted tubules in the main study P1 rats. The parental systemic NOAEL is 300 ppm (16.6 mg/kg bw/day in males). No toxicologically relevant effects were identified in P1 females or in the GD 17 satellite female groups at the highest dose tested (600 ppm; 40.2 mg/kg/day).

The thyroid toxicity NOAEL is established at 800/600 ppm (45.3 mg/kg/day in males and /40.2 mg/kg/day in females), the highest dose tested. The thyroid effects noted in the database were considered to be adaptive.

The offspring (F1 adults) LOAEL is 800/600 ppm (55.6 mg/kg bw/day in males and 46.7 mg/kg/day in females), based on kidney toxicity manifested as increased kidney weights and increased incidence of degeneration of the proximal convoluted tubules. The offspring NOAEL is 300 ppm (20.9/ mg/kg bw/day in males and 23.3 mg/kg/day in females).

The F1 offspring (PND 22) LOAEL is 800/600 ppm, based on decreased body weight observed throughout lactation. The offspring NOAEL is 300 ppm. The dose on a mg/kg/day basis for the PND 22 F1 offspring was not calculated.

The reproductive LOAEL is > 800/600 ppm (45.3 mg/kg bw/day in males, 40.2 mg/kg bw/day in females), based on the lack of effect on estrous cyclicity, (P1 females, satellite GD 17 dams, Set 3 F1 offspring) or reproductive indices (mating, fertility, time to mating, gestation length, pre- and post-implantation loss, number of corpora lutea (satellite GD 17 dams), sperm parameters, ovarian follicle counts, and reproductive organ histopathology). The reproductive NOAEL is

800/600 ppm (45.3 mg/kg bw/day in males, 40.2 mg/kg bw/day in females), the highest dose tested.

Chronic Oral Toxicity/Carcinogenicity in Rodents (Rat)

In a combined chronic toxicity/carcinogenicity study (MRID 43612001, 1995), 50 Fischer 344 rats/sex/group were administered 2,4-D (96.4%; Lot/Batch #909) in the diet for up to 24 months at target concentrations of 0, 5, 75, and 150 mg/kg/day. The achieved doses were respectively 4.77, 73.15, and 144.98 mg/kg/day in males and 4.89, 73.11, and 143.52 mg/kg/day in females. Additionally, 10 rats/sex/group were sacrificed at 12 months (interim sacrifice data were reported in MRID 43293901).

There were no treatment-related deaths or clinical signs of toxicity. Decreased ($p<0.05$) body weight was observed throughout the study in the high-dose group (males ↓4 to 8%; females ↓10 to 26%) and in mid-dose females (↓10 to 14%). At study termination, body weight decreases ($p<0.05$) in the high-dose males and females were 8 and 26%, respectively. The mid-dose females also displayed a decrease ($p<0.05$) in body weight at study termination of 14%. Body-weight gains were decreased ($p<0.05$) throughout the study in mid- and high-dose females, and high-dose males. Consistent with the decreased body-weight gains were decreases in food consumption in the same groups.

There were dose-related decreases ($p<0.05$) in T_4 values throughout the study in both sexes at the mid- and high-doses; decreases at 6- and 18-months in mid-dose males were NS. No treatment-related effects were observed on T_3 concentration at any dose. Thyroid weights were increased ($p<0.05$) in the mid-dose females and in both sexes at the high-dose at the 12-month interim sacrifice. At study termination, thyroid weights were increased ($p<0.05$) in both sexes at the mid- and high-doses, although the increase for mid-dose males was NS, and the increase at the mid-dose for both sexes was generally greater than at the high-dose. Decreased ($p<0.05$) testes weights were observed at the high-dose at both the interim and terminal sacrifices, and at the mid-dose at study termination (NS). Similarly, decreased (NS at 12 months; $p<0.05$ at 24 months) ovarian weights were observed at the high-dose at both sacrifice times and in the mid-dose females at study termination. The decreases in testes and ovarian weights are consistent findings in other studies on 2,4-D and its salts/esters.

Microscopically, there were increased incidences of lesions in the liver (altered tinctorial properties in mid-dose females, and both sexes at high dose), and thyroid (hyperplasia in high-dose males; hypertrophy and epithelial cells in high-dose females) at the interim sacrifice. At study termination, there was an increased incidence of increased size of the hepatocytes with altered tinctorial properties in both sexes at the high-dose. Tumor incidence was not affected by treatment.

The LOAEL is 75 mg/kg/day based on multiple endpoints, including decreased body-weight gain and food consumption in females, alterations in hematology and clinical chemistry parameters (including decreased T_4 in both sexes), increased thyroid weights (both sexes at study termination), and decreased testes and ovarian weights. There was no treatment-related increase in the incidence of any tumor. The NOAEL is 5 mg/kg/day.

Carcinogenicity in Rodents (Mouse)

In a carcinogenicity study (MRID 43879801 and 43597201, 1995), 50 B6C3F1 CRL BR mice/sex/group were administered 2,4-D (96.4% purity; Lot/Batch #909) in the diet for 104 weeks at concentrations of 0, 5 (both sexes), 62 (males)/150 (females), and 120 (males)/ 300 (females) mg/kg/day. Additionally, 10 mice/sex/group were sacrificed (interim) after 52 weeks. Body weights and food consumption were recorded throughout the study. The average achieved doses were 5.0, 61.9 or 128.6 mg/kg/day for male mice, respectively, and 5.01, 149.83 or 310.01 mg/kg/day for female mice, respectively.

There were no treatment-related deaths or clinical signs of toxicity in either sex. Body weight, body-weight gain, and food consumption were comparable among the male groups throughout the study. Females at the high-dose had a slightly lower body weight at the 3- and 6-month intervals ($p<0.05$; ↓4%). Body-weight gains were decreased ($p<0.05$) at all doses in the females at the 3-month interval (↓7%, ↓6%, and ↓14% at the low-, mid-, and high-dose, respectively), and the high-dose females had decreased ($p<0.05$) body-weight gain at the 6- and 12-month (↓9%) intervals. There were no consistent changes in food consumption in the female groups.

Thyroid parameters were not monitored, and reproductive organ weights were not evaluated. Gross pathology findings were comparable among the groups in both sexes. Microscopically, there was an increased incidence of lesions in the kidneys of both sexes in the mid- and high-dose groups. There was no treatment-related increase in any tumor type in either sex.

The LOAEL of 62 mg/kg/day (males)/150 mg/kg/day (females) is based on increased absolute and/or relative kidney weights, and an increased incidence of renal microscopic lesions. There was no treatment-related increase in the incidence of any tumor type. The NOAEL is 5 mg/kg/day.

Avian Reproduction Test (Quail) (OCSPP 850.2300)

Northern Bobwhite quail (*Colinus virginianus*) were fed diets containing 2,4-D (96.9% purity; Lot/batch not reported) at nominal concentrations of 0, 160, 400, and 1000 ppm (mean-measured concentrations of 0 (basal diet), 147, 382, or 962 ppm) for approximately 21 weeks in an avian reproduction study (MRID 45336401, 2000). There were no treatment-related mortalities, signs of toxicity, effects on adult body weight or feed consumption, or gross abnormalities observed upon necropsy. In addition, there were no treatment-related effects on reproductive parameters.

(The reviewer detected a significant reduction in eggs set/eggs laid at the highest treatment dose, 962 ppm. Despite being statistically significant, this endpoint was only reduced 4% and was not considered to be biologically significant by the reviewer.) The NOAEC was 962 ppm, the highest concentration tested, and the LOAEC was not determined (>962 ppm).

Fish Early Life-Cycle Toxicity Test (850.1400)

In an early life-cycle toxicity test with fathead minnow (*Pimphales promelas*), embyros (<24hrs old) were exposed to 2,4-D dimethylamine salt (2,4-D DMA; 99.3%) (MRID 41767701). Hatching success, larval survival, and growth were measured. The most sensitive parameter measured was length with effects observed at ≥28.4 mg a.i./L with no effect observed at 17.1 mg a.i./L (14.2 mg a.e./L).

In another early life-cycle toxicity test with fathead minnow (MRID 41737305), embryos were exposed to 2,4-D ethylhexyl ester (94.7%) for 32-days, at mean measured concentrations of 0.08, 0.13, 0.12, 0.22, 0.45 and 1.96 mg a.i./L. Hatching success, larval survival, and growth were measured. The most sensitive parameter measured was larval survival with a NOAEC of 0.12 mg a.i./L (0.0792 mg a.e./L) and a LOAEC of 0.22 mg a.i./L. However, large variability in measured test concentrations were observed.

APPENDIX 3: References Not Utilized in the 2, 4-D WoE Analysis

In 2009, after public review and comment, a final list of 67 chemicals and schedule for issuing Test Orders for the EDSP Tier 1 screening battery was made available in a Federal Register Notice issued October 21, 2009 (74 FR 54422). The agency's review of the initial data submitted as "other scientifically relevant information (OSRI) was provided in the Report of the Endocrine Disruptor Review Team (USEPA, 2010).

Beginning in 2011, the agency has reviewed data cited as "OSRI which included Part 158 studies previously submitted to the agency for registration/reregistration, published literature articles and/or Tier 1 assays. The agency also conducted a more recent search (2009 to 2014) of available scientific literature for any additional relevant information for their weight of evidence (WoE) evaluations. These articles were evaluated in accordance with the agencies Evaluation Guidelines for Ecological Toxicity Data in Open Literature, May 2011 (http://www.epa.gov/pesticides/science/efed/policy_guidance/team_authors/endangered_species_reregistration_workgroup/PDF_rot/esa_evaluation_open_literature.pdf) and the 2012 Guidance for considering and Using Open Literature Toxicity Studies to Support Human Health Risk Assessment (http://www.epa.gov/pesticides/science/lit-studies.pdf).

The following published and unpublished references were considered for use in the WoE analysis for 2,4-D but were not utilized due to one or more of the following reasons: 1) the article was not available in English; 2) the compound of interest was not used in the study; 3) the test material was not adequately described; 4) a formulated end-use product or mixture of chemicals was utilized as the test material; 5) only acute mortality toxicity data were provided; 6) the experimental conditions were not adequately described; 7) only an abstract of the study was available; 8) the reference is a review article or book chapter and does not contain primary study data; 9) insufficient information was available to adequately assess the validity of the study results; 10) the 40 CFR Part 158 guideline study was classified as unacceptable/inadequate; 11) the study dealt only with non-EDSP assay development; 12) no specific endocrine-related endpoints were assessed in the study; and 13) the study contained only data on invertebrates.

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