DRAFT FINAL REPORT

on

COMPARATIVE EVALUATION OF FATHEAD MINNOW ASSAYS FOR DETECTING ENDOCRINE-DISRUPTING CHEMICALS

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1.0 INTRODUCTION

The EPA has implemented an Endocrine Disruptor Screening Program (EDSP). In 1996, the Food Quality Protection Act and the Safe Drinking Water Act were enacted by Congress to authorize the EPA to implement a screening program to evaluate whether pesticides and other chemicals found in food or water could affect endocrine systems in humans. In this program, comprehensive toxicological and ecotoxicological screens and assays are being developed to identify and characterize the endocrine effects of environmental contaminants, industrial chemicals, and pesticides. A two-tiered approach is being utilized: Tier 1 employs a combination of in vivo and in vitro screens, and Tier 2 involves in vivo testing using two-generation reproductive studies. Validation of the individual screens and assays is required, and the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) will provide advice and counsel on the validation assays.

The Fish Screening Assay was selected as a component of the Tier 1 screening by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to evaluate the potential toxicity of chemicals and mixtures on the endocrine system (EDSTAC 1998). The Tier 1 screening assays were selected to obtain minimum, yet sufficient estimates of potential endocrine disrupting activity. The Committee has several stated goals for these assays. First, they should be relatively inexpensive, quick, and technically easy to perform. Second, they should be sensitive and specific, capture multiple endpoints, and be predictive across species, gender, and age. And third, they should be validated and standardized before they are used routinely by testing laboratories (EDSTAC 1998, Vol. 1, p 3-9). The purpose of using testing protocols within the EDSP is "to characterize the nature, likelihood of a dose-response relationship of endocrine disruption in humans and wildlife" (EDSTAC 1998; EPA 1997). Subsequently, the EPA has requested the development of a screening protocol that identifies compounds having the potential to affect selected endocrine processes in fish.

The recommended protocol to be used as part of a Tier 1 screening (T1S) battery includes a fish screen assay, which complements the information from assays using mammals and other ecologically significant animal classes. Therefore, the inclusion of the fish-screening assay in Tier 1 is important because estrogenic and androgenic controls in reproduction and development in fish differ enough from those in higher vertebrates that mammalian screening alone may not identify potential endocrine disrupting chemicals in this class of animals. It is expected that the fish-screening assay will complement the other screening assays such that through its completion, the following five criteria will be met.

1. The T1S battery should maximize sensitivity to minimize false negatives while permitting analysis of a yet undetermined, but acceptable, level of false positives. This criterion expresses the need to "cast the screening net widely" to not miss potential endocrine disruptors or estrogen-androgen-thyroid-active materials.
2. The T1S battery should include a range of organisms representing known or anticipated differences in metabolic activity. The battery should include assays from representative vertebrate classes to reduce the likelihood that important pathways for metabolic activation or detoxification of parent chemical substances or mixtures are not overlooked.
3. The T1S battery should be designed to detect all known modes of action for the endocrine endpoints of concern. All chemicals known to affect the action of estrogen, androgen, or thyroid hormones should be detected.
4. The T1S battery should include a sufficient range of taxonomic groups among the test organisms. Differences in endogenous ligands, receptors, and response elements among taxa can affect endocrine activity of chemical substances or mixtures.

5. The T1S battery should incorporate sufficient diversity among the endpoints and assays to reach conclusions based on "weight-of-evidence" considerations. Decisions based on the battery results will require weighing the data from several assays.

The Tier 1 screening must be relatively fast and efficient while meeting the criteria described above. The screening includes a fish reproductive assay, which fills important needs in the battery and complements the information from assays using mammals and other ecologically significant animal classes. Fish differ in steroid profiles from mammals. For example, 11-ketotestosterone (11-KT), as opposed to testosterone (T), is the most important androgen in fish, and the estrogen receptor (ER) in fish appears to differ structurally and functionally from the mammalian ER (Petit et al., 1995). In addition, steroid receptors in eggs and for hepatic vitellogenin (VTG) have no known analogous receptors in mammals, which would suggest sites of endocrine disruption unique to oviparous animals. Therefore, this assay is essential to address these known endocrine differences.

1.1 Purpose

The purpose of this study is to evaluate short-term screening assays designed to detect substances that interfere with the estrogen and androgen systems of fish. As previously stated, including the fish screening assay in Tier 1 is important because estrogenic and androgenic controls on reproduction and development in fish may differ enough from those on higher vertebrates that mammalian screening methods may not identify potential endocrine-disrupting chemicals in this class of animals.

The EDSTAC recommended a fish gonadal recrudescence assay for inclusion in the Tier 1 battery of assays (EDSTAC 1998). In subsequent developmental work, a gonadal recrudescence based approach with the fathead minnow did not prove to be very suitable. Instead, a short-term reproduction assay was determined to be more appropriate for investigating EDCs (Ankley et al. 2001). Also, at this time another approach using juvenile fish was suggested (Panter et al. 2001). The Organization for Economic Cooperation and Development established an Endocrine Disrupter Testing and Assessment Task Force which formed a Validation Management Group for ecotoxicity tests. This group recommended that a comparison study between the short-term reproduction assay (EPA 2002) and a modified version of the juvenile fish assay using non-spawning adults (OECD 2001) be undertaken.

U.S. EPA (2001) has described a short-term assay with the fathead minnow that considers reproductive fitness as an integrated measure of toxicant effects and enables measurement of a suite of histological and biochemical endpoints that reflect effects associated with [anti-] estrogens and androgens. The assay uses mature male and female fish. During a 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics are observed; and fecundity and fertilization success are monitored. At selected times during the assay, embryo hatching and larval survivability are measured. At termination of the assay, measurements are made of a number of endpoints reflective of the status of the reproductive endocrine system, including the gonadal-somatic index (GSI), gonadal histology, and plasma concentrations of vitellogenin and sex steroids (17β-estradiol, testosterone, 11-ketotestosterone).

The two primary study objectives are: 1) evaluate the transferability and sensitivity of short-term reproduction assays with the fathead minnow to identify specific modes of action of endocrine disruptors using four model compounds, and 2) conduct a side-by-side comparison of the 21-day fathead minnow short-term reproduction assay (EPA 2001) with two separate 14-day assays: one a shortened version of the 21-day assay with less intensive monitoring of reproductive performance and the other an assay using pre-spawning fathead minnows (OECD Draft 31 December 2001). This latter assay is simplified by not measuring reproductive performance parameters.

2.0 MATERIALS AND METHODS

2.1 Test Material and Exposure Regime

The test chemical concentrations series used for this project were determined in consultation with representatives of EPA and are summarized in Table 2.1. Each chemical was chosen based upon a suspected mode of action. Briefly methoxychlor is thought to be a weak estrogen and is believed to be metabolized into an active estrogen like metabolite. Trenbolone is an anabolic steroid that mimics 11-KT and testosterone to cause masculinization of females and perhaps enhance growth. Flutamide is thought to be metabolized into the 2-hydroxylated form which is a mammalian androgen receptor antagonist. If this effect occurs in fish, then the normal effectiveness of testosterone and 11-KT would be reduced. Fadrozole is an aromatase inhibitor. It is also an indirect anti-estrogen by block synthesis of estrogen (aromatase is the key synthetic step in E2 formation).

Table 2.1. Chemical concentrations used in the WA 2-18 program

	Exposure Concentration (µg/L)		
Test Chemical	**Low**	**Mid***	**High**
Methoxychlor	1	2.5	5
Trenbolone	0.1	0.5	1
Flutamide	6.0	350	650
Fadrozole	5	25	50

*Mid concentration used for non-spawning method only.

2.2 Test Material

Test-grade aliquots of each chemical were received from the EDSP chemical repository in Sequim, Washington. The chemicals were logged in for analysis following the procedures for sample receipt, handling, and storage: MSL-A-001, Sample Log-In Procedure, and MSL-A-002, Sample Chain-of-Custody. The test materials were stored under appropriate conditions until transferred to the toxicology laboratory for use in the diluter system. A copy of the chain-of-custody form accompanied all materials. Prior to conducting the chemical exposures, purity and stability experiments were conducted. A summary of those results is located in Appendix A.

2.3 Preparation and Sampling of Chemical Exposure Water

Methoxychlor and flutamide were prepared using a saturator column (Figure 2.1) similar to the method described in Kahl et al. (1999). A complete description of sample preparation and collection can be found in chemical stability plans included as appendices to this report. The saturator-column method was chosen because methoxychlor and flutamide were found to have low water solubility, and concentrated stock solutions approaching the limit of aqueous solubility were found not to be chemically stable over time. This was documented by chemical stability studies performed by the EDSP chemical repository.

Specifically, the saturator column was used to coat the chemical onto a large surface area (i.e., glass wool) and to expose the water to the surfaces until an equilibrium concentration was reached. Approximately 1 g of methoxychlor and in a later study, 5 g of flutamide, in an aliquot of acetone of predetermined volume were used in the column, followed by evaporation of the acetone by aspirator vacuum.

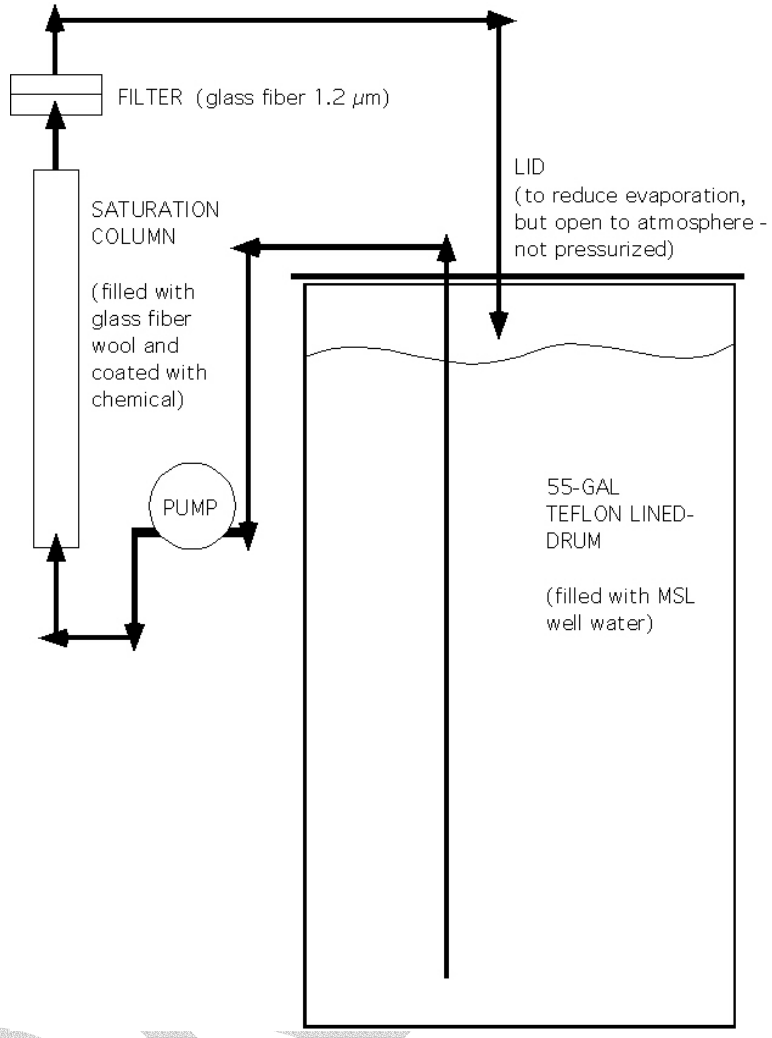

Figure 2.1 Saturator-column apparatus

To prevent the need for daily preparation of stock solutions, large volumes were prepared with either a 32-gal or a 55-gal stock solution in a high-density polyethylene (HDPE) drum lined with a removable Teflon bag, which provided an inert absorption surface. The stock solution was prepared by pumping water through the column with a fluid metering stainless-steel pump at a flow rate of about 0.1 L/min. The stock solution in the drum was recirculated through the saturator-column system. The stock solution was sampled daily until a stable concentration was reached (0.1 mg/L methoxychlor and 20 mg/L flutamide), then the drum was sampled twice per week to determine stability and duration of stability for each solution.

Stock-solution samples were generated by use of a saturator column as documented in the stability and testing plans (Appendix X). Saturator-column samples were collected to verify test concentrations as new columns were prepared to generate the large volumes of chemical-laden water required for the study.

Methoxychlor and flutamide sample concentrations were collected from the aquaria prior to beginning the test and weekly during the test to verify the concentration of the chemical solutions. Samples from replicate diluters provide a measure of precision.

The preparation of trenbolone stock solution did not require the use of a saturator column. A stock solution of 10 mg/L was initially prepared by the addition of 150 mg trenbolone into 15 L deionized water. Then, 2 L of this stock was added to 18 L deionized water, which resulted in a test solution of approximately 1 mg/L. Samples of the stock solution were analyzed prior to use. Test-solution samples were collected from each test aquarium and analyzed weekly throughout the duration of the test.

The preparation of the fadrozole stock solution also did not require the use of a saturator. A stock solution of 40 mg/L was prepared by the addition of approximately 800 mg fadrozole into 20 L deionized water. Samples of the stock solution were analyzed prior to use. Test-solution samples were collected from the aquaria and analyzed weekly throughout the duration of the test.

A continuous-flow proportional diluter was used to deliver chemical concentrations to the test aquaria (Figure 2.2). The diluter was a modified version of the Mount and Brungs adjusted to deliver three concentrations (including control) with four replicates per concentration for the EPA methods. A second diluter was modified for the non-spawning method to deliver four concentrations (including control) with two replicates per concentration.

The chemical stock solution was metered into the mixing cell of the diluter using a fluid metering pump. The diluter was set to add chemical-laden water to the test chamber every 12 min and was equal to six volume exchanges of water per day. The diluter casing was covered in black plastic to reduce biological activity, such as the development of algal growth, during the test. Prior to introduction of the chemical, each diluter was calibrated using sodium chloride as an easily measured surrogate test chemical. After completion of the salt calibrations, the chemical was added to the mixing chamber *via* a fluid metering pump, and concentrations in aquaria were checked prior to the introduction of organisms.

Figure 2.2. Continuous-flow proportional diluter system

2.4 Analytical Procedures

After preparation of the stock solutions for each of the chemicals, determinations of concentrations were made using the methods described below. The concentrations of the chemicals in the exposure chambers were measured prior to adding fish to verify that test concentrations were within 30% of target concentrations. Samples (up to 10 mL) were collected weekly from the test chambers and were hand-carried and delivered to the laboratory on the same day collected. Chain-of-custody forms accompanied all samples. Sample collection and extraction and analyses dates are included in the in-life chemistry data.

2.4.1 Methoxychlor

Water samples (approximately 10 mLs) were extracted using 1 mL hexane and analyzed using a gas chromatograph with an electron capture detector (GC-ECD). Next, an internal standard (300 ng iodonapthalene) was added to the water samples, and then mixed with a vortex mixer. The hexane layer was transferred to a GC autosampler vial and 1 µL injected onto the GC equipped with a Hewlett-Packard 30-m X 0.25-mm, DB-5 capillary column. The sample concentration was determined by comparing the peak heights in the chromatogram with the peak heights of the calibration standard five-point curve. The temperature program was set to start at 100ºC, and ramped at 20ºC/min to a final temperature of 300ºC. The injection port temperature was set at 250ºC and the detector at 325ºC. The autosampler was set to inject 1 µL of the matrix dilution every sample.

2.4.2 Trenbolone

Quantification by GC with mass selective detection (MSD) was used for the analysis of the low concentrations of trenbolone and control exposure water. Water samples (10 mL) were removed from the exposure tanks and stored in a 20-mL Pyrex culture tube, to which the internal standard, 17β-estradiol, was added. Next, approximately 1 g sodium chloride (NaCl) was added, the sample was agitated, and 2.0 mL hexane was added. This mixture was blended with a vortex mixer, and the hexane layer removed and placed in a new glass tube. This procedure was done in triplicate, and the hexane layers were pooled. The pooled hexane layers were then evaporated to dryness under helium (He) gas. Next, the evaporated residue was dissolved in 10 µL of n-methyl-n-trimethylsilyl-trifluoroacetamide (MSTFA) with catalytic amounts of resublimed iodine (1000:4 v:w) added as the derivitizing agent. A 1-µL quantity was injected onto the GC. The GC-MSD was operated in the selected ion-monitoring mode with the molecular ions m/z 442 (trenbolone /285 (internal standard, 17β-estradiol)) used for quantitative purposes. The observed retention time for trenbolone is approximately 18.29 minutes and for the internal standard, 14.55 minutes. A five-point calibration curve was prepared and used for quantification of the trenbolone in the water samples.

Analysis of the mid- and high-trenbolone-concentration exposure water was conducted by high-performance liquid chromatograph (HPLC) analysis using the fluorescence detector. Analyses of the trenbolone stock solution were done by HPLC analysis using the ultraviolet/visible (UV/VIS) detector at 347 nm.

2.4.3 Flutamide

The water samples (approximately 10 mLs) were mixed with 50:50 v:v ACN and analyzed using an HPLC with a UV/VIS detector at the 220-nm wavelength. A 60:40 ACN:water (v:v) mix was used for the eluent at 1.5 mL/min. Separation was attained using a Supelco polynuclear aromatic hydrocarbon (PAH) (25-cm X 4.8- mm, C-18 column). For samples analyzed using the HPLC system, data were

stored in MSL5, Room 219 on the computer with a property number of WV04738. Multipiers of 40 (1-mL total sample volume made from 0.025-mL of sample and 0.975 mL ACN:water) and 2 (0.5-mL sample with 0.5-mL ACN) were used in the Varian Star software.

2.4.4 Fadrozole

The samples were analyzed using an HPLC with a UV/VIS detector at the 236-nm wavelength with a five-point calibration curve. A 1.0-mL quantity of each sample was injected for separation and analysis. The eluent was a 25-mM sodium phosphate buffer solution and acetonitrile (lot # Y02820) at a 30:70 v:v proportion. Separation was attained using a Pherominex Synergy 4u Hydrus RP 80A 250-mm X 4.6-mm column. For samples analyzed using the HPLC system, data were stored in MSL5, Room 219, on the computer.

2.5 Animals and Husbandry

The requirements for age and size of the test species stated that the minnows must be sexually dimorphic, should be first-time spawning conditions, approximately 120 days old, and the minimum size must be 2.5 g for males and 1.5 g for females. These requirements were met by purchasing organisms from two suppliers: Environmental Consulting and Testing (EC&T), Superior, Wisconsin, and ABC Laboratories, Columbia, Missouri. Documentation of chain-of-custody, the condition of the animals when shipped and upon receipt, and environmental parameters (temperature) at the time of shipping for comparison with conditions encountered at the time of receipt, and verification of the taxonomy of the organisms (genus, species) and disease-free status were submitted with each batch of organisms. The vendor wrote an informal note stating that there was no reported incidence of disease during the care and maintenance of the minnows.

Approximately 1200, 4-month-old *P. promelas* were obtained from EC&T and used in the methoxychlor experiments. Because of questions regarding overall fish health that arose after the methoxychlor test, it was decided that younger *P. promelas* should be purchased and held for 2 to 3 months prior to the test. Most aquatic suppliers of *P. promelas* do not rear minnows to 120 days. Therefore, it is possible that any problem with the health of the fish became apparent only during their later more mature stages.

Approximately 2400, 30- to 60-day old *P. promelas* were purchased from ABC Laboratories and cultured to the stage of reproductive differentiation and sexual maturation following the guidance described in *Guidelines for the Culture of Fathead Minnows (Pimephales promelas) for Use in Toxicity Tests* (EPA 1987) and SOP Number EDSP.E-001-01. This group of *P. promelas* was used in experiments conducted with trenbolone, flutamide, and fadrozole.

Water conditions for both supplies of *P. promelas* were maintained at 24°C to 26°C. A flow-through system design provided adequate volume replacement for organism needs while maintaining the required constant temperature. A continuous, gentle aeration from an oil-free air supply was provided to the tanks. The minnows were housed in 30-gal tanks upon arrival and until sexually differentiated, whereupon they were separated by sex and transferred to clean, 10-gal aquaria until needed for testing. To establish breeding pairs, four females and two males were transferred at the time of assay to 5-gal containers, which contained spawning tiles made of terracotta. Table 2.2 provides the water-quality characteristics for culturing and testing the fathead minnow:

Table 2.2. Recommended ranges of water-quality characteristics for testing fathead minnows

Water Characteristic	Preferred Range
Temperature (^{o}C)	24°C - 26°C
Dissolved Oxygen (mg/L)	>4.9 mg/L (≥60% saturation)
pH	6.5 - 9.0 pH units
Total Alkalinity (mg/L as $CaCo_3$)	>20 mg/L
Total Organic Carbon (mg/L)	≤5 mg/L
Unionized Ammonia	≤35 mg/L

2.6 Study Schedule and Design

The design of this study was to evaluate the sensitivity of short-term reproduction and nonreproductive assays with the fathead minnow *(P. promelas)* to identify specific modes of action of endocrine disruptors using four model compounds, and to conduct a side-by-side comparison of the 21-day fathead minnow, short-term reproduction assay (EPA 2001) with two separate 14-day assays: a shortened version of the 21-day assay and an assay that does not measure reproductive performance (males and females are kept in separate chambers and do not spawn) (OECD 2001). Two different proportional flow diluters were used for a given chemical. One diluter was prepared to deliver three concentrations (low, medium, and high) and a control, and was used for the non-spawning method. The other was prepared to deliver two concentrations and a control, and was used for both the EPA 14- and 21-day assays. Two different stock solutions were prepared and monitored for each diluter. The testing schedule is presented in Table 2.3.

Table 2.3. Testing schedule for *P. promelas* using four chemicals and three assays

Chemical	Task	Date
Methoxychlor	EPA 14 Pre-exp.	9/29/2002
	EPA 21 Pre-exp.	9/29/2002
	EPA 14-day test	10/14/2002
	EPA 21-day test	10/14/2002
	Non-spawning	10/14/2002
Trenbolone	EPA 14 Pre-exp.	2/4/2003
	EPA 21 Pre-exp.	1/20/2003
	EPA 14-day test	2/11/2003
	EPA 21-day test	2/3/2003
	Non-spawning	2/10/2003
Flutamide	EPA 14 Pre-exp.	2/18/2003
	EPA 21 Pre-exp.	2/11/2003
	EPA 14-day test	2/25/2003
	EPA 21-day test	2/25/2003
	Non-spawning	3/10/2003
Fadrozole	EPA 14 Pre-exp.	3/17/2003
	EPA 21 Pre-exp.	3/10/2003
	EPA 14 test	3/27/2003
	EPA 21 day test	3/25/2003
	Non-spawning	4/1/2003

For the two EPA assays, the *P. promelas* were first held under a pre-exposure phase with no chemical present for 7 to 14 days to establish a record of spawning success and to measure viability of embryos. The assay units (5-gal aquaria with 4 females and 2 males) were then chosen for a 14- or 21-day chemical exposure. There was no pre-exposure phase for the non-spawning method prior to introduction of the chemical into the test system.

2.7 Description of Study Protocols

The experimental design included the comparison of three fish-screening protocols in side-by-side experiments using four different chemicals.

2.7.1 Summary of the 14-day Non-spawning Method

The 14-day non-spawning assay was based on a description in the OECD draft proposal–31 December 2001 (OECD 2001). The assay was intended to detect endocrine disruptor effects of specific chemicals with a mode of action that is either (anti)androgenic or (anti)estrogenic.

P. promelas chosen for this protocol were pre-spawning adults, which were separated by sex. The assay was initiated with healthy, sexually dimorphic adult fish (males and females contained in separate chambers to prevent induction of spawning). The chambers included two replicate tanks (one for each gender) per treatment with four treatments: a dilution water control, and low, medium, and high concentrations. The non-spawning assay was conducted at the same time as the EPA 14- and 21-day assays; however, a different chemical stock solution, proportional diluter, and water table were used for this method. The stock solutions and water chemistry parameters were monitored throughout testing, and observations conducted daily of test aquaria.

The measurement endpoints included survival, morphological, and histological parameters. At the conclusion of the test, blood samples were collected from the adults for determination of sex steroids and vitellogenin (VTG), and the gonads were sampled for measurement of the GSI and histological analyses. In addition, fork length measurements were taken and general gross morphological observations were made.

2.7.2 Summary of the 14-day EPA Method

The 14-day assay began with at least a 7-day pre-exposure phase. This phase was conducted using 5-gal aquaria containing three terracotta nesting tiles. Breeding pairs for each aquarium included four females and two males that were randomly assigned to an aquarium at each treatment concentration. Additional exposure chambers were set up to account for non-spawning or otherwise compromised breeding pairs in some chambers during this time.

The pre-exposure phase was conducted under conditions (temperature, photoperiod, feeding, and flow) identical to those used during the chemical exposure. The *P. promelas* were fed frozen *Artemia* up to three times daily *ad libitum* and were monitored daily for alterations in secondary sex characteristics (breeding tubercles in males; ovipositor in females), reproductive behavior, and spawning activity. No quantitative measures of fecundity were made during the pre-exposure phase. However, larval hatchability was assessed using a subset of normal eggs (approximately 50) incubated at test conditions for 3 to 6 days until eggs hatched.

The data collected from the pre-exposure phase were examined using criteria as recommended in EPA guidelines (page 37, EPA 2001): regular spawning occurs in each test chamber every 3 to 4 days, and a greater than 90% hatching rate of larvae is observed after 7 days.

Breeding pairs that passed the requirements from the pre-exposure phase were then transferred to the14-day chemical exposure phase. Test treatments included four replicate containers, a dilution-water control, and a low and high concentration that matched the low and high concentrations measured for the non-spawning assay. The 14-day assay measured the reproductive performance of groups of fathead minnows as the primary indicator for endocrine disruption. The fish's appearance, behavior, and fecundity were assessed daily, and routine water chemistry measurements were taken. Terracotta tiles and nytex screen were used as housing for fish during spawning. The screened terracotta dishes allowed enumeration of eggs that did not adhere to the underside of the tile. (Figure 2.3) Larval hatching as described above was conducted one time during the 14-day chemical exposure phase. At termination of the exposure, blood samples were removed from adults and analyzed for sex steroids and VTG. The gonads were also removed for GSI determination and later histological analyses. In addition, fork length measurements and general gross morphological conditions were noted (appearance of adults).

Figure 2.3 Terracotta dish with nytex screen

2.7.3 Summary of 21-day EPA Method

The 21-day EPA assay method paralleled the 14-day method with the exception that larval hatching was conducted once during the pre-exposure phase and three times during chemical exposure. Under this scenario, the pre-exposure phase continued for 14-days (rather than 7) and quantitative counts of fecundity were performed daily. The data generated from the pre-exposure phase were used to determine which spawning pairs were suitable for the 21-day chemical exposure. The aquaria chosen for chemical exposure were then transferred to the proportional diluter table in the same system/tanks as was used for the pre-exposure phase.

The 21-day chemical exposure was conducted using the same chemical stock solution, proportional diluter, and water table as the 14-day EPA assay. This side-by-side comparison using the same chemical concentrations and source of heated dilution water allowed for more robust statistical comparisons.

During the 21-day chemical-exposure period, the fish's appearance, behavior, and fecundity were assessed daily. Viability of resultant embryos (e.g., hatching success) was measured on Days 7, 14, and 21 by incubating approximately 50 eggs in dilution water for 3 to 6 days until the majority of eggs hatched.

At conclusion of the assay, blood samples were collected from the adults for determination of sex steroids and VTG, and the gonads were sampled for measurement of the GSI and for histological analyses. In addition, fork length measurements and general gross morphological conditions were noted (appearance of adults).

2.7.4 Summary of Assay Endpoints

Survival: Daily assessment of survival was made to provide a basis for expression and interpretation of reproductive output: that is, number of eggs per female per day.

Behavior of Adults: Abnormal behavior relative to controls, such as hyperventilation, loss of equilibrium, and feeding abstinence, was noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, were noted.

Figure 2.4 Staff members count eggs in support of fecundity measurements.

Fecundity/Fertilization Success: Egg production was determined daily. The terracotta spawning substrates were removed from the tanks, and the eggs were allowed to harden prior to enumeration of eggs. After hardening, the eggs were carefully rolled off the tile with a gentle circular motion of a gloved index finger and visually inspected under appropriate magnification. If no embryos were present, the substrate was left in the aquarium. If spawning occurred that morning, embryos typically underwent late cleavage, and determination of the fertility rate (number of embryos/number of eggs x 100) was easily achieved. Infertile eggs were opaque or clear with a white dot where the yolk precipitated; viable embryos remained clear for 36 to 48 hours until reaching the eyed stage. Fecundity was expressed on the basis of surviving females per reproductive (test) day per replicate. This approach was used for both the 14- and 21-day assays (Figure 2.4).

Hatchability and Larvae Appearance: This endpoint was assessed as follows: 14-day assay: once during pre-exposure (Day 7) and once during exposure (Day 7); 21-day assay: once during pre-exposure (Day 7) and three times during exposure (Days 7, 14, and 21).

Approximately 50 normal healthy looking embryos were transferred to incubation chambers (1-L flow-through jars with gentle flow and aeration) and held at 25°C (Figure 2.5). The larvae typically hatched in 3 to 6 days. Daily observations were conducted and the number of embryos, newly hatched larvae, and any dead embryos or larvae were scored. The data from the hatching test were used to determine the percentage of eggs that hatched and number of normal appearing larvae.

Figure 2.5. Flow-through embryo-incubation chambers

Appearance of Adults: The external appearance of the adults was assessed as part of the daily observations, and any unusual changes were noted. External features of particular importance included body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (dorsal nape pad, nuptial tubercles in males; ovipositor in females). The daily observation forms are included as appendices to this report.

Blood Sampling: At the conclusion of the exposure, the fish were anesthetized by transfer to an oxygenated solution of tricane sulfate (MS-222) (250 mg/L buffered with 200 mg $NaHCO_3$/L). Blood was collected from the caudal vein with a heparinized microhematocrit capillary tubule. Depending on the size of the fathead minnow, which usually is gender-dependent, blood volumes generally ranged from 30 µL to 80 µL. Plasma was separated from the blood *via* centrifugation (2 min) and stored with protease inhibitors at -80ºC until analyzed for VTG and sex steroids.

Vitellogenin: The measurement of VTG plasma samples was performed using an enzyme-linked immunoabsorbant test (ELISA). Vitellogenin levels were quantitated in plasma samples from each individual fish, for all treatments, using research quality test kits (product number V01003401) procured from Biosense Laboratories (AS – HIB-Thormohlengsgt. 55 N-5008, Bergen, Norway). The analyses were conducted on a Bio-Tek Synergy HT microtiter plate reader interfaced to a Dell computer, employing the Bio-Tek KC4 test analysis software.

Plasma samples were frozen in 5-µL aliquots and stored in 600-µL microcentrifuge tubes at -80°C until the day of analysis. For analyses, samples were removed from -80°C storage and placed on ice. The samples were rehydrated with 495 µL cold phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). Appropriate dilutions of the samples, beyond the initial 1:100 dilution, were carried out prior to the analysis. Dilutions ranged from 1:100 to $1:10^8$, determined by the sex of the fish and specific chemical exposure defined in the study. The test requires approximately 6 to 8 hours to complete, depending on the number of samples run and the number of dilutions of each sample required. A typical daily run would include 82 samples divided between two 96-well microtiter plates.

The test has a calibration range of 0.24 ng/mL to 250 ng/mL, with a viable quantitation range of approximately 0.6 ng/mL through 125 ng/mL. For data analyses, a value of zero (0) was assigned to samples in which vitellogenin was not detected. Data were natural-log transformed (ln (concentration +1)) before analyses were conducted.

The test required three 1-hour incubations, each with a different antibody-based reagent required to capture the sample analyte and create the detectable sandwich. The sandwich was made up of the capture antibody, the analyte, the detecting antibody, and the secondary antibody labeled with the horseradish peroxidase (HRP) enzyme. In the final step, the microtiter plate wells were thoroughly washed with a wash buffer, removing all test components, save the analyte captured within the bound sandwich. An HRP substrate was then added to the wells, and following a 30-min incubation, the absorbance of each well was read at 492 nm. Absorbance levels increased with increased calibrator or sample concentrations.

Sex Steroid: Plasma concentrations of β-estradiol, testosterone, and 11-ketotesosterone were measured using competitive enzyme immunoassays (EIAS) commercially available for each steroid of interest.

Gonad Size and Morphology: After blood was sampled, fish were weighed, and the gonads were removed and weighed to the nearest 0.1 mg to determine the GSI (GSI = 100 X gonad wt/body wt) (Figure 2.4). Typical GSI values for reproductively active fathead minnows range from 8% to 13% for females and from 1% to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes. After removal of the gonads, the remainder of the carcass of the fish was discarded.

Histology: Routine histological procedures were used to assess the condition of testes and ovaries from the fish. Gonads were placed in fixative (10% buffered formalin) and embedded in paraffin. Serial sections 4-to 5-µm thick were cut along the long axis of the gonad. At least two serial sections were collected from at least three steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of six tissue sections per sample (Figure 2.6). Sections were stained with hematoxylin and eosin, and were submitted to and evaluated by a board certified histologist without prior knowledge of the treatment regime associated with specific samples. A summary of the methods used by the histologist follows.

Figure 2.6. Collection of the liver and gonads

The histology method was previously described in Ankley et al. (2001); methods, original references, and photomicrographs of normal developmental stages of the reproductive tract of both female and male fathead minnows were included. The methods were developed for the systematic assessment of fathead minnow reproductive tracts and reference to pathological changes that may occur upon exposures to endocrine disruptor chemicals (EDCs). These methods were followed. The paraffin-embedding technique was selected over the glycol methacrylate-embedding technique for these analyses.

The following is an outline summary of the procedures that were used to evaluate the histological sections. Multiple measurements from each individual were taken from a variety of locations on the tissue sections.

Females:

1. The ovary was staged, that is, given a number from 1a, 1b, 2, 3, 4, or 5, based on the most advanced stage present (Table 2.4). The explanation of the stage numbering was provided in Ankley et al. (2001). This staging was done in six locations on each of the three histological slides.

2. Oogonia and oocytes were typed, and 100 cells from each of three sections were rated according to developmental stage (stages noted above, including atretic follicles and corpora lutea).

3. Abnormalities in the ovary were noted.

Males:

1. Testes were staged, that is, given a number from 1, 2a, 2b, 3a, 3 b, 4, or 5, based on the most advanced stage present (Table 2.5). This staging was done on four locations on each of the three slides.

2. Based on stages noted above, 100 spermatic cells were typed from each of the three slides. This typing is done by counting the cells along a straight line on an ocular grid in a predetermined pattern. More than one line on the grid is used if required to obtain the 100 cells.

3. The testicular lumen diameter (μm) was measured from six tubules on each of three slides.

4. Other changes were noted, including changes to the interstitial tissues, such as proliferation of Sertoli or Leydig cells. In addition, abnormal patterns of development were noted, such as premature shedding of spermatocytes into the tubule lumen or foci of necrotic spermatocytes. The presence of any ovatestes or patterns of testicular atrophy were noted.

Quality Assurance:

Over 300 randomly picked slides from the first data set were examined in detail for conformance with the descriptions provided in Ankley et al. (2001) prior to beginning the systematic examination of the histology slides. The raw data from systematic slide evaluation were recorded on hand-written sheets. These data were transferred to a Microsoft Excel spreadsheet. A second 100%-accuracy check of data transfer was conducted after the initial transfer of data to the spreadsheets. In addition, spot checks of comments and abnormal conditions were conducted by re-examining the slides for conformance with the original comments. In addition, a final examination of the spreadsheet for accuracy was made by the histology principal investigator.

Table 2.4. Histological stages of fathead minnow ovarian development.

Stage	**Characteristics**
1. Primary Growth	Oogonia and primary oocytes
	1a. Oocytes in nests; small cytoplasmic volume
	1b. Oocytes larger, out of nests, surrounded by follicle cells; many pleiomorphic nucleoli bordering the nuclear envelope
2. Cortical Alveolus	Appearance of cortical alveoli and possibly small lipid droplets
3. Early Vitellogenic	Appearance of yolk bodies; initially few and small, ultimately many and variably-sized; centrally-located germinal vesicle is round to oval with several peripheral nucleoli
4. Late Vitellogenic	Germinal vesicle loses nucleoli, moves towards the periphery and breaks down; yolk bodies frequently fill the entire center of the oocyte and a germinal vesicle may not be evident
5. Mature/Spawning Oocytes	Germinal vesicle breakdown complete; yolk bodies fuse and may become larger than cortical alveoli

Table 2.5. Histological stages of fathead minnow testicular development.

Stage	Characteristics
1. Resting Germ Cells	No development
2. Spermatogonia	2a. Primary Spermatogonia: Large cells near edges of tubule; have a lightly staining nucleus with a prominent nucleolus 2b. Secondary Spermatogonia: Clusters of medium-sized cells with a round, lightly basophilic nucleus; cluster or cyst is the result of several mitotic divisions of primary spermatocyte
3. Spermatocytes	3a. Primary Spermatocytes: Smaller cells with smaller, more basophilic nuclei than Spermatogonia; will undergo meiosis I to produce secondary spermatocytes 3b. Secondary Spermatocytes: Small cells with smaller, more basophilic nuclei than primary spermatocytes; will undergo meiosis II to produce spermatids
4. Spermatids and some spermatozoa in lumen of seminiferous tubules; small tubule lumen	Spermatids have a small, intensely basophilic nucleus; mature into spermatozoa
5. Abundant sperm in an expanded lumen	

Table 2.6 provides a detailed summary of each assay and the associated data collected during testing.

Table 2.6. Summary of the testing conditions for each assay

Parameters	Non-spawning 14-day	EPA 14-day	EPA 21-day
Age of organisms	Prebreeding adult (5-6 months) fathead minnows	Reproductive adult fathead minnows (120 day minimum)	Reproductive adult fathead minnows (120 day minimum)
Holding Conditions:	Temp: 25°C ±1°C D.O.[(a)] >4.9 mg/L Light: 16 h light:8 h dark with 400 – 500 lux Fed: live brine shrimp and ground salmon starter (automatic feeder) until able to eat frozen brine shrimp. Brine shrimp was given up to three times daily	Temp: 25°C ±1°C D.O. >4.9 mg/L Light: 16 h light:8 h dark with 400 – 500 lux Fed: live brine shrimp and ground salmon starter (automatic feeder) until able to eat frozen brine shrimp. Brine shrimp was given up to three times daily	Temp: 25°C ±1°C D.O. >4.9 mg/L Light: 16 h light:8 h dark with 400 – 500 lux Fed: live brine shrimp and ground salmon starter (automatic feeder) until able to eat frozen brine shrimp. Brine shrimp was given up to three times daily
Assay Conditions	Flow-through continuous dispersal of chemical concentrations using a proportional diluter	Flow-through continuous dispersal of chemical concentrations using a proportional diluter	Flow-through continuous dispersal of chemical concentrations using a proportional diluter
Duration:	14-day	14-day	21-day

Parameters	Non-spawning 14-day	EPA 14-day	EPA 21-day
Dilution water	Clean artesian well water monitored yearly for drinking water standards	Clean artesian well water monitored yearly for drinking water standards	Clean artesian well water monitored yearly for drinking water standards
material	EDC-chemical	EDC-chemical	EDC-chemical
chamber size	18 L	18 L (40 x 20 x 20 cm)	18 L (40 x 20 x 20 cm)
Volume:	10 L	10 L	10 L
# Exchanges/day	6 volumes	6 volumes	6 volumes
# Of conc./chemical	3	2	2
# Replicates:	2	4	4
Weight of each fish	Adult female: 1.5 ±10% Adult male: 2.5 ±10%	Not specified	Not specified
# Fish/replicate	10 (must be all one sex)	4 females and 2 males per test aquarium with nesting tiles	4 females and 2 males per test aquarium with nesting tiles
Feeding regime	Frozen brine shrimp, two to three times per day. Typically up to 1 mL per tank.	Frozen brine shrimp, two to three times per day. Typically up to 1 mL per tank.	Frozen brine shrimp, two to three times per day. Typically up to 1 mL per tank.
# Controls	Dilution water	Dilution water	Dilution water
# Replicates/control	2	4	4
# Fish/control	10 adult females 10 adult males (in separate vessels) = 20	4 adult females and 2 adult males per replicate = 24	4 adult females and 2 adult males per replicate = 24
Conditions:			
Photoperiod:	16 h light:8 h dark	16 h light:8 h dark	16 h light:8 h dark
Temperature:	25°C ±1°C- monitored continuously in one chamber and daily in one test replicate per concentration. Monitoring conducted using a min-max thermometer with readings recorded every 24 hours	25°C ±1°C- monitored continuously in one chamber and daily in one test replicate per concentration. Monitoring conducted using a min-max thermometer with readings recorded every 24 hours	25°C ±1°C- monitored continuously in one chamber and daily in one test replicate per concentration. Monitoring conducted using a min-max thermometer with readings recorded every 24 hours
Light intensity	540-1080 lux, monitored at the start and end of assay	540-1080 lux, monitored at the start and end of assay	540-1080 lux, monitored at the start and end of assay
Aeration:	D.O. >4.9 mg/L	D.O. >4.9 mg/L	D.O. >4.9 mg/L
pH	±0.5 pH units	Not Specified	Not Specified
Alkalinity:	>20 mg/L CaCO3	>20 mg/L CaCO3	>20 mg/L CaCO3
Hardness:	>140 mg/L CaCO3	>140 mg/L CaCO3	>140 mg/L CaCO3

Parameters	Non-spawning 14-day	EPA 14-day	EPA 21-day
Total ammonia	<0.5 mg/L	<0.5 mg/L	<0.5 mg/L
Monitoring:			
WQ Frequency:	Start and end of assay: Temperature, D.O, pH, and total ammonia in all replicates Daily: Temperature, pH and D.O. one replicate of each treatment Weekly total ammonia: 20% of samples Once during assay out of the water head tank – hardness and alkalinity	Start and end of assay: Temperature, D.O, pH, and total ammonia in all replicates Daily: Temperature, pH and D.O. one replicate of each treatment Weekly total ammonia: 20% of samples Once during assay out of the water head tank – hardness and alkalinity	Start and end of assay: Temperature, D.O, pH, and total ammonia in all replicates Daily: Temperature, pH and D.O. one replicate of each treatment Weekly total ammonia: 20% of samples Once during assay out of the water head tank – hardness and alkalinity
Corrective Actions:	Temperature: adjust controller Total ammonia: increase water flow and clean tanks Photoperiod: adjust controller Light intensity: adjust bulbs over tables pH: no action Alkalinity: no action Hardness: no action	Temperature: adjust controller Total ammonia: increase water flow and clean tanks Photoperiod: adjust controller Light intensity: adjust bulbs over tables pH: no action Alkalinity: no action Hardness: no action	Temperature: adjust controller Total ammonia: increase water flow and clean tanks Photoperiod: adjust controller Light intensity: adjust bulbs over tables pH: no action Alkalinity: no action Hardness: no action
Biological endpoints:	Survival Behavior Secondary sexual characteristics Gross morphology (GSI) and Gonadal history plasma VTG and sex steroids (β-estradiol, testosterone, 11-KT[b]) concentrations	Adult survival, reproductive behavior, fecundity, fertility, embryo hatch, secondary sexual characteristics, Gross morphology (GSI) and gonadal history, plasma VTG and sex steroids (β -estradiol, testosterone, 11-KT) concentrations	Adult survival, reproductive behavior, fecundity, fertility, embryo hatch, secondary sexual characteristics, Gross morphology (GSI) and gonadal history, plasma VTG and sex steroids (β -estradiol, testosterone, 11-KT) concentrations
Validity Criteria:	D.O. ≥60% saturation; Mean temp. 25°C ±2°C should be maintained and corrective action taken if water quality is outside of these limits Also, 90% survival in the controls	D.O. ≥60% saturation; Mean temp. 25°C ±2°C should be maintained and corrective action taken if water quality is outside of these limits Also, 90% survival in the controls	D.O. ≥60% saturation; Mean temp. 25°C ±2°C should be maintained and corrective action taken if water quality is outside of these limits Also, 90% survival in the controls

a) D.O. dissolved oxygen.
b) KT ketotestosterone.

2.8 Statistical Analyses

The screening assays as described were designed to detect potential EDCs with high power (minimum of 80%) and not to produce a precise estimate of toxicity. The statistical considerations were restricted to the demands of the screening test. The amount of information obtained from the screening test was limited to detecting effects on reproductive traits when both genders are exposed or in determining whether or not gender-specific differences are detected when gender-selective exposure was used.

Descriptive statistics, including the mean, standard deviation, minimum, maximum, and quartiles, were used to characterize each endpoint measured in the three tests. Statistical significance for each endpoint and chemical was evaluated based on the difference in the mean characteristics between the treated and control groups using analysis of variance, Tukey's multiple comparisons test, and the nonparametric Kruskal-Wallis test. Chemical-dosing regimes were considered classifications of fixed effects (i.e., control, low dose, mid dose, and high dose). Box plots were used to visually characterize the effect of each treatment.

Power analysis assuming a Type I error rate of $\alpha = 0.05$ was used to compare the sensitivity of selected endpoints. The minimum detectable difference assuming 80% power for the given sample size and standard deviation achieved during testing was calculated. The achieved power, given the observed maximum difference between means, was also calculated. Finally, the required sample size needed to achieve 80% power was calculated, given the achieved maximum mean difference.

Consistency between the screening test decisions across endpoints and chemicals were evaluated using Kendall's coefficient of concordance (Daniel 1978). The smallest difference between means detected at a power of 80% and $\alpha = 0.05$ for a given endpoint and protocol provided a quantitative measure of sensitivity. Recommendations as to potential changes to the number of replicates to achieve a given level of power are provided.

Appropriate data transformations were applied to maintain homogeneity of the within-class variances (i.e., data expressed as a percentage may be arcsine-square-root or light transformed, counts may be square-root or log transformed, and continuous data may be transformed to the natural logarithm) (Snedecor and Cochran 1980). A rank transformation or nonparametric statistics were used when the common data transformation was not successful in controlling heterogeneity (Daniel 1978).

Analysis may have been conducted both with and without suspected outliers (Chapman et al. 1996). Potential outliers may have been identified by values that exceed the median plus three times the interquartile range (i.e., the difference between the 75th and 25th percentiles). If an explanation could not be made for the divergence of data, then both analyses were presented, assuming that the results differed. If there were no changes to the results, then the analysis including the outliers was presented. If differences occurred, then the implications of removing the outliers were carefully documented. If an explanation could be made for the existence of outliers, the analysis excluding outliers may have been sufficient.

2.9 Quality Assurance

2.9.1 Technical Systems Audits

The Battelle, Sequim QA Unit performed assessments on activities and operations affecting data quality, the raw data and final report. Any findings were reported to the WA Project Manager and management to ensure that the requirements in relevant SOPs, WA protocol, QAPP, and the QMP were met. The

assessments for this study included technical systems audits (TSAs) and audits of data quality (ADQs) that included reviews of project notebooks, data base entry verifications from raw data sheets, and reviews of statistical analyses performed.

TSAs were performed at the start of the study, and for critical elements during the study such as:

- personnel training files for documentation that EDSP SOPs, the work plan and the WA QAPP have been read and understood by WA personnel before any activities begin
- calibration status of project instrumentation
- dosing and sample collection of dosing solutions, body and feed weights, and clinical observations
- chemical analysis of test chemicals
- and termination of each experiment

During TSA activities, the Battelle, Sequim QA Unit recorded observations to be used later in preparing the audit report. The Battelle, Sequim QA Unit observed completion of permitting requirements, implementation of procedures, data recording and record keeping, and equipment maintenance and calibration procedures and/or documentation, noting whether or not the activities adhered to the work plan, and the QAPP, applicable SOPs, and the QMP. Any findings were communicated to the technical personnel at the completion of the WA activity unless an error could compromise the WA (e.g., misdosing an animal). If necessary, the EDSP QA team members immediately notified the WA Leader/Study Director by telephone and/or e-mail of any adverse findings that could impact the conduct of the WA. This direct communication was also documented in the audit report.

2.9.2 Audits of Data Quality

Audits of data quality (ADQs) focused on the accuracy of data collection, recording, traceability, and calculations to ensure that the reported results were documented, traceable, and of high quality that accurately reflected the raw data; that the report accurately described the materials and methods used in the WA; and that conclusions were supported by the data. The assessment criteria for ADQs were that data collection, analysis, and reporting met the requirements of the applicable facility and program SOPs, the work plan, QAPP, and the EDSP QMP, and that deviations were documented as per the requirements of the procedure. Deviation reports relative to the work assignment were submitted to the WA Leader and included in the project records.

Direct and frequent communication between the Project Manager, Laboratory staff, and the QA Unit Manager was designed to provide for sufficient time to perform an ADQ so that the submission date of the audited final report met those specified in the work plan.

All data and records for review were submitted to the QA Unit Manager or delegate, who reviewed the data packages for completeness and, if incomplete, requested that the additional records needed for review be submitted. The EDSP QA team members reviewed a minimum of 10% of the raw data, depending on the level of prior technical review, the tabulated data, and WA records of performance and methods, to ensure compliance with planning documents mentioned previously. All tables and graphs were reviewed for completeness and accuracy of titles, headers, and footnotes. The EDSP QA team members checked all tabulated data designated as statistically significant. Findings were reported and corrective actions undertaken as described earlier. The EDSP QA team members reviewed the report using the audited data and corrected tables to ensure that the reported results were of high quality and

accurately reflected the raw data, and that the report accurately described the materials and methods used in the WA. Findings were reported and corrective actions undertaken as described earlier.

The eTSA and ADQ were conducted throughout the duration of this WA. Neither of these activities resulted in any major findings nor any stop work associated with the conduct of the experiments.

2.10 Storage of Records and Data Management

The data for this study were collected on preprinted data collection forms. The data forms included, as appropriate, the following items: study code, protocol number, tank number, treatment (Rx) code, and others. The forms had preprinted dates for collection of data when possible. Otherwise, the dates for data collection were hand printed on the forms as needed prior to or on the day of collection of the data. All data forms were initialed and dated by the person collecting the data, and all forms received documented technical review and signature approval. Corrections to data entries were made by drawing a single line through the error and recording the correct entry, initials, date, and error code that explained the reason for the correction.

The datasheets were clearly divided by chemical and protocol and placed in a workbook. These workbooks were kept next to the tanks until data entry into the database. The data were entered into a Microsoft Access database. The database forms corresponded to the datasheets in the workbooks. Data entry included transferring information on the written form to the database form. These database forms and tables associated with the forms had data integrity such that deletions were not allowed by the data entry personnel. Also, there was a quality control (QC) process during the data entry to identify and correct any obvious discrepancies in the data.

The original raw data collected on the data forms remain in the wet lab project file until there is a signed final report, at which time they are inventoried and archived on CD-ROMs for at least 2 years (longer if required by study protocol or government regulations), unless the sponsor requests that they be transferred to an archive location other than at Battelle.

All specimens and records remain the responsibility of Battelle PNWD and are retained in the Battelle archive for the length of time stipulated in the contract, which is typically 5 years. The archive is located at Battelle's facility in Sequim, Washington, and is maintained according to a policy of limited access. The Battelle sample custodian is responsible for archiving and retrieving work assignment materials. An archive inventory is maintained and storage capability is provided for the expedient retrieval of materials. Specimens and samples are disposed of only after assessing that they no longer afford evaluation.