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An evaluation of 2,4-dichlorophenoxyacetic acid in the Amphibian Metamorphosis Assay and the Fish Short-Term Reproduction Assay

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ABSTRACT

2,4-Dichlorophenoxyacetic acid (2,4-D) was evaluated in both the Amphibian Metamorphosis Assay (AMA) and the Fish Short Term Reproduction Assay (FSTRA). In the AMA, tadpoles were exposed to mean measured 2,4-D concentrations of 0 (water control), 0.273, 3.24, 38.0 and 113 mg acid equivalents (ae)/L for either seven or 21 days. In the FSTRA, fathead minnows were exposed to mean measured 2,4-D concentrations of 0 (water control), 0.245, 3.14, 34.0, and 96.5 mg ae/L for 21 days. The respective concentrations of 2,4-D were not overtly toxic to either *Xenopus laevis* tadpoles or fathead minnows (*Pimephales promelas*). In the AMA, there were no signs of either advanced or delayed development, asynchronous development, or significant histopathological effects of the thyroid gland among 2,4-D exposed tadpoles evaluated on either day seven or day 21 of the exposure. Therefore, following the AMA decision logic, 2,4-D is considered "likely thyroid inactive" in the AMA with a No Observable Effect Concentration (NOEC) of 113 mg ae 2,4-D/L. In the FSTRA, there were no significant differences between control and 2,4-D exposed fish in regard to fertility, wet weight, length, gonado-somatic indices, tubercle scores, or blood plasma concentrations of vitellogenin. Furthermore, there were no treatment-related histopathologic changes in the testes or ovaries in any 2,4-D exposed group. The only significant effect was a decrease in fecundity among fish exposed to 96.5 mg ae 2,4-D/L. The cause of the reduced fecundity at the highest concentration of 2,4-D tested in the assay was most likely due to a generalized stress response in the fish, and not due to a specific endocrine mode of action of 2,4-D. Based on fish reproduction, the NOEC in the FSTRA was 34.0 mg ae 2,4-D/L.

1. Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D; CAS No. 94-75-7) is an herbicide active ingredient that has been registered and in use since 1946. End-use products with 2,4-D active ingredient are generally formulated as amine salts or esters. The 2,4-D amine salts and esters rapidly convert to the 2,4-D acid under most environmental conditions (United States Environmental Protection Agency (USEPA), 2004). 2,4-D is a white crystalline solid at room temperature with a reported octanol/water partition coefficient (log K_{ow}) of 0.177 at pH of 7, a reported vapor pressure of 1.4×10^{-7} mm Hg at 25 °C, a reported water solubility of 569 mg/L at 20 °C, and an organic carbon/water partition coefficient (K_{oc}) of 61.7 (United States Environmental Protection Agency (USEPA), 2005). The mode of action of 2,4-D is via increased cell-wall plasticity and abnormal increases in biosynthesis of proteins and ethylene resulting in uncontrolled cell division and damage to the vascular tissue of plants (USEPA, 2005).

2,4-D is currently registered for use on field, fruit, and vegetable crops and for use on pasture, turf, lawns, rights-of way, as well as aquatic and forestry applications and can be applied prior to emergence, post-emergence, prior to harvest, or during the dormant season (USEPA, 2005). Application rates of 2,4-D to the soil environment are generally less than 2.0 pounds acid equivalent (ae) per acre per year, with maximum application rates for some crops and non-crops up to 4.0 pounds ae per acre per year (USEPA, 2005). For direct application of 2,4-D to floating weeds in aquatic areas, the current accepted application rate is 2.0 pounds ae per acre per application or 4.0 pounds ae per application per acre per crop or year. For submerged weeds in aquatic areas, the current 2,4-D application rate is 10.8 pounds ae per acre foot (USEPA, 2005).

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Due to its high water solubility and low soil adsorptivity, some 2,4-D can be expected to reach surrounding surface waters via flooding/runoff events (Franco and Trapp, 2010; Waite et al., 2002). 2,4-D can also reach water bodies via direct application to aquatic weeds. When 2,4-D was maximally applied in a lake via underwater injection, the calculated half life in the treated area was 3.2 days, and levels of 2,4-D in the application area were below the USEPA drinking water maximum contaminant level (MCL) of 70 parts per billion by day 21 (Wilson and Armbruster, 2007). When maximal spray application of 2,4-D was conducted in various aquatic habitats, the calculated half life in the treated area was 2.5 days, and levels of 2,4-D in the application area were below the MCL of 70 parts per billion by day eight (Wilson and Armbruster, 2007). It was observed in aquatic microcosm studies that degradation of 2,4-D follows a biphasic pattern with a slow decline in concentrations over a 4–6 day period followed by a rapid decrease in concentrations over the next few days, which is attributed to microbial acclimation followed by a rapid increase in microbial population (Wilson and Armbruster, 2007). Biodegradation in the aquatic sediment environment has also been investigated with reported half lives of 2,4-D in freshwater sediment ranging from fifteen to 25 days (Chinalia and Killham, 2006).

The maximum predicted concentrations of 2,4-D in aquatic habitats based on assumptions of peak use and direct application to either 50 to 100 percent of the water body range from either 2000 to 4000 μg/L, respectively (USEPA, 2005). Reported mean concentrations of 2,4-D in the freshwater aquatic environment range from 0.00842 to 1.45 μg/L, while reported maximal concentrations range from 0.015 to 75 μg/L (Waite et al., 2002; Donald et al., 2001; Woudneh et al., 2007; Sargeant et al., 2011; Mulder and Schmidt, 2011; USEPA, 2004; National Water Information Service, 2011).

Acute toxicity values for aquatic vertebrates, including both fish and amphibians, indicate that 2,4-D acid and amine salts are largely non-toxic with acute LC_{50} values ranging from >80 to 2780 mg ae/L (Alexander et al., 1985; Holcombe et al., 1995; Morgan et al., 1996; Palmer and Krueger, 1997a,b; USEPA, 2004). The No Observable Effect Concentrations (NOEC) from chronic fish studies with 2,4-D are in the range from 14 to 63 mg ae/L (USEPA, 2005; Mayes et al., 1990), and NOEC values for developing *Xenopus laevis* tadpoles are ≥ 40 mg ae/L 2,4-D (Lenkowski et al., 2010; Morgan et al., 1996; Cooke, 1972). Thus, the reported concentrations of 2,4-D in the aquatic environment are orders of magnitude below the levels known to cause adverse effects in both fish and amphibians.

In 2009, 2,4-D was included in the final list of chemicals for Tier 1 screening in the United States Environmental Protection Agency's Endocrine Disruptor Screening Program (EDSP). A total of 67 pesticide active ingredients and high production volume/pesticide inert chemicals were included on this final list based on exposure potential only, and not on any established suspicion of endocrine-like activity (Federal Register 74: 71 (April 15, 2009) p. 17579). Tier 1 screening in the EDSP is composed of a battery of eleven separate assays (United States Environmental Protection Agency (USEPA), 2009a). Through a weight of evidence approach, including the results of the entire Tier 1 screening battery and other scientifically relevant information, a test substance is evaluated for potential endocrine activity in the estrogen, androgen, and thyroid hormone systems. Two of the *in vivo* assays in the Tier 1 screening battery, the Amphibian Metamorphosis Assay (AMA) and the Fish Short-Term Reproduction Assay (FSTRA), are performed in the aquatic environment with aquatic vertebrates. The purpose of the present paper is to summarize the results of 2,4-D in the AMA and FSTRA Tier 1 screening assays, and to put the results of these standardized tests into context with the pre-existing toxicity and exposure data for 2,4-D in aquatic systems.

The AMA is a 21-day screening assay designed to evaluate test substances for potential interaction with the hypothalamus–pituitary–thyroid (HPT) pathway, and the tadpole of the African clawed frog, *X. laevis*, is used as a test species because thyroid hormone levels in the tadpole are essential for the progress of normal metamorphosis. The AMA is designed to expose developing *X. laevis* tadpoles to at least three waterborne concentrations of the test chemical plus a control water source for 21 days beginning with Nieuwkoop and Faber (NF) stage 51 tadpoles (Nieuwkoop and Faber, 1994). According to the USEPA guideline (United States Environmental Protection Agency (USEPA), 2009b), the study design includes four replicate tanks per treatment containing twenty tadpoles each. Five tadpoles per tank are euthanized and assessed on day seven and the remaining tadpoles are assessed on day 21. The endpoints of interest in this assay are survival, whole body wet weight, snout-vent length, hind-limb length (normalized by snout-vent length), developmental stage, and histology of the thyroid gland. Normalized hind limb length, developmental stage, and histology of the thyroid are specific endpoints that are intended to give direct information on potential thyroid perturbations via alterations in metamorphic rate, while survival, wet weight and snout-vent length serve as more generalized endpoints of growth and health. Results of the AMA should be used in weight of evidence approach with the other relevant assays in the EDSP Tier 7 battery to determine if there is potential for the test substance to interact with the thyroid hormone system.

The FSTRA is meant to provide information, in conjunction with other Tier 1 assays, on whether there is potential interaction of a chemical test substance with the hypothalamus–pituitary–gonadal (HPG) axis of fish (United States Environmental Protection Agency (USEPA), 2009c), and to help determine potential interactions with the estrogen, androgen and steroidogenesis pathways. The FSTRA is initiated with sexually mature fathead minnows that are exposed to the test substance for 21 days. The study design includes at least three concentrations of the test substance and a control treatment with four replicate vessels per treatment level containing two male and four female fathead minnows each. The endpoints in the FSTRA include both apical endpoints, including fecundity, fertility, survival, as well as fish weight and length, and secondary endpoints that are more specific to an endocrine mode of action, including gonadosomatic index, blood plasma concentrations of vitellogenin (VTG), estradiol and testosterone, as well as secondary sex characteristics (tubercle score), and gross morphology and histology of the gonads. Results of the FSTRA are used in weight of evidence approach with the other relevant assays in the EDSP Tier 1 battery to determine whether or not the observed responses in the assay are likely a result of either (anti) estrogenic, (anti)androgenic, steroidogenic, or HPG axis activity.

2. Materials and methods

2.1. Test chemical and analytical sampling

2,4-D acid (CAS No 94-75-7; Nufarm Americas, Inc., Burr Ridge, Illinois lot# 2006 2433 8006-USA; purity=98.6 percent) was the test chemical for both the AMA and FSTRA. For both assays, stock solutions of 2,4-D were prepared by dissolving 2,4-D in laboratory dilution water (LDW) and adjusting the pH with sodium hydroxide to a value near the pH of the lab dilution water. The pH adjustment with sodium hydroxide served to increase the solubility of 2,4-D and thus allowed for more concentrated stock solutions (Hopkins, 1987). In addition, this adjustment minimized potential pH variations among different 2,4-D treatment levels due to the acidic nature of the test material. Due to the relatively high water solubility and low toxicity of 2,4-D toward fish and amphibians, and in

accordance with guidance documents for the AMA and the FSTRA, the limit concentration of 100 mg 2,4-D/L was selected as the high test concentration in both the AMA and FSTRA. Thus, the nominal concentrations of 2,4-D that were used in both the AMA and the FSTRA were: 0 (water control), 0.4, 4.0, 40 and 100 mg 2,4-D/L. The selection of a total of four exposure concentrations (plus control) instead of the minimum requirement of three concentrations (plus control) was intended to allow for a better understanding of the dose–response curves, and the dose selection included a concentration (4 mg/L) consistent with a conservative, maximum, predicted environmental concentration from the use of 2,4-D as an aquatic herbicide (USEPA, 2005).

In both the AMA and FSTRA, water samples (approximately 2 ml) of the test solutions were removed from all replicate test vessels and stock solutions on a weekly basis throughout the exposure period in order to measure concentrations of 2,4-D. High Performance Liquid Chromatography with Ultraviolet detection (HPLC/UV) was used to measure concentrations of 2,4-D in the collected water samples. The Agilent 1100 HPLC system was used with a YMC ODS—AQ 2.0×50 mm, with 5 μm column for the analysis. A wavelength of 280 nm was used for detection. Analytical standards of 2,4-D over a concentration range from 0.1 to 20 μg 2,4-D acid/ml LDW were used to determine measured concentrations in the water samples.

2.2. Laboratory dilution water and test delivery system

The LDW used in both the AMA and FSTRA was Lake Huron water supplied to The Dow Chemical Company by the City of Midland Water Treatment Plant. Before use in the laboratory, the water was limed and flocculated with ferric chloride, sand-filtered, pH-adjusted with gaseous CO_2, carbon-filtered, and UV-irradiated. The LDW is routinely analyzed for pesticides, organics, metals and other inorganics twice per year. A continuous-flow diluter system was used to provide each concentration of 2,4-D and a negative (dilution water) control to the replicate vessels in the AMA and FSTRA.

2.3. Amphibian Metamorphosis Assay (AMA) methods

The Amphibian Metamorphosis Assay (AMA) with 2,4-D was conducted in accordance with the Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1100 and OECD 231 (Organization for Economic Cooperation and Development (OECD), 2009a; USEPA, 2009b). The exposure tanks were $30 \times 14.5 \times 20$ cm^3, and the water depth was maintained at 12 cm by a screen covered drain, allowing a holding capacity of approximately 5.2 L of water. A flow rate of 32 ± 3.2 ml/min provided a complete volume turnover approximately every 2.7 h for each replicate test vessel. Nieuwkoop and Faber stage 51 *X. laevis* tadpoles were used to initiate the study and were obtained from breeding pairs maintained in the laboratory. The breeding pairs were ordered through *Xenopus* Express (www.xenopus.com; Brooksville, Florida). The tadpoles were fed Sera Micron (Sera North America Montgomeryville, PA) twice a day according to the feeding schedule outline in the AMA guidance documents (Organization for Economic Cooperation and Development (OECD), 2009b; USEPA, 2009a). During the exposure, temperature, dissolved oxygen (DO), and pH were measured from select tanks on a daily basis. Temperature was also continuously monitored from one replicate tank. The target water temperature of the test solution was 22 ± 1 °C, and the target DO and pH levels were >40 percent air saturation (3.9 mg/L) and 6.5–8.5, respectively. Hardness, alkalinity, conductivity, and light intensity were measured at weekly intervals. The photoperiod was set at 12 h light:12 h dark. During the exposure period of the study, daily observations of tadpole survival were recorded. In addition, any abnormal tadpole behavior was noted when observed.

On day seven of the exposure, all tadpoles were netted and a total of five tadpoles from each replicate tank were randomly selected and euthanized with tricaine methesulfonate (MS-222) for assessment of wet weight, snout-vent length, hind limb length and developmental stage. These same endpoints were again assessed on day 21 following MS-222 euthanasia of the remaining tadpoles in each tank. Tadpole specimens collected on day 21 were additionally assessed for thyroid gland histopathology. Thyroid histopathology was assessed from five tadpole specimens per test vessel matching the median developmental stage of the controls. Thyroid histopathology was assessed across all treatments groups.

For assessment of thyroid histopathology, the lower jaw and portions of the tadpole head from each tadpole specimen were removed and placed in a histopathology cassette which was put into Davidson's solution for 48 to 72 h followed by long-term preservation in 10 percent neutral buffered formalin. For selected tadpole specimens, sections were acquired from central portions of the thyroid rather than peripheral areas to provide an accurate reflection of thyroid size. Serial sectioning was required to acquire central sections of the thyroid glands. Attempts were made to obtain at least two step sections, 30 μm apart, with sufficient amount of thyroid tissue present bilaterally. The slides were stained with hematoxylin and eosin (H&E) and cover slipped. The slides were examined by an American College of Veterinary Pathologists (ACVP) board certified veterinary pathologist with additional training in frog histopathology. Selected histopathological findings were graded to reflect the severity of specific thyroid lesions. The core criteria (*i.e.*, thyroid gland atrophy/hypertrophy, follicular cell hypertrophy, and follicular cell hyperplasia) were evaluated using the severity scoring system according to USEPA and OECD recommendations (USEPA, 2009b; Organization for Economic Cooperation and Development (OECD), 2007).

2.4. Fish Short Term Reproduction Assay methods

The Fish Short-Term Reproduction Assay (FSTRA) with 2,4-D was conducted in accordance with the Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1350 and OECD 229 (Organization for Economic Cooperation and Development (OECD), 2009b; USEPA, 2009c). The exposure tanks were $39 \times 20 \times 25$ ($L \times W \times H$) cm deep, and the water depth was maintained at 13 cm by a screen covered drain, allowing a holding capacity of approximately 10 L of water. A flow rate of 45 ± 4.5 ml/min provided at least six volume turnovers per day for each replicate test vessel. The spawning substrates (tiles) used in this test were constructed of ~9 cm long sections of ~10 cm diameter PVC pipe cut and sectioned lengthwise and inverted to form a semicircular arch. Each test vessel contained three spawning substrates. Each tile was positioned on a lipped tray of stainless steel (~13 cm long) to contain any eggs that do not adhere to the tile and would therefore fall to the bottom of the tank. The trays also contained a stainless steel screen to prevent fish from access to any fallen eggs.

Sexually dimorphic, reproductively mature, fathead minnows (*Pimephales promelas*) were used to initiate the study and were obtained from New England Bioassay, Inc. (Manchester, CT). The fish were approximately 5.5 months old at study initiation. Fish were fed thawed frozen brine shrimp (Brine Shrimp Direct, Ogden, UT) at least twice per day at a rate sufficient to promote active reproduction and maintain body condition.

During the exposure, temperature, DO, and pH were measured from all tanks on a weekly basis. Temperature was also continuously monitored from one replicate tank. The target water temperature of the test solution was 25 ± 1 °C, and the target DO and pH levels were >60 percent air saturation (4.9 mg/L) and 6.5–8.5, respectively. Hardness, alkalinity, conductivity, and light intensity were measured at weekly intervals. The photoperiod was set at 16 h light:8 h dark. During the study, daily observations of fecundity, fertility and fish survival were recorded. In addition, any abnormal behavior was noted when observed.

In the pre-exposure period, aquaria were exposed to the same experimental conditions (lighting, temperature, etc.) as in the exposure period, and they received the same water source at the same flow rate as in the exposure period; however, no test chemical was added to the diluter system during this period of time. The pre-exposure period was two weeks in duration. Following the two week pre-exposure period, fecundity was calculated for each spawning group based upon the egg counts for the previous seven days. Only test vessels that had no incidences of mortality, displayed the correct sex ratio (two males and four females), and exhibited acceptable spawning activity (*i.e.* spawning events at least two times in the immediately preceding seven days or egg production exceeding fifteen eggs/female/day/replicate vessel), were carried forward into the exposure period of the study.

On day 21 of the exposure, fish were observed *in situ* for behavioral and secondary sex characteristics prior to their removal from the test vessel. Fish were then euthanized with MS-222 and measured for weight and standard length. The caudal peduncle was then severed to collect blood in aprotinin-washed (9.5 TIU/ml), heparanized capillary tubes. Capillary tubes were kept on ice until they were centrifuged for the collection of blood plasma. Fish gonads were inspected to confirm sex and then removed, weighed, enclosed into pre-labeled plastic tissue cassettes and fixed in Davidson's solution. All tissues remained in Davidson's fixative for at least 24 h and were then transferred to 10 percent neutral buffered formalin for histopathologic evaluation. Male and female fish were then evaluated for secondary sex characteristics (*i.e.* the number and prominence of nuptial tubercles) as described in the relevant guidance documents (OECD, 2009b; USEPA, 2009c). Histopathologic evaluation of the stage and severity grading of the gonads was performed in general accordance with the procedures described in Appendix E of the USEPA test guideline (USEPA, 2009c). The sections were examined under a light microscope by an ACVP certified veterinary pathologist with experience in fish gonadal histopathology and expertise in toxicologic pathology.

Concentrations of vitellogenin (VTG) in the fish blood plasma were measured by enzyme-linked immunosorbant assay (ELISA) following the recommended procedures in Brodeur et al. (2006), and the recommendations of the manufacturer of the fathead minnow ELISA kit (Biosense Laboratories, Bergen, Norway). Each ELISA plate used for the VTG assays included at least six calibration standards covering the range of expected VTG concentrations and at least two non-specific binding assay blanks. Sample blanks and at least three separate dilutions of a plasma sample were conducted in replicate wells on two different plates. Any non-detect samples from the ELISA were recorded as 1/2 the limit of quantification (LOQ).

2.5. Statistical analyses for the AMA and FSTRA

In both the AMA and the FSTRA, statistical analyses of the continuous data set were analyzed with the Jonckheere–Terpstra test in a step down manner if the data were consistent with a monotonic dose–response (Hollander and Wolfe,

1973). If data were not consistent with a monotonic dose–response, the data were assessed for normality using the Shapiro–Wilk test and variance homogeneity using the Levene test. Where non-normality or variance heterogeneity was observed, normalizing and/or variance stabilizing transformations were applied. If the data were normally distributed with homogeneous variances, then a significant treatment effect was determined using the one-way ANOVA followed by Dunnett's test. Significant mortality was assessed, if necessary, using the Cochran–Armitage Linear Trend Test when the data were consistent with a monotonic dose–response, otherwise a Fisher's Exact test with a Bonferroni–Holm adjustment was used.

In the AMA, hind limb length was normalized by snout-vent length to account for the effects of growth. Length and weight data for tadpoles reaching stages greater than NF stage 60 were excluded from statistical analyses due to drastic morphological changes at this stage and above. In the AMA, a significant treatment effect for developmental stage was determined from the step-down application of the multi-quantal Jonckheere–Terpstra test from the 20th to the 80th percentile.

In the FSTRA, all biological response data, apart from mortality, were analyzed and reported separately by sex. A treatment effect for tubercle score was determined using the multiquantal Jonckheere–Terpstra test.

3. Results

3.1. AMA

In the 21-day AMA, the mean measured concentrations, calculated for each treatment level by taking the arithmetic average of the weekly measured concentrations, were 0.273, 3.24, 38.0, and 113 mg ae 2,4-D/L which equaled 68.3, 81.0, 95.0, and 113 percent of nominal concentrations, respectively (Table 1). Measured concentrations of 2,4-D dropped most noticeably in all replicate vessels at the lowest two nominal 2,4-D treatment groups between days seven and fourteen of the test. This phenomenon is likely the result of limited biodegradation which reduced 2,4-D concentrations to the same extent in all the test vessels, but proportionately this had a greater effect at the two lowest nominal concentrations. A large variety of micro-organisms are capable of degrading 2,4-D via aerobic oxidation, and this degradation is maximized at temperatures ranging from 21 to 25 °C (USEPA, 2005; Sinton et al., 1986). Biodegradation likely increased following the first week of exposure due to an increased microbial population in the test vessels that were capable of degrading 2,4-D. None of the analyses of the control vessels exhibited an elution peak at the retention time for 2,4-D at a concentration exceeding the lowest level quantified (LLQ), which was equivalent to 0.120 mg ae 2,4-D/L.

Table 1
Summary of results from analysis of test solutions for 2,4-dichlorophenoxyacetic acid (2,4-D) in the Amphibian Metamorphosis Assay.

Target concentration of 2,4-D (mg/L)	Average measured concentration of 2,4-D (mg/L) for each replicate tank	Coefficient of variation for each replicate tank	Average measured concentration of 2,4-D (mg/L) for each treatment group	Average percent of target concentration for each treatment group
0.0	< LLQ[a]	NA	< LLQ	NA
	< LLQ	NA		
	< LLQ	NA		
	< LLQ	NA		
0.4	0.302	0.520	0.273	68.3
	0.288	0.576		
	0.252	0.623		
	0.292	0.599		
4.0	3.15	0.262	3.24	81.0
	3.09	0.266		
	3.32	0.171		
	3.39	0.184		
40	38.2	0.157	38.0	95.0
	38.0	0.162		
	37.6	0.172		
	38.1	0.162		
100	113	0.174	113	113
	113	0.165		
	113	0.166		
	114	0.168		

[a] < LLQ = less than the lowest level quantified of 0.120 mg 2,4-D/L.

Dissolved oxygen, pH, temperature, and light intensity met the specified values over the 21-day exposure period ranging from 5.3–8.2 mg/L, 7.0–7.8, 21.5–23.0 °C, and 618–882 lx, respectively. Throughout the 21-day exposure period, hardness and alkalinity ranged from 64 to 76 mg/L $CaCO_3$ and from 30 to 40 mg/L $CaCO_3$, respectively. Iodide, which is necessary for thyroid function, was not detectable in the LDW; however, iodide was quantified at 53.7 μg/g in the Sera Micron feed.

Throughout the exposure, no abnormal behaviors were noted among control or 2,4-D exposed tadpoles, and only one tadpole from the 3.24 mg ae/L 2,4-D treatment group died during the 21-day exposure period. Mean and median wet weights, snout-vent lengths, normalized hind-limb lengths, and developmental stages for day seven and day 21 tadpoles are reported in Table 2. The mean wet weights, snout-vent lengths, and normalized hind limb lengths among control and 2,4-D exposed tadpoles were not statistically different on either day seven or day 21 of the exposure (Table 2). According to the multi-quantal Jonckheere–Terpstra test, there was no significant difference in developmental stage among either day seven or day 21 control and 2,4-D-exposed tadpoles (Table 2). The median developmental stage among control tadpoles on day 21 was NF 58.5. The 10th and 90th percentiles for NF developmental stage in the control group on day 21 were 58 and 60.5, respectively.

There were no treatment-related histopathologic changes in the thyroid gland in any of the treatment groups, including no evidence of glandular atrophy or hypertrophy or follicular cell hyperplasia. The incidence of tall columnar cells lining the follicles (follicular cell hypertrophy) did not show any treatment-related differences and was interpreted to be within normal limits at all concentrations of 2,4-D. There was no evidence of any inflammatory or degenerative changes in the thyroid glands examined in any treatment group. All other histopathologic criteria, such as the overall size of the gland, the follicular lumen area, amount and type of colloid, and the follicular cell type and arrangement, of all 2,4-D exposed tadpoles were comparable to those of the controls.

The present study met the requirements for test validity in accordance with the USEPA guideline (USEPA, 2009b). Furthermore, the present study achieved most of the performance criteria for the AMA (USEPA, 2009b). The only performance criterion that was not met consistently in the present study was that a total of six replicate test vessels in the lower two treatment groups had measured concentrations of 2,4-D with coefficients of variation that exceeded 20 percent (Table 1). This was likely due to biodegradation of the test material in the test vessels. Since the validity criteria and most of the performance criteria were fulfilled, the present study is considered valid, of high quality, and useful to demonstrate that the test material, 2,4-D, did not exhibit thyroid activity in the AMA.

3.2. FSTRA

In the 21-day FSRTA, the mean measured concentrations, calculated for each treatment level by taking the arithmetic average of the weekly measured concentrations, were 0.245, 3.14, 34.0, and 96.5 mg ae 2,4-D/L which equaled 61.3, 78.5, 85.0, and 96.5 percent of nominal concentrations, respectively (Table 3).

Table 2
Mean ± standard deviation of wet weight, snout-vent length, normalized hind limb length, and developmental stage of *X. laevis* exposed to 2,4-dichlorophenoxyacetic acid (2,4-D) (median value in parentheses)[a].

Mean measured 2,4-D (mg/L)	N	Wet weight (g)		Snout-vent length (mm)		Normalized hind limb length[b]		Developmental stage	
		Day 7	Day 21	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21
< LLQ[c] (control)	4	0.526 ± 0.042 (0.482)	1.63 ± 0.168 (1.65)	18.5 ± 0.541 (18.2)	26.8 ± 1.40 (27.2)	0.125 ± 0.0076 (0.122)	0.647 ± 0.0457 (0.628)	54.0 ± 0.252 (54)	58.9 ± 0.329 (58.5)
0.273	4	0.479 ± 0.042 (0.490)	1.73 ± 0.117 (1.72)	18.0 ± 0.488 (18.0)	27.4 ± 0.538 (27.7)	0.120 ± 0.0170 (0.122)	0.661 ± 0.0423 (0.674)	53.8 ± 0.163 (54)	59.3 ± 0.357 (59)
3.24	4	0.493 ± 0.049 (0.485)	1.63 ± 0.251 (1.76)	18.3 ± 0.584 (18.3)	27.0 ± 1.62 (27.5)	0.121 ± 0.0104 (0.120)	0.626 ± 0.0338 (0.630)	54.0 ± 0.191 (54)	59.1 ± 0.441 (59)
38.0	4	0.488 ± 0.022 (0.489)	1.75 ± 0.057 (1.73)	18.1 ± 0.209 (18.3)	27.7 ± 0.319 (27.9)	0.119 ± 0.0060 (0.116)	0.635 ± 0.0417 (0.627)	53.8 ± 0.100 (54)	58.9 ± 0.274 (58.5)
113	4	0.496 ± 0.042 (0.488)	1.91 ± 0.218 (1.94)	18.4 ± 0.308 (18.5)	28.0 ± 0.408 (28.6)	0.121 ± 0.0030 (0.116)	0.580 ± 0.0469 (0.591)	53.9 ± 0.191 (54)	58.8 ± 0.175 (58)
p Value		0.5649[d]	0.2625[d]	0.4591[d]	0.3950[d]	0.7799[d]	0.0723[d]	0.6372[e]	0.1042[e]

[a] Tadpoles stage 60 and above were dropped from length and weight calculations due to overt morphological changes.
[b] Hind limb length is normalized by snout-vent length.
[c] Lowest level quantified (LLQ) = 0.120 mg 2,4-D/L.
[d] *p* Value according to the one-way ANOVA followed by Dunnett's *t*-test.
[e] Median *p* value for the 20th to 80th percentiles according to the multi-quantal Jonckheere–Terpstra test.

Table 3
Summary of results from analysis of test solutions for 2,4-dichlorophenoxyacetic acid (2,4-D) in the Fish Short Term Reproduction Assay.

Target concentration of 2,4-D (mg/L)	Average measured concentration of 2,4-D (mg/L) for each replicate tank	Coefficient of variation for each replicate tank	Average measured concentration of 2,4-D (mg/L) for each treatment group	Average percent of target concentration for each treatment group
0.0	< LLQ[a]	NA	< LLQ	NA
	< LLQ	NA		
	< LLQ	NA		
	< LLQ	NA		
0.4	0.240	0.492	0.245	61.3
	0.258	0.380		
	0.242	0.442		
	0.241	0.427		
4.0	3.29	0.128	3.14	78.5
	3.45	0.078		
	2.59	0.467		
	3.21	0.172		
40	34.1	0.106	34.0	85.0
	32.8	0.139		
	35.3	0.050		
	33.8	0.142		
100	95.7	0.063	96.5	96.5
	95.2	0.024		
	97.1	0.064		
	97.5	0.060		

[a] < LLQ = less than the lowest level quantified of 0.10 mg 2,4-D/L.

As observed in the AMA, 2,4-D concentrations decreased most noticeably in the lowest dose groups, and this phenomenon was likely due to limited biodegradation. None of the analyses of the control vessels exhibited an elution peak at the retention time for 2,4-D at a concentration exceeding the lowest level quantified (LLQ), which was equivalent to 0.10 mg ae 2,4-D/L.

During the exposure phase of the study, dissolved oxygen, pH, temperature, and light intensity met the specified values of the 21-day exposure period, ranging from 6.2–8.3 mg/L, 7.0–7.8, 24.5–25.5 °C, and 591–770 lx, respectively. Throughout the 21-day exposure period, hardness and alkalinity ranged from 62 to 68 mg/L $CaCO_3$ and from 30 to 46 mg/L $CaCO_3$, respectively.

Table 4
Mean ± standard deviation of fecundity and percent fertility in the Fish Short Term Reproduction Assay with 2,4-dichorophenoxyacetic acid (2,4-D) (median values in parentheses).

Mean measured 2,4-D (mg/L)	N	Mean fecundity (# eggs/female/day)	Mean percent fertility
< LLQ[a] (control)	4	29.8 ± 6.40 (28.3)	96.9 ± 0.50 (97.1)
0.245	4	29.0 ± 6.47 (29.1)	96.3 ± 3.08 (97.8)
3.14	4	25.9 ± 5.27 (27.2)	96.1 ± 1.54 (96.6)
34.0	4	22.9 ± 8.72 (22.9)	97.7 ± 0.78 (97.7)
96.5	4	19.7* ± 5.97 (18.4)	95.8 ± 2.68 (95.3)

[a] < LLQ = less than the lowest level quantified = 0.10 mg/L 2,4-D.
* Jonckheere–Terpstra test indicated a significant difference at $\alpha = 0.05$.

Only one fish died during the 21-day exposure period. This fish was from the 96.5 mg ae 2,4-D/L treatment group. No abnormal behavior (e.g. hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence or feeding abstinence) was noted among control or 2,4-D-exposed fish and no dose-dependent changes in appearance were observed during the exposure. According to the Cochran–Armitage Linear Trend Test, there was no statistically significant difference in mortality among the treatment groups ($p = 0.1556$).

According to the Jonckheere–Terpstra test, fecundity was significantly reduced at the highest test concentration of 96.5 mg ae 2,4-D/L compared to controls ($p = 0.0444$, Table 4). When the highest dose level of 96.5 mg ae 2,4-D/L was removed from the analysis, there were no significant differences in fecundity among the remaining treatment groups ($p = 0.1912$). There were no significant differences between treatment groups in the percentage of fertile eggs (ANOVA followed by Dunnett's test, $p = 0.6974$, Table 4).

According to the ANOVA followed by Dunnett's *t* test, there were no statistically significant differences in male or female length, weight, GSI, or VTG plasma levels between control and 2,4-D exposed fathead minnows (Table 5). There were no tubercles noted among female fathead minnows at the end of the exposure period, and male tubercle scores were not found to be significantly different among control and 2,4-D-exposed fish (Table 5).

There were no treatment-related histopathologic changes either in the testes or ovaries of fathead minnows exposed to 2,4-D at any concentration tested. The pattern of predominant

Table 5
Mean ± standard deviation of wet weight, length, gonado-somatic index, tubercle score and vitellogenin concentrations in male and female fathead minnows exposed to 2,4-dichlorophenoxyacetic acid (2,4-D) in the Fish Short Term Reproduction Assay (median value in parentheses).

Mean measured 2,4-D (mg/L)	N	Wet weight (g)		Length (mm)		Gonado-somatic index		Vitellogenin (mg/ml)		Male tubercle score
		Male	Female	Male	Female	Male	Female	Male	Female	
< LLQ[a] (control)	4	3.57 ± 0.141 (3.53)	1.59 ± 0.169 (1.59)	51.5 ± 1.54 (51.6)	42.4 ± 1.37 (49.2)	1.20 ± 0.144 (1.25)	11.9 ± 1.65 (11.9)	$1.34 \times 10^{-3} \pm 1.73 \times 10^{-3}$ (0.493×10^{-3})	27.6 ± 21.5 (20.6)	32.0 ± 4.02 (31.3)
0.45	4	3.72 ± 0.308 (3.69)	1.64 ± 0.074 (1.65)	53.0 ± 1.37 (52.6)	42.7 ± 0.562 (42.7)	1.23 ± 0.227 (1.30)	12.9 ± 2.29 (13.3)	$1.77 \times 10^{-3} \pm 1.25 \times 10^{-3}$ (1.77×10^{-3})	55.1 ± 47.5 (25.3)	31.3 ± 3.88 (31.0)
3.14	4	3.69 ± 0.144 (3.64)	1.62 ± 0.173 (1.54)	52.2 ± 0.887 (52.0)	42.1 ± 0.924 (42.0)	1.19 ± 0.102 (1.21)	11.2 ± 2.58 (11.3)	$0.93 \times 10^{-3} \pm 0.78 \times 10^{-3}$ (0.81×10^{-3})	19.8 ± 7.69 (19.2)	33.6 ± 4.96 (34.5)
34.0	4	3.93 ± 0.488 (3.85)	1.62 ± 0.173 (1.62)	54.5 ± 2.20 (54.8)	42.4 ± 1.47 (42.7)	1.19 ± 0.228 (1.21)	11.8 ± 3.44 (12.2)	$0.91 \times 10^{-3} \pm 0.59 \times 10^{-3}$ (0.75×10^{-3})	21.0 ± 14.4 (13.9)	33.1 ± 1.97 (32.5)
96.5	4	3.73 ± 0.408 (3.75)	1.62 ± 0.082 (1.56)	53.8 ± 2.45 (54.5)	42.5 ± 0.777 (43.0)	1.30 ± 0.097 (1.31)	13.3 ± 2.02 (14.1)	$1.29 \times 10^{-3} \pm 0.98 \times 10^{-3}$ (1.18×10^{-3})	31.6 ± 12.7 (26.4)	34.5 ± 3.08 (34.3)
p Value		0.9613[b]	0.9946[b]	0.1771[b]	0.6503[b]	0.8654[b]	0.7462[b]	0.8185[b]	0.5280[b]	1.000[c]

[a] < LLQ = less than the lowest level quantified = 0.10 mg/L 2,4-D.
[b] *p* Value according to the one-way ANOVA followed by Dunnett's *t*-test.
[c] Median *p* value for the 20th to 80th percentiles according to the multi-quantal Jonckheere–Terpstra test.

germ cell distribution (staging) in ovaries or testes was comparable between the controls and all treated groups. All histopathologic findings were considered background spontaneous changes and unassociated with exposure to 2,4,D due to lack of dose–response relationships.

The present study met the requirements for test validity in accordance with the USEPA guideline (USEPA, 2009c). Furthermore, the present study achieved most of the performance criteria for the FSTRA (USEPA, 2009c). The only performance criterion that was not met consistently in the present study was that a total of five replicate test vessels had measured concentrations of 2,4-D with coefficients of variation that exceeded 20 percent (Table 3). This was likely due to biodegradation of the test material in the test vessels. The present study is considered valid, of high quality, and useful to determine that the test material, 2,4-D, does not interact with the estrogen, androgen, and steroidogenic pathways or with the HPG axis in fathead minnows.

4. Discussion

4.1. AMA

There were no signs of overt toxicity among exposed tadpoles in the present study. Throughout the entire exposure period, there was only one incidence of tadpole mortality, and no indications of developmental delay or abnormal behavior. Thus, the NOEC in this study was 113 mg ae 2,4-D/L (highest dose tested and assay limit dose). The lack of mortality and overt signs of toxicity in the present study were consistent with previous studies on 2,4-D and amphibian toxicity, which reported that concentrations of 2,4-D acid ranging from 50 to 226 mg ae/L were not acutely toxic to various frog species (Morgan et al., 1996; Palmer and Krueger, 1997a; Cooke, 1972). Furthermore, aquatic microcosms containing various tadpole species were not adversely affected by 2,4-D applied at the manufacturers' recommended application rate (0.117 ml/m^2), indicating no foreseeable risk toward amphibians from 2,4-D exposures in the field (Relyea, 2005).

The results of the AMA with 2,4-D indicated that the examined endpoints among developing *X. laevis* were not altered in response to 2,4-D. In the AMA with 2,4-D, there were no signs of advanced or delayed development or asynchronous development among 2,4-D exposed tadpoles relative to control tadpoles on either day seven or day 21 of the exposure. In addition, compared to thyroid glands from controls, there were no significant histopathological effects observed among thyroid glands from 2,4-D exposed tadpoles. Therefore, following the AMA decision logic as presented in the relevant guidelines (USEPA, 2009b; OECD, 2009a), 2,4-D is considered "likely thyroid inactive" in the AMA at concentrations $\leq$113 mg ae/L. In contrast to these results, there is some evidence that 2,4-D may have the potential to interact with the HPT axis in experimental screening level assays (Raldua and Babin, 2009) or at relatively high concentrations exceeding the saturation threshold for renal clearance in mammalian test systems (Charles et al., 1996; Neal et al., 2010). In an experimental three-day screening level bioassay investigating thyroxine immunoreactivity in the developing thyroid gland of zebrafish embryos, exposure to a single concentration of 5.5 mg/L 2,4-D caused a slight but significant decrease in thyroxine immunoreactivity in the thyroid gland in comparison to controls (Raldua and Babin, 2009). The authors of this study clarify that this screening assay is limited in that it only indicates thyroid gland status and does not address adverse effects or the specificity of the modality of action with respect to HPT interactions (Raldua and Babin, 2009). Furthermore, only a single concentration of 2,4-D was evaluated for thyroxine immunoreactivity, prohibiting a dose–response evaluation for this endpoint. In more standard subchronic studies with rats (Charles et al., 1996), decreases in thyroid hormone levels and increases in thyroid weight were observed only at doses $\geq$100 mg/kg/day 2,4-D, but these dose levels were well above a dose level exceeding saturation of renal clearance of 2,4-D (>50 mg/kg/day). Toxicity observed at dose levels exhibiting non-linear toxicokinetic behavior due to saturation of clearance mechanisms overestimate potential human health risks when there is a sufficient margin of exposure (Barton et al., 2006; Bus and Hammond, 2007). Likewise, in an extended one-generation reproduction toxicity test with 2,4-D, adaptive changes in thyroid histopathology (smaller thyroid follicles with small vacuoles) were observed among 25 percent of dams on gestation day (GD) seventeen following seven weeks of exposure to 600 ppm (40.2–45.3 mg/kg/day); however, these alterations occurred at a dose level known to be above the limit of renal toxicokinetic saturation in female rats (Neal et al., 2010). No other thyroid-related effects in the extended one-generation reproduction test (altered hormone levels, thyroid weights and/or histopathology) were noted at lower dose levels on GD seventeen or at other life stages at doses up to $\leq$800 ppm (82 mg/kg/day in male offspring) (Neal et al., 2010). Given the large margin of exposure for 2,4-D (human exposures are <10,000× the existing NOEL in animal studies), thyroid changes occurring at dose levels exceeding saturation of renal clearance are not relevant for human risk assessment (Barton et al., 2006; Bus and Hammond, 2007).

The minimal and high-dose specific evidence of thyroid effects noted only in the non-linear toxicokinetic range in high quality mammalian toxicity studies is consistent with the absence of an interaction of 2,4-D with the HPT in the AMA screening assay. Thus, the results of both the AMA and other toxicity data indicate 2,4-D has only a minimal potential to modulate thyroid endocrine function and would not be predicted at environmentally relevant exposure levels.

4.2. FSTRA

The lack of mortality and overt signs of toxicity in the FSTRA were consistent with the concentrations of 2,4-D reportedly required to elicit acute lethality among various fish species (Mayes et al., 1990; Alexander et al., 1985; Doe et al., 1987; Holcombe et al., 1995). Compared to controls, the only significant effect in the FSTRA was a decrease in fecundity among fish exposed to 96.5 mg ae 2,4-D/L. The NOEC and LOEC values (based on fecundity) in the present study were therefore 34.0 mg ae 2,4-D/L and 96.5 mg ae 2,4-D/L, respectively. These results were similar to the reported NOEC values of 39.2–42.5 mg ae 2,4-D/L in a 28-day chronic early life stage test with medaka fish (Holcombe et al., 1995) and a NOEC and MATC of 63.4 and 80.4 mg ae 2,4-D/L, respectively, in an early life stage toxicity test with fathead minnows (Mayes et al., 1990).

There were no significant treatment related effects on the more specific endocrine-responsive endpoints in the present FSTRA, such as VTG concentrations, gonado-somatic indices, gonadal histopathology, or tubercle scores. Therefore, the FSTRA with 2,4-D did not indicate specific changes in estrogen or androgen-responsive endpoints or potential interactions with these pathways. The lack of significant alterations in VTG concentrations in the FSTRA is in contrast to results in juvenile rainbow trout reported in Xie et al. (2005), which indicated that following seven days of exposure to concentrations of 0.164 and 1.64 mg ae 2,4-D/L, VTG levels were significantly increased relative to controls. However, in the study reported by Xie et al. (2005) VTG levels were analyzed in juvenile male and female fish in response to 2,4-D exposure without regard to potential sex differences in this measure. The inclusion of both male and female immature fish, without differentiating the sex of these individuals, in the Xie et al. (2005) study may have contributed to the variability in the resultant VTG measurements and also the measured responses, because immature female rainbow trout have higher levels of VTG than immature males (Bon et al., 1997). Other reasons for differences in the VTG measurements in the present study and the Xie et al. (2005) study could also be due to species sensitivity differences, the chemical form of the 2,4-D tested (acid *versus* salt), or differences in experimental design. A total of three replicate tanks with $n=2$ fish each ($n=6$ fish per treatment level) were used in the Xie et al. (2005) study, whereas a greater degree of replicates ($n=4$) and fish ($n=$eight males and sixteen females per treatment level) were assayed for VTG concentrations in the present study. The failure to observe an increased level of VTG, an estrogen receptor responsive marker, in the FSTRA was not unexpected, in that 2,4-D did not show signs of competitive binding with either the human or rainbow trout estrogen receptor in yeast cell systems (Petit et al., 1997; Orton et al., 2009). The FSTRA results are also consistent with another available wildlife toxicity study in which 2,4-D showed no activity on the HPG axis of alligators (Spiteri et al., 1999). In conclusion, it is most likely that the observed decrease in fecundity at the highest concentration tested in the present FSTRA was due to some other stress response, and not due to a specific interaction with the HPG axis of fish. For example, alterations in oxidative stress biomarkers have been demonstrated in short term toxicity studies with several fish species exposed to nominal concentrations of 87 mg ae 2,4-D/L, indicating that oxidative stress pathways are activated in fish exposed to these relatively high concentrations of 2,4-D (Oruc et al., 2004). This type of stress response is not predicted at environmentally relevant concentrations of 2,4-D.

5. Conclusion

The results of the AMA indicate that 2,4-D is not thyroid active in the AMA. The results of the FSTRA indicate that 2,4-D activity is not consistent with estrogen, androgen, or steroidogenisis pathway interactions, nor are interactions with the HPG axis considered likely based on the very limited and high exposure nature of the observed response. The NOEC values observed in the present AMA and FSTRA are well above both predicted maximal concentrations and actual measured concentrations of 2,4-D in the aquatic environment, including when it is applied as an aquatic herbicide.

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References

Alexander, H.C., Gersich, F.M., Mayes, M.A., 1985. Acute toxicity of four phenoxy herbicides to aquatic organisms. Bull. Environ. Contam. Toxicol. 35, 314–321.

Barton, H.A., Pastoor, T.P., Chambers, J.E., Diliberto, J., Doerrer, N.G., Driver, J.H., Hastings, C.E., Iyengar, S., Krieger, R., Stahl, B., Timchalk, C., 2006. The acquisition and application of absorption, distribution, metabolism, and excretion (ADME) data in agricultural chemical safety assessments. Crit. Rev. Toxicol. 36, 9–35.

Bon, E., Barbe, U., Rodriguez, N., Cuisset, B., Pelissero, C., Sumpter, J.P., Menn, F.L., 1997. Plasma vitellogenin levels during the annual reproductive cycle of the female rainbow trout (*Oncorhynchus mykiss*): establishment and validation of an ELISA. Comp. Biochem. Physiol. 117B, 75–84.

Brodeur, J.C., Woodburn, K.B., Zhang, F., Bartels, M.J., Klecka, G.M., 2006. Plasma sampling and freezing procedures influence vitellogenin measurements by enzyme-linked immunoassay in the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 25, 337–348.

Bus, J.S., Hammond, L.E., 2007. Regulatory progress, toxicology, and public concerns with 2,4-D: where do we stand after two decades? Crop. Prot. 26, 266–269.

Charles, J.M., Cunny, H.C., Wilson, R.D., Bus, J.S., 1996. Comparative subchronic studies on 2,4-dichlorophenoxyacetic acid, amine and ester in rats. Fundam. Appl. Toxicol. 33, 161–165.

Chinalia, F.A., Killham, K.S., 2006. 2,4-Dichlorophenoxyacetic acid (2,4-D) biodegradation in river sediments of Northeast-Scotland and its effect on the microbial communities (PLFA and DGGE). Chemosphere 64, 1675–1683.

Cooke, A.S., 1972. The effects of DDT, dieldrin, and 2,4-D on amphibian spawn and tadpoles. Environ. Pollut. 3, 51–68.

Doe, K.G., Ernst, W.R., Parker, W.R., Julien, G.R.J., Hennigar, P.A, 1987. Influence of pH on the acute lethality of fenitrothion, 2,4-D, and aminocarb and some pH-altered sublethal effects of aminocarb on rainbow trout (*Salmo gairdneri*). Can. J. Fish. Aquat. Sci. 45, 287–293.

Donald, D.B., Gurprasad, N.P., Quinnett-Abbott, L., Cash, K., 2001. Diffuse geographic distribution of herbicides in northern prairie wetlands. Environ. Toxicol. Chem. 20, 273–279.

Franco, A., Trapp, S., 2010. A multimedia activity model for ionizable compounds: validation study with 2,4-dichlorophenoxyacetic acid, aniline, and trimethoprim. Environ. Toxicol. Chem. 29, 789–799.

Holcombe, G.W., Benoit, D.A., Hammermeister, D.E., Leonard, E.N., Johnson, R.D., 1995. Acute and long-term effects of nine chemicals on the Japanese medaka (*Oryzias latipes*). Arch. Environ. Contam. Toxicol. 28, 287–297.

Hollander, M., Wolfe, D.A., 1973. Nonparametric Statistical Methods. John Wiley and Sons, New York.

Hopkins, D.L., 1987. 2,4-Dichlorophenoxyacetic Acid: Determination of the Water Solubility. Unpublished Report of The Dow Chemical Company. Laborotory Report No.: ES-994. MRID No. 41332002. November 25, 1987.

Lenkowski, J.R., Sanchez-Bravo, G., McLaughlin, K.A., 2010. Low concentrations of atrazine, glyphosate, 2,4-dichlorophenoxyacetic acid, and triadimefon exposures have diverse effects on *Xenopus laevis* organ morphogenesis. J. Environ. Sci. 22, 1305–1308.

Mayes, M.A., Gorzinski, S.J., Potter, R.B., Richardson, C.H., 1990. 2,4-Dichlorophenoxyacetic Acid: Evaluation of the Toxicity to Early Life Stages of the Fathead Minnow, *Pimephales promelas*. Report of Toxicology and Environmental Research and Consulting. The Dow Chemical Company, Midland, Michigan.

Morgan, M.K., Scheuerman, P.R., Bishop, C.R., Pyles, R.A., 1996. Teratogenic potential of atrazine and 2,4-D using FETAX. J. Toxicol. Environ. Health 48, 151–168.

Mulder, R., Schmidt, C., 2011. Groundwater, Surface Water, and Sediment Monitoring for Pesticides and Nitrate in Billings, Montana.

National Water Information System (NWIS) Water-Quality Web Services. U.S. Geological Survey. http://qwwebservices.usgs.gov/. Accessed March 2011.

Neal, B., Bus, J.S., Zablotny, C.L., Yano, B.L., Passage, J., Marty, M.S., 2010. 2,4-Dichlorophenoxyacetic Acid (2,4-D): Evaluation of Reproductive/Endocrine Endpoints in a Dietary Extended One-generation Study in Crl:CD(SD) Rats. Abstract No. 420. 2010 Itinerary Planner. Society of Toxicology, Salt Lake City, UT.

Nieuwkoop, P.D., Faber, J., 1994. Normal Table of *Xenopus laevis* (Daudin). Garland Publishing, New York.

Organization for Economic Cooperation and Development (OECD), 2007. Guidance Document on Amphibian Thyroid Histology. Series on Testing and Assessment. No. 82. Environmental Health and Safety Publications, Paris.

Organization for Economic Cooperation and Development (OECD), 2009a. OECD Guideline for the Testing of Chemicals: The Amphibian Metamorphosis Assay. OECD 231. Adopted 7 September 2009.

Organization for Economic Cooperation and Development (OECD), 2009b. OECD Guideline for Testing of Chemicals: Fish Short Term Reproduction Assay. OECD 229. Adopted 7 September 2009.

Orton, F., Lutz, I., Kloas, W., Routledge, E.J., 2009. Endocrine disrupting effects of herbicides and pentachorophenol: *in vitro* and *in vivo* evidence. Environ. Sci. Technol. 43, 2144–2150.

Oruc, E.O., Sevgiler, Y., Uner, N., 2004. Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl. Comp. Biochem. Physiol. Part C 137, 43–51.

Palmer, S.J., Krueger, H.O., 1997a. 2,4-D Dimethylamine Salt: A 96-hour Static Acute Toxicity Test With the Leopard Frog Tadpoles (*Rana pipiens*). Study ID 467-103. MRID No. 44517306. Wildlife International Ltd., Easton, Maryland.

Palmer, S.J., Krueger, H.O., 1997b. 2,4-D (2,4-Dichlorophenoxyacetic acid): A 96-hour Static Acute Toxicity Test With the Leopard Frog Tadpoles (*Rana pipiens*). Study ID 467-102. MRID No. 44517307. Wildlife International Ltd., Easton, Maryland.

Petit, F., Le Goff, P., Cravedi, J.-P., Valotaire, Y., Pakdel, F., 1997. Two complimentary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. J. Mol. Endocrinol. 19, 321–335.

Raldua, D., Babin, P.J., 2009. Simple, rapid zebrafish larva bioassay for assessing the potential of chemical pollutants and drugs to disrupt thyroid gland function. Environ. Sci. Technol. 43, 6844–6850.

Relyea, R.A., 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecol. Appl. 15, 618–627.

Sargeant, D., Dugger, D., Anderson, P., Newell, E., 2011. Surface Water Monitoring Program for Pesticides in Salmonid-bearing Streams. 2010 Data Summary. Publication No. 11-03-021. Washington State Departments of Ecology and Agriculture. April 2011. Available at: 〈http://agr.wa.gov/PestFert/natresources/swm/〉.

Sinton, G.L., Fan, L.T., Erickson, L.E., Lee, S.M., 1986. Biodegradation of 2,4-D and related xenobiotic compounds. Enzyme Microb. Technol. 8, 395–403.

Spiteri, I.D., Guillette, L.J., Crain, D.A., 1999. The functional and structural observatons of the neonatal reproductive system of alligators exposed in ovo to atrazine, 2,4-D, or estradiol. Toxicol. Ind. Health 15, 181–186.

United States Environmental Protection Agency (USEPA), 2004. Environmental Date and Effects Division's Risk Assessment for the Reregistration Eligibility Document for 2,4-Dichlorophenoxyacetic Acid (2,4-D).

United States Environmental Protection Agency (USEPA), 2005. Reregistration Eligibility Decision for 2,4-D. EPA 738-R-05-002. June 2005.

United States Environmental Protection Agency (USEPA), 2009a. Endocrine Disruptor Screening Program (EDSP) FFDCA §408(p) Order/FIFRA §(3)(c)(2)(B) Data Call-IN (DCI). Order # EDSP-030001-12. Chemical # PC Code: 030001. CAS # 94-75-7, Chemical Name: 2,4-D. Issued on 10/29/2009.

United States Environmental Protection Agency (USEPA), 2009b. Endocrine Disruptor Screening Program Test Guidelines. OPPTS 890.1100: Amphibian Metamorphosis (Frog). EPA 740-C-09-002. October 2009.

United States Environmental Protection Agency (USEPA), 2009c. Endocrine Disruptor Screening Program Test Guidelines. OPPTS 890.1350: Fish Short-term Reproduction Assay. EPA 740-C-09-007. October 2009.

Waite, D.T., Cessna, A.J., Grover, R., Kerr, L.A., Snihura, A.D., 2002. Environmental concentrations of agricultural herbicides: 2,4-D and triallate. J. Environ. Qual. 31, 129–144.

Wilson, R.D., Armbruster, J.A., 2007. The Dispersion and Dissipation of 2,4-D Concentrations When Used for Typical Aquatic Applications. Available at: 〈http://www.24d.org/scientificstudies/Environmental.aspx〉.

Woudneh, M.B., Sekela, M., Tuominen, T., Gledhill, M., 2007. Acidic herbicides in surface waters of Lower Fraser Valley, British Columbia, Canada. J. Chromatogr. A 1139, 121–129.

Xie, L., Thrippleton, K., Irwin, M.A., Siemering, G.S., Mekerbri, A., Crane, D., Berry, K., Schlenk, D., 2005. Evaluation of estrogen activities of aquatic herbicides and surfactants using an rainbow trout vitellogenin assay. Toxicol. Sci. 87, 391–398.