SPONSOR

U.S. Environmental Protection Agency
1200 Pennsylvania Ave., NW
Washington DC 20460

USEPA TASK ORDER/BATTELLE CONTRACT NO.

TO 14/ EP-W-11-063

TEST ITEM

2-Ethylhexyl 4-Hydroxybenzoate

STUDY TITLE

21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

DATA REQUIREMENT

U.S. EPA, Endocrine Disruptor Screening Program Test Guidelines, OPPTS 890.1100 Amphibian Metamorphosis (Frog) (October 2009)

STUDY DIRECTOR AND AUTHOR

Douglas J. Fort, Ph.D.

STUDY INITIATION DATE

January 7, 2016

STUDY COMPLETION DATE

March 28, 2018

AMENDED STUDY COMPLETION DATE

May 21, 2018

TESTING FACILITY

Fort Environmental Laboratories, Inc. (In-Life Testing)
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REPORT NUMBER

BATT01-00388

Total Pages: 252

1.1. GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with the following GLP principles:

- United States Environmental Protection Agency, (FIFRA), Good Laboratory Practice Standards, Title 40 Code of Federal Regulations Part 160 (effective October 16, 1989) with the following exceptions:
  - Analysis of the laboratory dilution water for organics, pesticides and metals at Red River Laboratory (Oklahoma City, Oklahoma) using standard EPA methods was not GLP-compliant;
  - Per Sponsor-mandated exception, the test substance was not chemically-characterized in a GLP-compliant manner;
  - Per Sponsor-mandated exception, range-finding studies conducted as a component of FEL study (BATT01-00385) used to determine test concentrations for the present study, BATT01-00388, were not performed in a GLP-compliant manner;
  - Per Sponsor-mandated exception, following study finalization, specimens remaining at FEL, and embedded tissues or specimens maintained by EPL will be disposed of in accordance with QMP, QAPP, and respective facility SOPs.

Since the analyses of the dilution water was conducted following standard validated methods, this exception will not be expected to impact on the study results. Lack of GLP-compliant chemical characterization or GLP-compliant range-finding studies will not be expected to impact study results, nor change the conclusions drawn from the study.

GLP Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

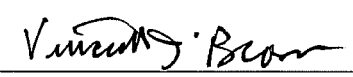
FEL Study No.: BATT01-00388

Test Substance: 2-Ethylhexyl 4-Hydroxybenzoate (CAS No. 5153-25-3)

5/21/2018

Douglas J. Fort, Ph.D.
Fort Environmental Laboratories, Inc.
(Study Director)

Date



5/21/2018

Vincent J. Brown, Ph.D.
Battelle
(Sponsor Representative)

Date

1.2. STATEMENT OF QUALITY ASSURANCE UNIT

This study was conducted in accordance with Standard Operating Procedures (SOPs) and the protocol as approved by the Sponsor. The Fort Environmental Laboratories, Inc. (FEL) Quality Assurance Unit conducted the inspections detailed below:

Type of Inspection	Dates		
	Inspection	Reported to Study Director	Reported to Management
Protocol Review	1/7/2016	1/7/2016	1/7/2016
Technical Systems Audit	2/8/2016	2/8/2016	2/8/2016
Audit of Data Quality	8/22/2016	8/22/2016	8/22/2016
Draft Report Audit	2/24/2017	2/24/2017	2/24/2017
Final Report Audit	3/28/2018	3/28/2018	3/28/2018
Amended Final Report Audit	5/21/2018	5/21/2018	5/21/2018

This report accurately reflects the raw data obtained during the performance of this study. The protocol and all protocol amendments and deviations are presented as an attachment (Appendix A). Two protocol deviations occurred during the course of this study. Procedures pertinent to this study are described in this report. The primary goal of the Quality Assurance Unit (QAU) is to ensure the accuracy and precision of routine laboratory operations, data generated reports, and presentation of studies conducted by FEL. The final report is considered to constitute an accurate and complete reflection of the data generated from this study.

Mike Mathis — 5/21/2018

Michael Mathis, QAU Manager, FEL — Date

1.3. CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of the data obtained from this study

Study Director:

Douglas J. Fort, Ph.D., Study Director, FEL — 5/21/2018 Date

Performing Laboratory Management:

Deanne Fort, Facility Manager, FEL — 5/21/18 Date

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2. EXECUTIVE SUMMARY

Guidelines:	U.S. EPA, Endocrine Disruptor Screening Program Test Guidelines, OPPTS 890.1100 Amphibian Metamorphosis (Frog) (3).
Nominal Test Concentrations:	100.0, 33.0, 10.9, 3.6 µg/L 2-Ethylhexyl 4-Hydroxybenzoate (2-EHHB), dilution water control
Mean Measured Test Concentrations:	62.0, 25.0, 10.3, 4.58 µg/L 2-EHHB, <0.208 µg/L 2-EHHB detected in dilution water control (MQL=0.208 µg/L)
Replicate Mean CV (%):	6, 2, 3, 13, NA (<MQL) (Appendix E)
Age of Test Organisms:	NF stage 51
Source of Test Organisms:	Frog larvae were from breeding of adults cultured at FEL. Adult frogs were originally obtained from *Xenopus* 1 (Dexter, MI).

2.1. Method

Under the guidance of a sponsor-approved Quality Assurance Project Plan (QAPP) (1), in compliance with United States Environmental Protection Agency, (FIFRA), Good Laboratory Practice Standards, Title 40 Code of Federal Regulations Part 160 (effective October 16, 1989) (2) with exceptions noted (page 2), and based EPA Test Guidelines OPPTS 890.1100 (3) using four test concentrations and a control, an Amphibian Metamorphosis (Frog) Assay using 2-ethylhexyl 4-hydroxybenzoate [2-EHHB] was performed. The in-life exposure phase of the study was initiated on February 5, 2016 and concluded February 26, 2016. In the conduct of the present study, FEL relied on internal SOPs, per the GLP regulations and documented QAU practices subject to sponsor and other government audit. These SOPs are not available to the public. Randomly selected NF stage 51 larvae were exposed to four test concentrations and a dilution water control and were evaluated in quadruplicate, with 20 organisms per replicate. Once larvae were placed in the exposure system, mortality observations were made. On study day (SD) 7, developmental stage, hind limb length, and wet weight were determined on larvae randomly selected (5/replicate), euthanized, and preserved for possible histology. The test was terminated on day 21, at which time all test animals were staged, measured, weighed, and visually observed for dysmorphology. Euthanized larvae were randomly selected (5/replicate) and preserved for possible histology. Temperature was measured daily; and pH, DO, and light intensity (lux) were measured three times per week. Total hardness and alkalinity of water were measured in the control and one replicate of the highest concentration once per week.

On SD 0, healthy and normal looking tadpoles of the stock population were pooled in a single vessel containing an appropriate volume of dilution water. Once the staging was completed, the larvae were randomly distributed to exposure treatment tanks until each tank contained 20 larvae. Each treatment tank was then inspected for animals with abnormal appearance (e.g., injuries, abnormal swimming behavior, etc.). Overtly unhealthy tadpoles were removed from the treatment tanks and replaced with larvae newly selected from the pooling tank. Five randomly selected stage 51 pre-exposed tadpoles were humanely euthanized in 150 to 200 mg/L buffered 3-aminobenzoic acid ethyl ester (MS-222) and preserved to verify stage upon in-life test setup. On SD 7, 5 randomly chosen tadpoles per replicate were removed from each test tank and humanely euthanized in 150 to 200 mg/L MS-222, appropriately buffered with sodium bicarbonate. Tadpoles were rinsed in water and blotted dry, followed by body weight determination to the nearest mg. Hind limb length and snout-to-vent length (SVL), along with developmental stage (using a binocular dissection microscope), were determined for each tadpole. At test termination (SD 21), the remaining tadpoles were removed from the test tanks and humanely euthanized in 150 to 200 mg/L MS-222, appropriately buffered with sodium bicarbonate. Tadpoles were rinsed in water and blotted dry, followed by body weight determination to the nearest mg. Developmental stage, hind limb length, and SVL were measured for each tadpole.

All larvae were then placed in Davidson's fixative for 48 to 72 hours as whole body samples for histological assessments. Larvae were rinsed in dechlorinated tap water and preserved in 10% (w/v) neutral buffered formalin (NBF). For histopathology, a total of 5 tadpoles were sampled from each replicate tank. Since follicular cell height is stage dependent, the most appropriate sampling approach for histological analyses was to use stage-matched individuals, when possible. Animals selected for histopathology (n=5 from each replicate) were matched to the median stage of the controls (pooled replicates) whenever possible. If replicate tanks with more than five larvae at the appropriate stage existed, then five larvae were randomly selected. If replicate tanks with fewer than five larvae at the appropriate stage existed, randomly selected individuals from the next lower or upper developmental stage were sampled to reach a total sample size of five larvae per replicate. The decision to sample additional larvae from either the next lower or upper developmental stage was made based on an overall evaluation of the stage distribution in the control and chemical treatments. If the test article induced retardation of development, additional larvae were sampled from the next lower stage. Alternatively, if the chemical treatment was associated with an acceleration of development, then additional larvae were sampled from the next upper stage.

2.2. Results and Conclusions

- Results of present study met the performance criteria established for the OPPTS 890.1100 Amphibian Metamorphosis Assay (3) and were considered valid (Table 16). The following decision logic was applied to the present study to determine if 2-EHHB affected thyroid activity.

- No significant differences between the median developmental stage or normalized HLL between the control and the treatments were observed on exposure day 7 or at the conclusion of the study.

- Asynchronous development was not noted in the control or treatments during the conduct of the study.

- Although mild to moderate histopathological lesions were observed in the control and the 2-EHHB treatments, there was no clear relationship between test article concentration and response.

- Larvae exposed to 2-EHHB in the 33 and 100 µg/L 2-EHHB treatments weighed significantly more than the controls both at study day (SD) 7 and 21 (conclusion). Thus, 2-EHHB appeared to impact growth (weight).

- No effect of 2-EHHB exposure on SVL at either study day was observed.

- No impact on hind limb length (HLL) was noted in any of the 2-EHHB treatments based on SVL-normalized HLL, although unnormalized HLL at SD 7 in larvae in the 100 µg/L 2-EHHB treatment was significantly greater than the control (Jonckheere-Terpstra test, p=0.0355). In contrast, there was as an impact on HLL observed at SD 21 (conclusion).

- No significant effects on behavior or signs of overt toxicity were noted.

- Using the decision criteria in the AMA test guideline (OCSPP 890.1100), 2-EHHB does not appear to affect amphibian metamorphosis or affect the thyroid axis directly based on the endpoints measured at the concentrations tested.

3. INTRODUCTION

FEL was contracted by Battelle Memorial Institute to perform the Amphibian Metamorphosis (Frog) Assay under EPA Test Guidelines OPPTS 890.1100 (3) using 2-ethylhexyl 4-hydroxybenzoate [2-EHHB] (test substance) as directed by USEPA Task Order (TO) 14 under USEPA/Battelle Memorial Institute contract EP-W-11-063. This study was conducted in accordance under the guidance of a sponsor-approved Quality Assurance Project Plan (QAPP) (1); in compliance with United States Environmental Protection Agency, (FIFRA), Good Laboratory Practice Standards, Title 40 Code of Federal Regulations Part 160 (effective October 16, 1989) (2) with exceptions noted (page 2). The present study was performed under the FEL Quality Assurance Management Plan (QAMP) (4); relevant facility standard operating procedures (SOPs); and the following Study Protocol No. BATT01-3, prepared for FEL Study No. BATT01-00388 and associated protocol amendments, with the exceptions of deviations noted (Appendix A). In the conduct of the present study, FEL relied on internal SOPs, per the GLP regulations and documented QAU practices subject to sponsor and other government audit. These SOPs are not available to the public. The in-life exposure phase of the study was initiated on February 5, 2016 and concluded February 26, 2016.

An amphibian metamorphosis assay was performed in which Nieuwkoop and Faber (NF) (5) stage 51 *Xenopus laevis* larvae were exposed to different concentrations of the test substance for 21-days. The full in-life phase schedule is provided in Table 1. In contrast to that specified in EPA Test Guidelines OPPTS 890.1100 (3), which require testing of three independent concentrations of test substance, the general experimental design entailed exposing tadpoles to 4 different concentrations of the test chemical and dilution water control. Each test chemical concentration and dilution water control was comprised of 4 replicates tanks (experimental unit) containing 20 organisms per replicate tank. The treatment tanks were randomly assigned to a position in the exposure system in order to account for possible variations in temperature and light intensity. The primary endpoints were hind limb length (HLL), body length (snout to vent length [SVL]), developmental stage, wet weight, thyroid histology, and daily mortality.

4. STUDY PERSONNEL

- Dr. Vincent Brown, Battelle Memorial Institute – Study Monitor
- Dr. Douglas Fort, FEL – Study Director
- Ms. Deanne Fort, FEL – Manager, In-life study facility
- Mr. Michael Mathis, FEL – QAU Manager
- Dr. Tom Leak, ABC Laboratories, Inc. – Principal Investigator (PI), analytical chemistry
- Dr. Jeffrey Wolf, Experimental Pathology Laboratories (EPL), Inc. – PI, histopathology
- Mr. Kevin Todhunter, FEL – Technician
- Ms. Alex Oppenborn, FEL – Technician
- Ms. Jennifer Staines, FEL – Technician
- Ms. Franchesca Rollerson, FEL – Technician
- Mr. Troy Fort, FEL – Technician

5. ANIMAL WELFARE ACT COMPLIANCE

This study complied with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR). The Sponsor should make particular note of the following:

- The Sponsor signature on the protocol documented for the Study Director the Sponsor's assurance that the study described in this protocol does not unnecessarily duplicate previous experiments.

- Whenever possible, procedures used in this study were designed to avoid or minimize discomfort, distress or pain to animals. All methods were described in the study protocol or in written laboratory standard operating procedures.
- By design, this study killed and/or resulted in the pain and distress of test organisms. Euthanasia of test organisms prior to completion of the test would interfere with study objectives. Upon completion of the test all distressed amphibians were painlessly euthanized in a timely manner.
- Methods of euthanasia used during this study were in conformance with the above referenced regulation and were consistent with EPA Test Guidelines OPPTS 890.1100 (3).

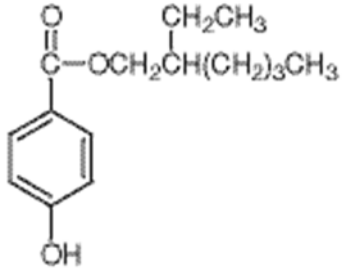
6. MATERIALS AND METHODS

6.1. Test Substance

2-EHHB (TCI America, Portland, OR, lot number 7CZZO, expiration date and re-test date not provided, 99.3% (HPLC) with 98.0% minimum specification (w/w) pure [w/w] per Certificate of Analysis produced by TCI America) was received from TCI America (Appendix B). Physicochemical information is provided below:

Name: 2-ethylhexyl 4-hydroxybenzoate
IUPAC Name: 2-ethylhexyl 4-hydroxybenzoate

Structure:



Phase/Color: liquid, colorless, pale yellow
Specific Gravity: 1.04 g/mL at 25°C
Solubility (water): 6.0 mg/L at 25°C
Boiling Point: 270.0°C

Synonyms:	2-ethylhexylparaben, 4-hydroxybenzoic acid 2-ethylhexyl ester, 4-hydroxybenzoic acid octyl ester, octyl 4-hydroxybenzoate, octylparaben
CAS number:	5153-25-3
Molecular formula:	$C_{15}H_{22}O_3$
Molecular weight:	250.3 g/mole

Due to limited water solubility, a solid phase saturator column was used to maximize 2-EHHB concentrations in water and was used to prepare a master stock solution ultimately used to prepare each test concentration. The solid phase saturator columns were prepared by FEL. The test substance was stored in the chemical repository at room temperature (RT) while not in use as recommended by the Certificate of Analysis supplied by TCI America. The definitive test concentrations to be used were determined by a range-finding study designed to identify the concentration of test chemical that causes lethality and morbidity.

6.1.1. Dilution and Laboratory Control Water

Dechlorinated (charcoal-filtered) tap water was used as the dilution water for this study. The dechlorinated laboratory water was prepared by passing tap water through a 4-filter system; a multimedia filter to remove suspended solids in the feed water; a 10-inch pre-treatment filter (5 µm) to remove any additional solids; a 3.6 ft^3 activated virgin carbon treatment filter to remove chlorine, ammonia, and higher molecular weight organics; and a 5 µm polishing filter to remove any carbon particles from the carbon treatment phase. The dechlorinated tap water also served as the laboratory control water. Facility water quality characteristics of the laboratory water were monitored bimonthly for pH, dissolved oxygen (DO), conductivity, hardness, alkalinity, ammonia, residual oxidants; and at least annually for iodide (I-), polyaromatic hydrocarbons (PAHs), pesticides, and metals. The culture water was most recently analyzed for pesticides, PAHs, and metals on February 1, 2016, and all water quality measurements cited above met the U.S. EPA criteria for aquatic toxicity test culture/dilution water.

Sufficient iodine (I-) needs to be available to the larvae through a combination of aqueous and dietary sources for the thyroid gland to synthesize thyroid hormones to support normal metamorphosis. Based on previous work (3), the amphibian metamorphosis assay has been demonstrated to work well when test water I- concentrations ranged between 0.5 and 10 µg/L. I- was measured in facility dilution and during the study using ion-selective electrode (ISE) analysis in accordance with facility SOP. I- levels in the dilution water fell within the acceptable range (see section 7.2. Water Quality Measurements and Test System Performance). Thus, no I- supplementation was necessary.

6.2. Test System

The test system was the African Clawed Frog (*Xenopus laevis)*, from which NF stage 51 larvae were used in the metamorphosis assay (3). *Xenopus laevis* is a well-studied laboratory animal that exhibits ease of use in the laboratory, rapid development, and simplicity of observation due to transparency during embryo-larval development (6). In addition, *Xenopus laevis* is routinely cultured in laboratories worldwide and is easily obtainable through commercial

suppliers. Reproduction can be easily induced in this species throughout the year using human chorionic gonadotropin (hCG) injections and the resultant larvae can be routinely reared to selected developmental stages in large numbers to permit the use of stage-specific test protocols. It was also the test organism required in the OPPTS 890.1100 test guideline (3).

6.2.1. Origin, Handling and Feeding

The *X. laevis* larvae used for this study were obtained from an in-house culture (originally purchased from *Xenopus* I, Dexter, MI) where adults were injected with human chorionic gonadotropin (hCG) to induce reproduction. Details for adult frog care and breeding, specific to FEL, but consistent with OPPTS 890.1100 test guideline (3), are found in facility SOPs. Fertilized egg collection was performed as described in ASTM E1439-98 (6) and OPPTS 890.1100 Amphibian Metamorphosis (Frog) test guideline (3). All tadpoles that were used as test organisms were derived from the same clutch (spawn). In addition, 2 to 3 clutches were collected to evaluate the quality of the spawns and determine which produced the highest quality larvae for the initiation of the study. Embryos were cultured at 22ºC ± 1º for 4 days to allow for hatching and development to NF stage 45/46, at which time they were randomly divided into groups of approximately 200 and maintained in tanks containing 50 L of culture (dilution) water. For the present study (BATT01-00388), this culture yielded a population density of 4 larvae/L and was maintained at a constant flow rate (50 mL/minute) and water temperature (22º ± 1ºC) until they reached developmental NF stage 51 within 12 -17 days.

Tadpoles were fed Sera Micron® (Sera GmbH, Heinsberg, Germany) throughout the pre-exposure period (after NF stage 45/46) and during the entire test period of 21 days. Sera Micron®, a commercially available tadpole food that has been shown to support proper growth and development of *X. laevis* tadpoles, is a fine particulate that stays suspended in the water column for a long period of time. Therefore, the total daily amount of food was divided into smaller portions and fed twice daily, except on weekends. Initially, 300 mg Sera Micron® per tank was fed twice per day (total = 600 mg/d) for the first 4 days of exposure. During the course of the study, the total daily food ration increased according to the rations specified in Table 2. Feeding frequency was twice per day on Monday through Friday, and once per day at twice the weekday volume on weekends. Sera Micron® was fed as a stock solution (density of 60 mg/mL dilution water). The Sera Micron® stock solution was freshly prepared every other day.

6.3. Exposure System

The route of exposure was aqueous, which was the most appropriate method for aquatic organisms and readily water soluble test materials. A flow-through diluter system (Benoit Mini-Diluter; ECT, Superior, WI) was used in the performance of the amphibian metamorphosis assay exposure. The system contained water-contact components of glass (aquaria), stainless steel (diluter housing and water bath), and Teflon® (tubing responsible for test material delivery). Exposure tanks were glass aquaria (with approximate measurements of 22.5 x 14.0 x 16.5 cm deep) equipped with standpipes that result in an actual tank volume of 4.0 L and minimum water depth of 10 to 15 cm. Each dilution cell within the head box of the diluter was labeled with color-coded laboratory tape with the test concentration. The replicate splitter cell and aquaria delivery tubing were labeled with the same color tape as the appropriate dilution cell and the replicate for each test concentration. Each aquarium was labeled with identical tape color and

specifically denoted test concentration and replicate. Test system calibration was checked prior to study start, weekly during the study, and at test termination. The system was capable of supporting up to 5 exposure concentrations and a control, with up to 4 replicates per treatment. The flow rate to each tank was 25 mL/min, which provides a complete volume replacement every 2.7 h. Fluorescent lighting was used to provide a photoperiod of 12 h light and 12 h dark at an intensity that ranged from 600 to 2,000 lux (lumens/m2) at the water surface. Water temperature was maintained at 22º ± 1°C, pH maintained between 6.5 to 8.5, and the dissolved oxygen (DO) concentration > 3.5 mg/L (> 40% of the air saturation) in each test tank. 2-EHHB feed stock was prepared and pumped to the master mixing cell of the diluter using continuous flow dual solid-liquid saturator columns in which ca. 10 g of 2-EHHB was loaded on each column in acetone. The acetone was evacuated prior to use using a vacuum pump and 2-3 week continuous column flushing during the equilibration phase. The columns were plumbed in series and dechlorinated tap water was pumped through the columns at a rate of ca. 4 mL/min. to produce one stock solution pumped into the master mixing cell of the diluter. The columns were equilibrated for at least two weeks prior to use and equilibration was noted by the production of a consistent stock concentration. Since the stock was produced by the saturator columns, the nominal concentration is estimated based on solubility and diluter operation (flows) were based strictly on measured stock concentrations. Diluter operation was based on stock concentration based on PD=HCxDD/SC, where PD is pump delivery rate, HC is high test concentration, DD is the diluter delivery rate (volume), and SC is the stock concentration.

Temperatures were measured daily; and pH, DO, and light intensity (lux) were measured three times per week. Total hardness and alkalinity were measured in the control and one replicate of the highest concentration once per week. Test solution from each replicate tank at each concentration was sampled for chemical analysis during the equilibration phase, on SD 0, 7, 14, and 21 at test termination. Thus, during the in-life study 4 sets of samples were analyzed. In addition, stock solutions were collected for analysis at each weekly sampling point. Duplicate samples representing a split of the original samples were collected in the event of sample loss during shipment or analysis; or if confirmatory analyses were warranted based on result as determined by the Study Director. Duplicate samples were stored 4ºC (1-9 ºC). At each sampling point, 20 mL of sample was collected from each replicate of the control and treatments, and placed in 40 mL VOC vial. Twenty mL of methanol was added to each sample for extraction. The vials were sealed with appropriate caps and tightly sealed for shipment to EAG Laboratories.

6.3.1. Test Substance Analyses - EAG Laboratories Test Site (Columbia, MO)

Test substance analysis of the diluter test solutions and stock solutions was performed by EAG Laboratories (Columbia, MO). The analytical measurement method (7) for test substance determined by the chemical laboratory in conjunction with the Study Director and Study Monitor was performed in accordance with procedures and SOPs in place at EAG Laboratories and in accordance with method validation performed at EAG Laboratories. Complete details of the methods used and analysis of test substance in samples submitted from the study are provided in Appendix C.

6.3.2. Test Animal Selection

When a sufficient number of the pre-exposure population reached developmental stage 51 (14 to 17 d post-hatch), larvae were transferred to a pooling tank containing dilution water. All larvae used in the in-life study were from the same clutch of offspring. Individual larvae were randomly removed from the pooling tank by scooping with a small strainer. Animals were carefully handled during this transfer in order to minimize handling stress and to avoid any injury.

The developmental stage of the animals was determined using a binocular dissection microscope. The primary developmental landmark for selecting stage 51 organisms was hind limb morphology (3). The morphological characteristics of the hind limbs was examined under the microscope. The morphological appearance of the hind limbs at stage 51 differed markedly from the limb morphology at stages 50 and 52, making it possible to correctly distinguish the different stages of the larvae.

Animals that met the stage criteria were transferred to a holding tank containing 100% dilution water. The selected larvae were randomly distributed to exposure treatment tanks (including the control) containing 4.0 L of treatment solution until each tank contained 20 larvae (5 larvae/L density). Each treatment tank was then inspected for animals with abnormal appearance (*e.g.*, injuries, abnormal swimming behavior, etc.). Overtly unhealthy looking tadpoles were removed from the treatment tanks and replaced with larvae newly selected from the holding tank. Treatment tanks were labeled with the study, treatment, and replicate identification at a minimum. The treatment tanks were randomly assigned to a position in the exposure system in order to account for possible variations in temperature and light intensity.

6.4. Study Design and Additional Experimental Conditions

The randomly selected NF stage 51 larvae were exposed to 4 test concentrations and a dilution water control. Each test concentration and control were evaluated in quadruplicate, with 20 organisms per replicate. Once larvae were placed in the exposure system, mortality observations were made daily and any dead larvae were immediately removed. On d 7, body length (SVL), developmental stage, hind limb length, and wet weight were determined on larvae randomly selected (5/replicate), euthanized, and preserved for possible histology. The test was terminated on SD 21, at which time all test animals were staged (NF), measured (cm), weighed (g), and visually observed for dysmorphology. Euthanized larvae were randomly selected (5/replicate) and preserved for possible histology. Critical test parameters and experimental conditions for the in-life study are presented in Table 3.

6.4.1. Day 0 Test Initiation and Sample Collection

On SD 0, healthy and normal looking tadpoles of the stock population were pooled in a single vessel containing an appropriate volume of dilution water. For developmental stage determination, tadpoles were individually removed from the pooling tank using a small net or strainer and transferred to a transparent measurement chamber containing dilution water. No anesthesia was used. Animals were carefully handled during this transfer to minimize handling

stress and to avoid injury. The developmental stage of the animals was determined using a binocular dissection microscope.

Tadpoles that met the stage criteria described above in the protocol were held in a tank of clean culture water until the staging process was completed. Once the staging was completed, the larvae were randomly distributed to exposure treatment tanks until each tank contained 20 larvae. Each treatment tank was then inspected for animals with abnormal appearance (e.g., injuries, abnormal swimming behavior, etc.). Overtly unhealthy tadpoles were removed from the treatment tanks and replaced with larvae newly selected from the pooling tank. Five randomly selected stage 51 pre-exposed tadpoles were humanely euthanized in 200 mg/L buffered MS-222 and preserved to verify stage upon in-life test setup.

6.4.2. Day 7 Measurements and Sample Collection

On day 7 of the study, 5 tadpoles per replicate were randomly chosen, removed from each test tank and humanely euthanized in 150 to 200 mg/L MS-222, appropriately buffered with sodium bicarbonate to achieve pH 7. Tadpoles were rinsed in water and blotted dry, followed by body weight determination to the nearest mg. Hind limb length and SVL, along with developmental stage (using a binocular dissection microscope), were determined for each tadpole.

6.4.3. Day 21 Measurements

At test termination (day 21), the remaining tadpoles were removed from the test tanks and humanely euthanized in 150 to 200 mg/L MS-222, appropriately buffered with sodium bicarbonate to achieve pH 7. Tadpoles were rinsed in water and blotted dry, followed by body weight determination to the nearest mg. Developmental stage, hind limb length, wet body weight, and SVL were measured for each tadpole.

All larvae were then placed in Davidson's fixative for 48 to 72 hours as whole body samples for histological assessments. Larvae were then rinsed in dechlorinated tap water and preserved in 10% (w/v) neutral buffered formalin (NBF). For histopathology, a total of 5 tadpoles were sampled from each replicate tank. Since follicular cell height is stage dependent, the most appropriate sampling approach for histological analyses was to use stage-matched individuals, when possible. Animals selected for histopathology (n=5 from each replicate) were matched to the median stage of the controls (pooled replicates) whenever possible. If replicate tanks with more than five larvae at the appropriate stage existed, then 5 larvae were randomly selected. If replicate tanks with fewer than five larvae at the appropriate stage existed, randomly selected individuals from the next lower or upper developmental stage were sampled to reach a total sample size of five larvae per replicate. The decision to sample additional larvae from either the next lower or upper developmental stage was made based on an overall evaluation of the stage distribution in the control and chemical treatments. If the test substance induced retardation of development, additional larvae were sampled from the next lower stage. Alternatively, if the chemical treatment was associated with an acceleration of development, then additional larvae were sampled from the next upper stage.

6.5. Observations

Test data and daily observations were recorded in the study records. Study records included study tracking sheets, test information sheets, study calendars identifying major events, study logs for recording detailed observations and comments, daily mortality and developmental stage data sheets, and test termination data sheets. The primary endpoints of the metamorphosis assay were mortality, developmental stage (NF), hind limb length, snout-vent length (SVL), wet body weight, and thyroid histology. Gross morphology (physical appearance at test takedown) was a secondary endpoint. During the 21-day exposure phase, determination of selected endpoints was performed on SD 7 and day 21. Table 4 provides an overview of the measurement endpoints and the corresponding observation time points.

6.5.1. Mortality

All test tanks were checked daily for dead tadpoles and the numbers were recorded for each tank. Dead animals were removed from the test tank as soon as observed.

6.5.2. Developmental Stage

The developmental stages of *X. laevis* tadpoles were determined by using the staging criteria of Nieuwkoop and Faber (5). Developmental stage data were used to determine if development was accelerated, asynchronous, delayed, or unaffected. Acceleration or delay of development was determined by making a comparison between the median stage achieved by the control and treated groups. Asynchronous development was reported when the tissues examined were not malformed or abnormal, but the relative timing of the morphogenesis or development of different tissues was disrupted within a single tadpole. Developmental stage data were reported at SD 7 and in-life test termination (SD 21).

6.5.3. Hind Limb Length

Hind limb development is typically used qualitatively in the determination of developmental stage, but was also used in this study as a quantitative endpoint. All length measurements (millimeters) were based on digital photographs of the surviving organisms from each treatment. For consistency, hind limb length was measured on the left hind limb. Hind limb length was evaluated both at SD 7 and at in-life test termination (SD 21).

6.5.4. Body Length (SVL)

SVL was the first of two endpoints used to assess tadpole growth. SVL (millimeters) was used to help assess generalized toxicity of the test substance. All length measurements were based on digital photographs of the surviving organisms from each treatment. SVL was evaluated both at SD 7 and at in-life test termination (SD 21).

6.5.5. Wet Body Weight

Determinations of wet body weight were used to assess possible effects of test substance on the growth rate of tadpoles in treatment groups relative to the control group. Wet weight measurements were performed on organisms euthanized for collection of SD 7 endpoints and on surviving organisms on in-life SD 21 at test termination.

6.5.6. Thyroid Gland Histopathology

While developmental stage and hind limb length were important endpoints to evaluate exposure-related changes in metamorphic development, developmental delay cannot, by itself, be considered a diagnostic indicator of anti-thyroidal activity. Some changes can only be observed based on routine histopathological analysis.

EPL, under direction of the sponsor, will perform the tissue preparation and histology in accordance with appropriate facility guidance documents (SOPs) and the relevant guidance documents on histology for the AMA (3,8). In accordance with USEPA (3) and OECD guidelines (8), the paraffin blocks were not be sealed as per Wolf (2015) (9). Following the conclusion of exposure (SD 21), FEL sent 5 stage-matched NBF preserved larvae per replicate (20 per treatment or control) to EPL via overnight courier for histopathological processing and analyses.

Histological evaluation of the thyroid included, but was not limited to: thyroid gland hypertrophy/atrophy, follicular cell hypertrophy, follicular cell hyperplasia, and as additional qualitative criteria: follicular lumen area, colloid quality and follicular cell height/shape Severity grading will be reported in accordance with USEPA and OECD guidelines (3,8). Overt and significant changes in apical endpoints indicating developmental acceleration or asynchrony could preclude the necessity to perform histopathological analysis of the thyroid glands. However, absence of overt morphological changes or evidence of developmental delay warrant histological analyses.

6.6. Additional Observations

All cases of abnormal behavior (e.g. uncoordinated swimming, hyperventilation, atypical quiescence, non-feeding, etc.) and grossly visible malformations were recorded in the study records and included in the final study report.

6.7. Data Analysis and Statistics

All data were tabulated in data entry spreadsheet templates (DEST) by FEL. The DESTs were then used by FEL to prepare the final report. The histopathology report (EPL, Sterling, VA), raw data (DEST), and statistical report (Battelle) are provided in Appendices D through F, respectively. In an effort to present the most realistic estimation of exposure concentrations, analytical chemistry analysis results of duplicate analytical samples were averaged with the original sample result and both the individual results and average were reported in the DESTs. In the event a result from an original sample or its duplicate was found to be an outlier as defined as being outside the interquartile range (IQR), both results were included in the DEST, but the outlier (either original or duplicate) was not used in the analysis of the mean measured concentration used to estimate exposure concentration in the report. Outliers were determined by (Q1 – 1.5*IQR) and (Q3 + 1.5*IQR), where IQR was the interquartile difference defined as Q3 minus Q1. In the event both the original and duplicate were found to be outside the bounds of the IQR, the original and duplicate results were averaged and reported. Statistical analyses of the data were performed by Battelle and were consistent with the OPPTS 890.1100 test guideline (3), the TO 14 QAPP (1), and generally followed procedures described in the document Current

Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application (10). For all continuous quantitative endpoints (HLL, SVL, wet weight) that followed a monotonic concentration-response, the Jonckheere-Terpstra test was applied in step-down manner to establish significant treatment effects. For continuous endpoints that were not consistent with a monotonic concentration-response, the data were evaluated for normality (Shapiro-Wilk's test) and homogeneity (Levene's test). If the data sets are normally distributed with heterogeneous variance following data transformation, the Tamhane-Dunnett, (T3 test) or the Mann-Whitney-Wilcoxon U test with a Bonferroni-Holm adjustment to the p-values was used to evaluate the data. Where no normalizing transformation can be found, the Mann-Whitney-Wilcoxon U test using a Bonferroni-Holm adjustment to the p-values was used to evaluate the data sets. A test termed RSCABS (Rao-Scott Cochran Armitage by Slices) that uses a step-down Rao-Scott adjusted Cochran-Armitage trend test on each level of severity in a histopathology response was used to evaluate histopathology data (11).

A significant treatment effect for developmental stage was determined on the replicate median values using the Jonckheere-Terpstra or Mann-Whitney U test with a Bonferroni-Holm adjustment to the p-values. Concentration-response monotonicity was assessed visually from the replicate and treatment medians or means. The statistical significance of all tests indicated was assessed at $p = 0.05$.

7. RESULTS

7.1. Range-Finding Test

The range-finding study was conducted separately (FEL Study No. BATT01-00385) and was not required to be GLP-compliant. Based on range-finding, the maximum tolerable concentration (MTC) (1) was determined to be 100 µg/L (Appendix G). The test concentration series was 1x, 0.33x, 0.11x, and 0.04x, where x is the MTC value. Therefore, the sponsor-approved test concentrations were 100.0, 33.0, 10.9, and 3.6 µg/L.

7.2. Water Quality Measurements and Test System Performance

Results of water quality measurements and test system performance are presented in Table 5. I- levels in the dilution water were measured on Study Day (SD) 0 and 21 (in-life conclusion) of the 00389 study and contained 9.3 (±0.03) and 9.2 (±0.05) µg/L I-, respectively, which fell within the acceptable range. All physicochemical water quality parameters in study were within acceptable ranges (Table 5).

7.3. Confirmation of Test Concentrations

Nominal 2-EHHB concentrations selected for the Amphibian Metamorphosis Assay study were 0.0 (control), 3.6, 10.9, 33.0, and 100 µg/L. The dilution water control solutions showed no detectable levels of 2-EHHB with the exception of SD 0, replicate D, which had trace levels (0.210 µg/L) (Table 6). Since time interval between sample collection events was consistent throughout study, the mean measured concentration represented an accurate estimation of exposure concentration. Because a continuous stock solution was prepared using

solid-liquid phase saturator columns, stock concentrations varied somewhat during the course of the study. However, since these concentrations were used to adjust stock pump flow to the diluter master mixing cell, impact on test solution concentrations was minimal. The mean measured concentration represented the average of each data point from SD 0, 7, 14, and 21 for each replicate (A-D, represents intra-replicate mean) of the control and each treatment per facility SOP. IQRs determined for the 0.0 (control), 3.6, 10.9, 33.0, and 100 µg/L 2-EHHB treatments were 0.104-0.104, -4.75-12.2, -10.7-28.3, -9.70-55.9, and -34.7-157 µg/L 2-EHHB, respectively. Based on IQR analysis of the control and each treatment group, 0.0 µg/L replicate D duplicate sample from Study Week (SW) 0 was considered an outlier (Appendix E). The corresponding mean measured concentrations in the definitive study were <0.208 (control), 4.58, 10.3, 25.0, and 62.0 µg/L 2-EHHB (Table 7). Lower test substance recovery was noted in the two highest test concentrations, potentially due to the complexity of the environment within each replicate tank regardless of a high-level attention to water quality maintenance and diluter performance. In some cases, variability in analytical measurement was noted with duplicate samples. The coefficient of variation (CV) [(Standard deviation/mean)100] was based on the standard deviation of the four replicate means (n=4) for the control and the four replicate means (n=4) for each treatment per facility SOP. The CVs of the intra-replicate means of the measured test concentrations for the 3.6, 10.9, 33.0, and 100 µg/L treatments were 13%, 3%, 2%, and 6%, respectively; which were acceptable based on the criteria establish in the test guidance (3) and protocol BATT01-3 for study BATT01-00388.

7.4. Mortality

Test organism survival during the study is presented in Table 8. No mortality was observed during the study. Since all larvae survived during the study, no statistical analyses were performed.

7.5. Development

7.5.1. Developmental Stage

Larval developmental stages on SD 7 and 21 are provided in Table 8. The median developmental stage on exposure SD 7 was 54 for the control and each treatment, as well as all replicates of all treatments. At test conclusion (day 21), the median developmental stage for the control ranged from 59 in replicate B to 58 in the remaining replicates. The median developmental stage for the <0.208 (control), 3.6, 10.9, 33, and 100 µg/L 2-EHHB treatments were 58, 58, 58, 59, and 59, respectively. The IQR and the number of different stages occurring (in parentheses) for each treatment and the control on SD 7, was 54 (1). At the conclusion of the study, the IQR values for the control and each treatment concentration were 58 to 59 (2). The median developmental stage attained at SD 7 was not evaluated statistically, since all larvae were recorded as NF stage 54. The median developmental stage attained at SD 21 in the 100 µg/L 2-EHHB treatment was not significantly different from the control (Jonckheere-Terpstra test, p=0.1962), and this test was thus not performed on the remaining lower treatments. No signs of asynchronous development were noted.

7.5.2. Hind Limb Development

Larval hind limb development results for SD 7 and SD 21 are provided in Table 9. SVL results (Table 10) was used to normalize hind limb length and is presented in Table 11. On exposure day 7, the mean normalized HLLs were each 0.10 in the control and each treatment with the exception of 3.6 µg/L 2-EHHB, which was 0.12. At test termination (SD 21), the mean normalized HLLs were 0.24, 0.36, 0.34, 0.38, and 0.35 in the control, 3.6, 10.9, 33, and 100 µg/L 2-EHHB treatments. Normalized HLL in each of the 2-EHHB treatments were not significantly different than the control at SD 7 or SD 21 (test termination) (Mann-Whitney Wilcoxon U test with Bonferroni-Holm adjustment, $p>0.05$).

7.6. Growth

7.6.1. Snout-Vent Length (SVL)

The effect of 2-EHHB exposure on SVL is provided in Table 10. SVL, one of two measures of larval growth, ranged from 15.5 mm in the control to 16.3 mm in the 100 µg/L 2-EHHB treatment on exposure day 7. At exposure day 21, SVL ranged from 27.3 mm in the control to 28.4 mm in the 3.6 µg/L 2-EHHB treatment. SVLs measured in each of the treatments on SD 7 or 21 were not significantly different from the control (Dunnett's test, $p>0.05$ for SD 7, and Mann-Whitney Wilcoxon U test with Bonferroni-Holm adjustment, $p>0.05$ for SD 21).

7.6.2. Body Weight

The effect of 2-EHHB exposure on body weight is provided in Table 12. Body weight, the second measure of larval growth, ranged from 0.2061 g in the control to 0.3131 g in the 100 µg/L 2-EHHB treatment on exposure day 7. At exposure day 21, body weight ranged from 1.1260 g in the control to 1.6426 g in the 33 µg/L 2-EHHB treatment. Body weights measured on SD 7 in the 33 and 100 µg/L 2-EHHB treatments were significantly greater than the control (Jonckheere-Terpstra test, $p=0.0399$ and 0.0079, respectively). Body weights measured on SD 21 in the 33 and 100 µg/L 2-EHHB treatments were significantly greater than the control (Jonckheere-Terpstra test, $p=0.0196$ and 0.0053, respectively).

7.7. Thyroid Gland Histopathology

The results of histopathological evaluation of the thyroid glands are provided in Tables 13 and 14. The histopathology report (EPL, Sterling, VA) is provided in Appendix D. There were two histopathological findings recorded in this study: follicular cell hypertrophy and follicular cell hyperplasia. "*The former was characterized by a relative increase in the proportion of follicular epithelial cells that exhibited increased cell height (i.e., columnar shape relative to cuboidal), and the latter by a proportional increase in stratification, crowding, or papillary in-folding of follicular epithelial cells.*" (EPL, see Appendix D). The rationale for this response is that anuran metamorphosis is considered to be a thyroid-dependent process; therefore, basal levels of follicular cell hypertrophy and hyperplasia are anticipated findings in control frogs at the developmental stage at which they were sacrificed in the study (*i.e.,* median Stage 59). Larvae preparing for metamorphic climax (NF stage 61) require a large surge of thyroid hormone to initiate the final cascade of metamorphic processes, including resorption of the tail. This process significantly taxes the thyroid during the assay, which results in follicular

hypertrophy and in some cases hyperplasia. This stress diminishes at stage 62 as metamorphic climax proceeds.

"There were slight, non-dose-responsive increases in the incidence and/or severity of follicular cell hypertrophy (mild to moderate), and in the incidence of follicular cell hyperplasia (mild), in some treated groups compared to controls; however, these relative differences were too insubstantial to conclude that they represented treatment effects." (EPL, see Appendix D). The control thyroid histopathology was acceptable in the present study, and histopathological findings in the thyroid gland were not significantly more prevalent or severe in 2-EHHB treated frogs as compared to controls.

7.8. Clinical Signs of Toxicity

Clinical signs of toxicity were not observed during the conduct of the present study (Table 15).

8. DISCUSSION

8.1. Performance Criteria and Validity

Performance of the present study and the relationship to the performance criteria and test validity established in protocol BATT01-3 and study guidance document (3) are provided in Table 16. The coefficients of variation (CV, expressed as %) for the measured test concentrations between each replicate for the control or each treatment concentration at a given measurement point (study day 0, 7, 14, and 21) and overall were <20%. Control mortality was <10% in each replicate of the control. The median developmental stage of the control was >57. The interquartile range (10th and 90th percentile) for the control was <4. The range of pH measured in the control and treatments was between 6.5 and 8.5, the temperature in the study was maintained at 22±1ºC, and the inter-replicate range in temperature was maintained at ≤0.5 ºC. None of the test concentrations demonstrated overt toxicity and none of the test concentrations including the control had compromised replicates. In summary, the present study met all performance criteria established for the OPPTS 890.1100 Amphibian Metamorphosis Assay (3) (Table 16). Further, the present study met all validity criteria for a test article that does not have thyroid axis activity.

9. ASSESSMENT OF THE AMPHIBIAN METAMORPHOSIS ASSAY RESULTS

Results of present study met the performance criteria established for the OPPTS 890.1100 Amphibian Metamorphosis Assay (3) and were considered valid (Table 16). The following decision logic was applied to the present study to determine if 2-EHHB affected thyroid activity.

- No significant differences between the median developmental stage or normalized HLL between the control and the treatments were observed on exposure day 7 or at the conclusion of the study.

- Asynchronous development was not noted in the control or treatments during the conduct of the study.

- Although mild to moderate histopathological lesions were observed in the control and the 2-EHHB treatments, there was no clear relationship between test article concentration and response. *"The stimulus for both follicular cell hypertrophy and hyperplasia in larvae* (from the control or the various treatments) *is increased circulating levels of thyroid stimulating hormone (TSH) (Tietge et al., 2010)[1], concentrations of which are highest in the X. laevis pituitary between Nieuwkoop and Faber (NF) stages 58-62 (Korte et al., 2011)[2]. For reasons that are not yet completely clear, the rapid elevation in TSH that is associated with metamorphic climax occurs despite a concomitant rise in circulating thyroid hormones (TH), which would otherwise be expected to suppress pituitary TSH production via the classic hypothalamus-pituitary-thyroid (HPT) negative feedback mechanism (Buckbinder and Brown, 1993; Sternberg et al., 2011)[3]. Following metamorphic climax (e.g., NF stage 66), levels of TSH and TH decrease, at which point the histological appearance of the thyroid glands becomes more quiescent (Grim et al., 2009)[4]."*

- Larvae exposed to 2-EHHB in the 33 and 100 µg/L 2-EHHB treatments weighed significantly more than the controls both at study day (SD) 7 and 21 (conclusion). Thus, 2-EHHB appeared to impact growth (weight).

- No effect of 2-EHHB exposure on SVL at either study day was observed.

- No impact on hind limb length (HLL) was noted in any of the 2-EHHB treatments based on SVL-normalized HLL, although unnormalized HLL at SD 7 in larvae in the 100 µg/L 2-EHHB treatment was significantly greater than the control (Jonckheere-Terpstra test, p=0.0355). In contrast, there was as an impact on HLL observed at SD 21 (conclusion).

- No significant effects on behavior or signs of overt toxicity were noted.

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[1] Tietge JE, Butterworth BC, Haselman JT, Holcombe GW, Hornung MW, Korte JJ, Kosian PA, Wolfe M, Degitz SJ. (2010). Early temporal effects of three thyroid hormone synthesis inhibitors in *Xenopus laevis*. Aquat. Toxicol., 98:44-50.

[2] Korte JJ, Sternberg RM, Serrano JA, Thoemke KR, Moen SM, Lillegard KE, Hornung MW, Tietge JE, Degitz SJ. (2011). Thyroid-stimulating hormone (TSH): measurement of intracellular, secreted, and circulating hormone in *Xenopus laevis* and *Xenopus tropicalis*. Gen. Comp. Endocrinol.,171:319-325.

[3] Buckbinder L, Brown DD. (1993). Expression of the *Xenopus laevis* prolactin and thyrotropin genes during metamorphosis. Proc. Natl. Acad. Sci. U S A, 90:3820-3824 and Sternberg RM, Thoemke KR, Korte JJ, Moen SM, Olson JM, Korte L, Tietge JE, Degitz SJ Jr. (2011). Control of pituitary thyroid-stimulating hormone synthesis and secretion by thyroid hormones during *Xenopus* metamorphosis. Gen Comp Endocrinol, 173:428-437.

[4] Grim KC, Wolfe M, Braunbeck T, Iguchi T, Ohta Y, Tooi O, Touart L, Wolf DC, Tietge J. (2009). Thyroid Histopathology Assessments for the Amphibian Metamorphosis Assay to Detect Thyroid-active Substances. Toxicol. Pathol., 37(4):415-424.

10. CONCLUSIONS

Results from the USEPA OPPTS 890.1100 Amphibian Metamorphosis Assay (3) indicated that growth (weight) was impacted by 2-EHHB exposure relative to the control. However, the significance of increases in body weight at SD 7 and SD 21 following exposure to 2-EHHB at 33 and 100 µg/L cannot be deduced from this study. No treatment-related effects on thyroid-mediated development were noted during the study. There were no significant histopathologic findings in the thyroid related to 2-EHHB exposure in this study. Using the decision criteria in the AMA test guideline (OCSPP 890.1100), 2-EHHB does not appear to affect amphibian metamorphosis or affect the thyroid axis directly based on the endpoints measured at the concentrations tested.

11. SAMPLE HANDLING AND CUSTODY

All samples received, generated during the course of testing, and submitted to EAG Laboratories (Columbia, MO) and EPL (Sterling, VA) in this study were accompanied by an appropriately signed chain of custody and handled in accordance with facility SOPs. Samples were entered into a sample check-in logbook and assigned a unique sample tracking number. Each sample was also properly labeled with its assigned sample tracking number. Sets of test solution samples collected by FEL were preserved as described by EAG Laboratories (Columbia, MO) and shipped to EAG Laboratories by commercial carrier. Whole body tissue samples collected at the conclusion of the in-life phase were shipped to EPL (Sterling, VA) via commercial carrier. Samples, when not in use, were properly preserved and stored, based on sample matrix.

12. RECORD MAINTENANCE AND ARCHIVAL

Test facility-related records (personnel training, equipment calibration and maintenance, storage temperature records, etc.) were retained at the Test Facility. No records were disposed of without the authorization of the Sponsor. The records were organized and included an index.

Certified exact copies of the original raw data, derived data (DEST), QA reports, study guidance documents, correspondence, and draft and final reports were electronically maintained at the In-life test facility in accordance with facility SOPs until study finalization. All original raw data and the original Final Study Report were kept in designated file cabinets located in a secured file room at the Test Facility. After final approval of all reports and conclusion of the study, all electronic files will be transferred to compact discs (CDs) and verified as exact copies of the original. Copies of the electronic disc and the Final Study Report will be sent to the Sponsor. Immediately following finalization of the final report, all original handwritten raw data, original raw data files, the original Final Study Report, protocol and protocol amendments associated with the study will be maintained in the archive at FEL until shipped to the archive location below per facility SOP. In addition, all EPL-generated histology data records will be shipped to the Sponsor for archiving. Original raw analytical data and original analytical reports from EAG Laboratories (Columbia, MO) will be sent to the Sponsor for archiving. The archive location is:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2696
Attn: Vincent J. Brown, Ph.D.
614-424-5928
brownv@battelle.org

13. SPECIMENS ARCHIVAL

The preserved test specimens were labeled and stored at FEL until study finalization in accordance with facility SOPs. Per Sponsor mandated exception, following study finalization, specimens remaining at FEL, and embedded tissues or specimens maintained by EPL will be disposed of in accordance with QMP, QAPP, and respective facility SOPs. All slides produced during the histopathological analyses will be stored at EPL until study finalization. After study finalization, all slides were shipped to the sponsor at the address below.

Sharlene R. Matten, Ph.D.
Senior Biologist, US EPA
Exposure Assessment Coordination and Policy Division
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14. TEST SUBSTANCE WASTE DISPOSAL

Disposal of waste material generated by the study was performed in accordance with those requirements provided in the Material Safety Data Sheets (MSDS) and facility SOPs. The test substance were returned to the Sponsor in accordance with those requirements provided in the facility SOPs.

15. STUDY PROTOCOL ADHERENCE

The study was performed in accordance with Study Protocol No. BATT01-3 (Appendix A). There were nine amendments to and two deviations from the Study Protocol (Appendix A).

16. REFERENCES

1. QAPP, Endocrine Disruptor Screening Program (EDSP) Tier 1 and Tier 2 *In Vivo* Testing of Selected Chemicals for Potential Endocrine Effects in Non-Mammals. EPA Contract No. EP-W-11-063 Task Order 14, version 1.2, August 28, 2015.

2. USEPA, Federal Insecticide, Fungicide, Rodenticide Act (FIFRA). Good Laboratory Practice Standards, Title 40 Code of Federal Regulations Part 160 (effective October 16, 1989).

3. OPPTS. 890.1100 Amphibian Metamorphosis (Frog), United States Environmental Protection Agency, Washington DC, EPA 740-C-09-002, October 2009.

4. FEL Quality Assurance Management Plan (QAMP), Fort Environmental Laboratories, Stillwater, OK, July 23, 2010 (current version).

5. Nieuwkoop, P.D. and Faber, J. Normal Tables of *Xenopus laevis* (Daudin). Garland Publishing, London, 1994.

6. Standard Guide for Conducting the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX). American Society for Testing and Materials (ASTM), E 1439-98, 1998 (Reapproved 2004).

7. EAG Laboratories (formerly ABC Laboratories). Validated Analytical Chemistry Test Method for 2-Ethylhexyl 4-Hydroxybenzoate in Aquatic Assays for EPA EDSP. Under subcontract with Battelle for U.S. EPA Prime Contract No. EP-W-11-063, Task Order 14, 2015.

8. OECD (2007). Guidance Document on Amphibian Thyroid Histology. Environmental Health and Safety Publications. Series on Testing and Assessment. No. 82. Paris, France.

9. Wolf, J. (12 Aug 2015). Draft Procedures for EDSP Studies. EPL (Experimental Pathology Laboratories, Inc.) internal document.

10. OECD. Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. Environmental Health and Safety Publications. Series on Testing and Assessment, No. 54, Paris, France, 2006.

11. Green J.W., Springer T.A., Saulnier A.N., Swintek J. 2014. Statistical analysis of histopathology endpoints. Environmental Toxicology and Chemistry. 33(5):1108-1116.

TABLES

Table 1 In-life Phase Schedule

Study Phase	Study Day	Study Activity
Range-Finding	---	• Range Finding (5,6)
Main Study Pre-Exposure	PE -1	• Breed *X. laevis* • Calibrate diluter (salt water)
	PE 0	• Sort / count test embryos • Prepare test substance stock solution • Collect / store test substance stock • Start diluter equilibration with test substance
	PE 2	• Collect test solution samples / ship test substance stock and test solutions to ABC (EAG) Labs
	PE 6	• Breed back-up *X. laevis*
	PE 7	• Sort / count back-up test embryos
	PE 12-16	• *X. laevis* larvae should be at NF stage 51
Main Study Exposure	E 0	• Introduce test larvae to exposure system • Collect test solution samples / ship test substance stock and test solutions to ABC (EAG) Labs
	E 7	• Collect test solution samples / ship test substance stock and test solutions to ABC (EAG) Labs • Collect / fix tissue specimens required for day 7 endpoints
	E 14	• Collect test solution samples / ship test substance stock and test solutions to ABC (EAG) Labs
	E 21	• Test takedown • Collect test solution samples / ship test substance stock and test solutions to ABC (EAG) Labs • Collect/fix tissue specimens for all test termination endpoints

Table 2 Sera Micron® Feeding Rate for Tadpoles during In-Life Phase of AMA using Flow-Through Conditions

Study Day	Food Ration (mg Sera Micron®/animal/day)
0 - 4	30
5 - 7	40
8 - 10	50
11 - 14	70
15 - 21	80

Table 3 Experimental Conditions for In-Life Study

Test substance		2-ethylhexyl 4-hydroxybenzoate	
Test System (species)		*Xenopus laevis* Larvae	
Initial Larval Stage		NF Stage 51	
Exposure Period		21 d	
Larvae Selection Criteria		Developmental Stage and Optional Total Length	
Test Chemical Concentration (µg/L)		0.0 (control), 3.6, 10.9, 33.0, and 100.0	
Exposure System		Flow-Through Mini-Diluter	
Exposure Route		Abiotic Exposure via Culture Media	
Flow-Rate		25 mL/min	
Primary Endpoints / Determination Days		Mortality	Daily
		Developmental Stage	Study Days 7 and 21
		Hind Limb Length	Study Days 7 and 21
		Snout-Vent Length	Study Days 7 and 21
		Wet Body Weight	Study Days 7 and 21
		Thyroid Histology	Study Day 21
Additional Observations		Morphology/Behavior	Study Days 7 and 21
Dilution Water / Laboratory Control		Dechlorinated Tap Water (charcoal-filtered)	
Larval Density		20 Larvae / Test Vessel (5 / L)	
Test Solution / Test Vessel		4 L (10-15 cm water height)	
Replication		4 Replicates / Test Concentration and Control	
Acceptable Mortality Rate in Controls		≤10%	
Thyroid Fixation	Number Fixed	5 / Replicate (randomly selected, stage matched)	
	Region	Head	
	Fixation Fluid	Davidson's Fixative	
Feeding	Food	Sera Micron®	
	Frequency / Amount	Twice daily / see Table 2	
Lighting	Photoperiod	12 h Light : 12 h dark	
	Intensity	600 to 2,000 lux (Measured at Water Surface)	
Water Temperature		22º ± 1ºC	
pH		6.5 – 8.5	
Dissolved Oxygen (DO) Concentration		>3.5 mg/L (>40% Air Saturation)	
Analytical Chemistry Sample Schedule		Equilibration phase and 4 Events (d 0, d 21, and 2 events between d 0 and d 21)	

Table 4 Observation Time Points for Endpoints

Endpoints:	Daily	Study Day 7	Study Day 21
Primary[1]:			
Mortality	●		
Developmental Stage		●	●
Hind Limb Length		●	●
Snout-Vent Length		●	●
Wet Body Weight		●	●
Thyroid Gland Histology			●
Secondary:			
Gross Morphology		●	●

[1] Statistical evaluation will be considered for each of the primary endpoints.

Table 5 Summary of Water Quality Characteristics in the Test System

Parameter	Treatment (µg/L)	Replicate	Minimum	Maximum	Measurement Interval
pH (s.u.)	0.0	A	7.2	8.1	3x Weekly
		B	7.3	8.1	3x Weekly
		C	7.3	7.9	3x Weekly
		D	7.3	7.9	3x Weekly
	3.6	A	7.4	7.9	3x Weekly
		B	7.5	7.9	3x Weekly
		C	7.5	7.9	3x Weekly
		D	7.6	7.9	3x Weekly
	10.9	A	7.5	7.9	3x Weekly
		B	7.4	7.9	3x Weekly
		C	7.4	7.8	3x Weekly
		D	7.5	7.8	3x Weekly
	33.0	A	7.5	7.9	3x Weekly
		B	7.5	7.9	3x Weekly
		C	7.5	7.9	3x Weekly
		D	7.5	7.9	3x Weekly
	100	A	7.5	7.9	3x Weekly
		B	7.5	8.0	3x Weekly
		C	7.4	8.0	3x Weekly
		D	7.5	8.0	3x Weekly
Dissolved oxygen (mg/L)	0.0	A	6.8	8.3	3x Weekly
		B	6.9	8.0	3x Weekly
		C	6.2	8.1	3x Weekly
		D	6.4	7.8	3x Weekly
	3.6	A	5.5	7.7	3x Weekly
		B	5.6	7.6	3x Weekly
		C	4.9	7.9	3x Weekly
		D	4.2	7.6	3x Weekly
	10.9	A	4.8	8.1	3x Weekly
		B	5.3	7.9	3x Weekly
		C	5.4	7.7	3x Weekly
		D	5.9	8.0	3x Weekly
	33.0	A	4.6	7.7	3x Weekly
		B	5.4	7.8	3x Weekly
		C	5.4	7.7	3x Weekly
		D	5.8	7.7	3x Weekly
	100	A	5.6	7.6	3x Weekly
		B	5.9	7.5	3x Weekly
		C	5.4	7.6	3x Weekly
		D	5.4	7.6	3x Weekly

Table 5 (*continued*)
Summary of Water Quality Characteristics in the Test System

Parameter	Treatment (µg/L)	Replicate	Minimum	Maximum	Measurement Interval
Temperature (ºC)	0.00	A	22.3	22.6	Daily
		B	22.3	22.8	Daily
		C	22.3	22.7	Daily
		D	22.3	22.7	Daily
	3.6	A	22.3	22.8	Daily
		B	22.3	22.7	Daily
		C	22.3	22.7	Daily
		D	22.3	22.7	Daily
	10.9	A	22.3	22.7	Daily
		B	22.3	22.7	Daily
		C	22.3	22.7	Daily
		D	22.3	22.7	Daily
	33.0	A	22.3	22.7	Daily
		B	22.3	22.7	Daily
		C	22.3	22.7	Daily
		D	22.4	22.7	Daily
	100	A	22.3	22.7	Daily
		B	22.3	22.7	Daily
		C	22.3	22.7	Daily
		D	22.3	22.7	Daily
Light Intensity[1] (lux)	0.00	A	631	818	3x Weekly
		B	632	769	3x Weekly
		C	615	851	3x Weekly
		D	603	912	3x Weekly
	3.6	A	654	912	3x Weekly
		B	664	901	3x Weekly
		C	661	923	3x Weekly
		D	642	854	3x Weekly
	10.9	A	615	812	3x Weekly
		B	631	831	3x Weekly
		C	602	894	3x Weekly
		D	613	883	3x Weekly
	33.0	A	615	796	3x Weekly
		B	638	823	3x Weekly
		C	638	852	3x Weekly
		D	605	832	3x Weekly
	100	A	615	813	3x Weekly
		B	645	812	3x Weekly
		C	641	825	3x Weekly
		D	614	816	3x Weekly

[1] Measured at water level.

Table 5 (*continued*)
Summary of Water Quality Characteristics in the Test System

Parameter	Treatment (µg/L)	Minimum	Maximum	Measurement Interval
Total Hardness (mg/L as CaCO3)	0.0	128	140	1x Weekly
	100	128	148	1x Weekly
Alkalinity (mg/L as CaCO3)	0.0	48	68	1x Weekly
	100	52	72	1x Weekly
Iodide (µg/L)	N/A	9.2	9.3	2x during study
Ammonia[1] (µg/L)	N/A	<0.06	<0.06	2x during study
Chlorine (µg/L)	N/A	<0.05	<0.05	2x during study

[1] Expressed as nitrogen.

Table 6 Summary of Treatment Concentrations in the AMA with 2-EHHB

Study Day	Nominal Concentration (µg/L)	Replicate	Sample ID[1]	Measured Concentration[2] (µg/L)	CV[3] (%)
0	0.0	A	034	<MQL	---
		B	035	<MQL	
		C	036	<MQL	
		D	037	<MQL	
	3.6	A	038	3.63	2.3
		B	039	3.58	
		C	040	3.60	
		D	041	3.77	
	10.9	A	042	9.04	6.4
		B	043	8.66	
		C	044	9.52	
		D	045	10.0	
	33.0	A	046	22.8	5.7
		B	047	22.3	
		C	048	25.3	
		D	049	22.8	
	100	A	050	60.0	13.3
		B	051	59.2	
		C	052	78.2	
		D	053	66.6	
7	0.0	A	162	<MQL	---
		B	163	<MQL	
		C	164	<MQL	
		D	165	<MQL	
	3.6	A	166	4.47	19.4
		B	167	7.28	
		C	168	6.04	
		D	169	5.94	
	10.9	A	170	13.3	6.4
		B	171	14.7	
		C	172	12.6	
		D	173	13.5	
	33.0	A	174	35.4	7.5
		B	175	30.5	
		C	176	31.0	
		D	177	30.5	
	100	A	178	83.3	4.6
		B	179	86.5	
		C	180	87.1	
		D	181	78.6	

[1] Results are based on values reported in the DEST, Analytical & Water Quality tab (Appendix E).
[2] Minimum Quantitation Level (MQL) = 0.208 µg/L.
[3] Coefficient of variation = (Standard deviation / mean)100. Represents inter-replicate CV at each sampling point.

Table 6 *(continued)* Summary of Treatment Concentrations in the AMA with 2-EHHB

Study Day	Nominal Concentration (µg/L)	Replicate	Sample ID[1]	Measured Concentration[2] (µg/L)	CV[3] (%)
14	0.0	A	187	<MQL	---
		B	188	<MQL	
		C	189	<MQL	
		D	190	<MQL	
	3.6	A	191	5.08	19.8
		B	192	8.05	
		C	193	5.86	
		D	194	6.50	
	10.9	A	195	14.9	12.8
		B	196	10.9	
		C	197	12.9	
		D	198	13.6	
	33.0	A	199	26.8	7.5
		B	200	28.9	
		C	201	31.6	
		D	202	31.2	
	100	A	203	51.3	7.9
		B	204	58.3	
		C	205	62.3	
		D	206	57.0	
21	0.0	A	511	<MQL	---
		B	512	<MQL	
		C	513	<MQL	
		D	514	<MQL	
	3.6	A	515	2.42	3.4
		B	516	2.31	
		C	517	2.27	
		D	518	2.43	
	10.9	A	519	5.19	3.3
		B	520	5.37	
		C	521	5.24	
		D	522	4.95	
	33.0	A	523	14.6	5.6
		B	524	16.4	
		C	525	14.7	
		D	526	15.8	
	100	A	527	41.5	2.5
		B	528	41.1	
		C	529	41.2	
		D	530	39.2	

[1] Results are based on values reported in the DEST, Analytical & Water Quality tab (Appendix E).
[2] Minimum Quantitation Level (MQL) = 0.208 µg/L.
[3] Coefficient of variation = (Standard deviation / mean)100. Represents inter-replicate CV at each sampling point.

Table 7. Summary of Mean Measured Concentrations in the AMA with 2-EHHB

Study Day	Nominal Concentration (µg/L)	Mean Measured Concentration (µg/L) [1]	CV[2] (%)
Mean Measured Concentrations	0.0	<MQL[3]	---
	3.6	4.58	13
	10.9	10.3	3
	33.0	25.0	2
	100	62.0	6

[1] Since time interval between sample collection events was consistent throughout study, the mean measured concentration represents an accurate estimation of exposure concentration. The mean measured concentration represents the average of each data point from SD 0, 7, 14, and 21 for each replicate (A-D) of the control and each treatment per facility SOP.

[2] Coefficient of variation = (Standard deviation / mean)100. Standard deviation of the mean of four replicates (n=4) for the control and each treatment divided by the mean of the mean measured concentrations of each replicate per facility SOP. Designated as intra-replicate mean CV used in assessment variability of estimated exposure concentration and test acceptability.

[3] Minimum Quantitation Level (MQL) = 0.208 µg/L.

Table 8 Effect of 2-EHHB Exposure on Mortality and Developmental Stage[1]

Treatment [Mean Measured Conc.] (µg/L)	Replicate	Mortality (Study Day 7)		Mortality (Study Day 21)		NF Stage (Study Day 7)			NF Stage (Study Day 21)			Jonckheere-Terpstra Test on Day 21 NF Stage (p-value)
		N	Dead	N	Dead	N	Median	IQR[2]	N	Median	IQR	
0.0 [<MQL]	A	20	0	15	0	5	54	54-54	15	58	58-58	
	B	20	0	15	0	5	54	54-54	15	59	58-59	
	C	20	0	15	0	5	54	54-54	15	58	58-59	
	D	20	0	15	0	5	54	54-54	15	58	58-59	
	Overall	80	0	60	0	20	54	54-54	60	58	58-59	
3.6 [4.58]	A	20	0	15	0	5	54	54-54	15	58	58-59	NP[3]
	B	20	0	15	0	5	54	54-54	15	58	58-59	
	C	20	0	15	0	5	54	54-54	15	58	57-59	
	D	20	0	15	0	5	54	54-54	15	59	58-59	
	Overall	80	0	60	0	20	54	54-54	60	58	58-59	
10.9 [10.3]	A	20	0	15	0	5	54	54-54	15	58	58-59	NP
	B	20	0	15	0	5	54	54-54	15	58	57-59	
	C	20	0	15	0	5	54	54-54	15	58	58-59	
	D	20	0	15	0	5	54	54-54	15	58	57-59	
	Overall	80	0	60	0	20	54	54-54	60	58	58-59	
33 [25.0]	A	20	0	15	0	5	54	54-54	15	59	58-59	NP
	B	20	0	15	0	5	54	54-54	15	59	58-59	
	C	20	0	15	0	5	54	54-54	15	59	58-59	
	D	20	0	15	0	5	54	54-54	15	58	58-59	
	Overall	80	0	60	0	20	54	54-54	60	59	58-59	
100 [62.0]	A	20	0	15	0	5	54	54-54	15	59	58-59	0.1962
	B	20	0	15	0	5	54	54-54	15	59	58-59	
	C	20	0	15	0	5	54	54-54	15	58	58-59	
	D	20	0	15	0	5	54	54-54	15	58	58-59	
	Overall	80	0	60	0	20	54	54-54	60	59	58-59	

[1] Jonckheere-Terpstra tests were conducted on NF stage Day 21 replicate median values in a stepdown fashion at the 0.05 level (2-sided). The test was not conducted for SD 7 since all tadpoles were recorded as NF stage 54 on Day 7.
[2] Interquartile range, 10th to 90th percentiles.
[3] Jonckheere-Terpstra step-down test was not performed since the highest treatment group at 100 µg/L was not statistically significant at p=0.05. NP=not performed.

Table 9 Effect of 2-EHHB Exposure on Hind Limb Length (mm) on Study Days 7 and 21

Treatment [Mean Measured Conc.] (µg/L)	Replicate	Hind Limb Length (mm) Study Day 7					Hind Limb Length (mm) Study Day 21				
		N	Replicate Mean	Mean	SEM[1]	CV (%)[2]	N	Replicate Mean	Mean	SEM	CV (%)
0.0 [<MQL]	A	5	1.80	1.66	0.06	7.16	15	6.18	6.83	0.23	6.60
	B	5	1.62				15	6.87			
	C	5	1.52				15	7.06			
	D	5	1.70				15	7.19			
3.6 [4.58]	A	5	1.70	1.86	0.12	12.81	15	10.87	10.39	1.41	27.13
	B	5	1.94				15	11.92			
	C	5	1.64				15	6.29			
	D	5	2.16				15	12.50			
10.9 [10.3]	A	5	1.82	1.73	0.12	13.57	15	9.23	8.83	0.59	13.43
	B	5	1.90				15	9.67			
	C	5	1.80				15	9.35			
	D	5	1.38				15	7.07			
33 [25.0]	A	5	1.82	1.81	0.05	5.52	15	11.97	10.51	0.88	16.80
	B	5	1.94				15	9.69			
	C	5	1.78				15	11.96			
	D	5	1.70				15	8.41			
100 [61.9]	A	5	1.88	2.02[3]	0.08	8.24	15	10.47	9.72	0.36	7.48
	B	5	2.04				15	10.10			
	C	5	1.90				15	9.50			
	D	5	2.24				15	8.81			

[1] Standard error of the mean

[2] Coefficient of variation = (standard deviation / mean) × 100

[3] Significantly greater than control (Jonckheere-Terpstra test, p=0.0180).

Table 10 Effect of 2-EHHB Exposure on Snout-to-Vent Length (mm) on Study Days 7 and 21

Treatment [Mean Measured Conc.] (µg/L)	Replicate	Snout-to-Vent Length (mm) Study Day 7					Snout-to-Vent Length (mm) Study Day 21				
		N	Replicate Mean	Mean	SEM[1]	CV (%)[2]	N	Replicate Mean	Mean	SEM	CV (%)
0.0 [<MQL]	A	5	15.44	15.48	0.28	3.62	15	25.34	27.32	0.71	5.21
	B	5	15.70				15	27.28			
	C	5	14.72				15	28.56			
	D	5	16.04				15	28.11			
3.6 [4.58]	A	5	13.74	14.40	0.62	8.59	15	30.53	28.36	1.09	7.66
	B	5	14.24				15	29.57			
	C	5	13.42				15	25.61			
	D	5	16.18				15	27.71			
10.9 [10.3]	A	5	14.44	14.04	0.63	8.97	15	27.07	25.88	0.46	3.59
	B	5	14.06				15	25.55			
	C	5	15.32				15	26.04			
	D	5	12.32				15	24.85			
33 [25.0]	A	5	15.38	14.97	0.49	6.61	15	26.54	27.67	0.41	2.97
	B	5	15.88				15	28.25			
	C	5	15.04				15	28.30			
	D	5	13.58				15	27.57			
100 [61.9]	A	5	16.34	16.30	0.24	2.89	15	27.49	27.90	0.30	2.13
	B	5	16.68				15	27.64			
	C	5	15.62				15	27.70			
	D	5	16.54				15	28.79			

[1] Standard error of the mean
[2] Coefficient of variation = (standard deviation / mean) × 100

Table 11 Effect of 2-EHHB Exposure on Normalized Hind Limb Length (ratio of HLL:SVL) on Study Days 7 and 21

Treatment [Mean Measured Conc.] (µg/L)	Replicate	Normalized Hind Limb Length (ratio of HLL:SVL) Study Day 7					Normalized Hind Limb Length (ratio of HLL:SVL) Study Day 21				
		N	Replicate Mean	Mean	SEM[1]	CV (%)[2]	N	Replicate Mean	Mean	SEM	CV (%)
0.0 [<MQL]	A	5	0.10	0.10	0.00	0.00	15	0.25	0.24	0.00	1.58
	B	5	0.10				15	0.24			
	C	5	0.10				15	0.24			
	D	5	0.10				15	0.25			
3.6 [4.58]	A	5	0.12	0.12	0.01	8.70	15	0.35	0.36	0.04	23.67
	B	5	0.12				15	0.40			
	C	5	0.10				15	0.25			
	D	5	0.12				15	0.45			
10.9 [10.3]	A	5	0.10	0.10	0.00	0.00	15	0.33	0.34	0.02	12.58
	B	5	0.10				15	0.38			
	C	5	0.10				15	0.35			
	D	5	0.10				15	0.28			
33 [25.0]	A	5	0.10	0.10	0.00	0.00	15	0.46	0.38	0.04	20.10
	B	5	0.10				15	0.33			
	C	5	0.10				15	0.42			
	D	5	0.10				15	0.30			
100 [61.9]	A	5	0.10	0.10	0.00	0.00	15	0.38	0.35	0.02	9.81
	B	5	0.10				15	0.36			
	C	5	0.10				15	0.35			
	D	5	0.10				15	0.30			

[1] Standard error of the mean
[2] Coefficient of variation = (standard deviation / mean) × 100

Table 12 Effect of 2-EHHB Exposure on Wet Body Weight (g) on Study Days 7 and 21

Treatment [Mean Measured Conc.] (µg/L)	Replicate	Body Weight (g) Study Day 7					Body Weight (g) Study Day 21				
		N	Replicate Mean	Mean	SEM[1]	CV (%)[2]	N	Replicate Mean	Mean	SEM	CV (%)
0.0 [<MQL]	A	5	0.2070	0.2061	0.0080	7.7446	15	0.9296	1.1260	0.0716	12.7242
	B	5	0.2142				15	1.1100			
	C	5	0.1834				15	1.2427			
	D	5	0.2196				15	1.2216			
3.6 [4.58]	A	5	0.2440	0.2638	0.0303	22.9956	15	1.6203	1.4581	0.1845	25.3048
	B	5	0.2398				15	1.4865			
	C	5	0.2182				15	0.9367			
	D	5	0.3532				15	1.7889			
10.9 [10.3]	A	5	0.2596	0.2531	0.0296	23.3964	15	1.4731	1.3281	0.0643	9.6833
	B	5	0.2462				15	1.3003			
	C	5	0.3254				15	1.3719			
	D	5	0.1810				15	1.1672			
33 [25.0]	A	5	0.3248	0.2911[3]	0.0274	18.8170	15	1.4983	1.6426[4]	0.0598	7.2788
	B	5	0.3286				15	1.6669			
	C	5	0.2996				15	1.7871			
	D	5	0.2112				15	1.6182			
100 [61.9]	A	5	0.3234	0.3131[5]	0.0218	13.9443	15	1.5794	1.6284[6]	0.0222	2.7267
	B	5	0.3544				15	1.6057			
	C	5	0.3230				15	1.6499			
	D	5	0.2514				15	1.6787			

[1] Standard error of the mean
[2] Coefficient of variation = (standard deviation / mean) × 100
[3] Significantly greater than control (Jonckheere-Terpstra test, p=0.0399)
[4] Significantly greater than control (Jonckheere-Terpstra test, p=0.0196)
[5] Significantly greater than control (Jonckheere-Terpstra test, p=0.0079)
[6] Significantly greater than control (Jonckheere-Terpstra test, p=0.0053)

Table 13 Summary of Histopathologic Findings for Follicular Cell Hypertrophy

Treatment [Mean Measured Conc.] (µg/L)	Replicate	Mild[1]		Moderate[2]	
		No. Findings/ No. in Group	Proportion	No. Findings/ No. in Group	Proportion
0.0 [<MQL]	A	2/5	0.40	0/5	0.00
	B	3/5	0.60	0/5	0.00
	C	1/5	0.20	0/5	0.00
	D	4/5	0.80	0/5	0.00
	Overall	10/20	0.50	0/20	0.00
3.6 [4.58]	A	3/5	0.60	0/5	0.00
	B	0/5	0.00	0/5	0.00
	C	1/5	0.20	0/5	0.00
	D	2/5	0.40	1/5	0.20
	Overall	6/20	0.30	1/20	0.05
10.9 [10.3]	A	4/4[3]	1.00	0/4[3]	0.00
	B	4/5	0.80	0/5	0.00
	C	2/5	0.40	0/5	0.00
	D	3/5	0.60	0/5	0.00
	Overall	13/19[1]	0.68	0/19[3]	0.00
33 [25.0]	A	2/5	0.40	0/5	0.00
	B	2/5	0.40	0/5	0.00
	C	4/5	0.80	1/5	0.20
	D	4/5	0.80	0/5	0.00
	Overall	12/20	0.60	1/20	0.05
100 [61.9]	A	3/5	0.60	0/5	0.00
	B	3/5	0.60	1/5	0.20
	C	1/5	0.20	0/5	0.00
	D	4/5	0.80	0/5	0.00
	Overall	11/20	0.55	1/20	0.05

[1] No significant difference between treatments and control (RSCABS, p=0.1501).
[2] No significant difference between treatments and control (RSCABS, p=0.2025).
[3] Thyroid gland tissue was not recovered from one tadpole in replicate A of treatment group 10.9 µg/L

Table 14 Summary of Histopathologic Findings for Follicular Cell Hyperplasia

Treatment [Mean Measured Conc.] (µg/L)	Replicate	Mild[1]	
		No. Findings/ No. in Group	Proportion
0.0 [<MQL]	A	0/5	0.00
	B	0/5	0.00
	C	0/5	0.00
	D	1/5	0.20
	Overall	1/20	0.05
3.6 [4.58]	A	0/5	0.00
	B	0/5	0.00
	C	0/5	0.00
	D	1/5	0.20
	Overall	1/20	0.05
10.9 [10.3]	A	0/4[2]	0.00
	B	0/5	0.00
	C	0/5	0.00
	D	0/5	0.00
	Overall	0/19[2]	0.00
33 [25.0]	A	0/5	0.00
	B	0/5	0.00
	C	1/5	0.20
	D	2/5	0.40
	Overall	3/20	0.15
100 [61.9]	A	0/5	0.00
	B	1/5	0.20
	C	1/5	0.20
	D	0/5	0.00
	Overall	2/20	0.10

[1] No significant difference between treatments and control (RSCABS, p=0.1553).
[2] Thyroid gland tissue was not recovered from one tadpole in replicate A of treatment group 10.9 µg/L.

Table 15 Clinical Signs of Toxicity in *Xenopus laevis*

Treatment [Mean Measured Conc.] (µg/L)	Replicate	Clinical Signs[1]		
		Type	n	Incidence
0.0 [<MQL]	A	None	15	0
	B	None	15	0
	C	None	15	0
	D	None	15	0
3.6 [4.58]	A	None	15	0
	B	None	15	0
	C	None	15	0
	D	None	15	0
10.9 [10.3]	A	None	15	0
	B	None	15	0
	C	None	15	0
	D	None	15	0
33 [25.0]	A	None	15	0
	B	None	15	0
	C	None	15	0
	D	None	15	0
100 [61.9]	A	None	15	0
	B	None	15	0
	C	None	15	0
	D	None	15	0

[1] Includes abnormal swimming behavior, lethargy, loss of equilibrium, curvature of the spine (e.g., "bent tail"), other malformations, and lesions.

Table 16 General Test Performance Criteria[1]

Criterion	Acceptable Limits	Criteria Passed
Test concentrations	≤20 % CV of measured test concentration[2]	√
Control mortality	≤ 10 % in any replicate of the control	√
Minimum median control developmental stage at test termination	57	√
Range of control developmental stages	≤ 4 for the 10th and 90th percentile	√
DO	≥ 40 % of air saturation	√
pH	6.5 – 8.5	√
Water temperature	22 ± 1°C with inter-replicate variability ≤ 0.5°C	√
Test concentrations without overt toxicity (excluding control)	≥ 2	√

[1] Based on Protocol BATT01-3 for study BATT01-00388.
[2] CVs of the intra-replicate means of the measured test concentrations.

Appendix A

PROTOCOL, PROTOCOL AMENDMENTS, AND PROTOCOL DEVIATIONS

FEL Protocol No.:	**BATT01-3**
GLP Study Title:	**21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethyhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis***
Test Guideline:	**U.S. EPA, OPPTS 890.1100 (October 2009)**
FEL Study No.:	**BATT01-00388**
Test System:	***Xenopus laevis***
Test Substance:	**2-Ethyhexyl 4-Hydroxybenzoate**
USEPA Task Order:	**TO 14**
USEPA/Battelle Contract No.:	**EP-W-11-063**
Sponsor:	**U.S. Environmental Protection Agency 1200 Pennsylvania Ave., NW Washington DC 20460C**
Testing Facility:	**Fort Environmental Laboratories, Inc. (FEL) 515 South Duncan Street Stillwater, OK 74074**
PI Support Site: (Analytical Chemistry)	**ABC Laboratories 7200 East ABC Lane Columbia, MO 65202**
PI Support Site: (Histopathology)	**Experimental Pathology Laboratories (EPL) 45600 Terminal Drive Sterling, VA 20166**

Amendments:

Number	Date	Section(s)	Page(s)
1			
2			
3			
4			

1. SIGNATURE PAGE

Title/Name	Signature	Date
STUDY DIRECTOR: Douglas J. Fort, Ph.D.		1/7/2016
SPONSOR REPRESENTATIVE:[1] Vincent J. Brown, Ph.D.		1/7/2016

[1]Study Monitor.

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2. GOOD LABORATORY PRACTICE

This study will be conducted in compliance with the following GLP principles:

- United States Environmental Protection Agency, (FIFRA), Good Laboratory Practice Standards, Title 40 Code of Federal Regulations Part 160 (effective October 16, 1989) with the following exceptions:
  - Analysis of the laboratory dilution water for organics, pesticides and metals at Red River Laboratory (Oklahoma City, Oklahoma) using standard EPA methods will not be GLP-compliant,
  - The test substance will not be chemically-characterized in a GLP-compliant manner,
  - Range-finding studies conducted as a component of FEL study (BATT01-00385) used to determine test concentrations for the present study, BATT01-00388, were not performed in a GLP-compliant manner.

Since the analyses of the dilution water will be conducted following standard validated methods, this exception will not be expected to impact on the study results. Lack of GLP-compliant chemical characterization or GLP-compliant range-finding studies will not be expected to impact study results, nor change the conclusions drawn from the study. The final report will contain a statement indicating whether the study data complies with the above GLP guidelines. The statement will be signed by the Study Director.

3. QUALITY ASSURANCE

A GLP-compliant QAU is present at the test facility (FEL), each Principal Investigator (PI) support facility (ABC Labs and EPL), and the Battelle Memorial Institute (Sponsor Representative). A sponsor-approved Quality Assurance Project Plan (QAPP) (1) is in place for the study. All independent QA activities will be performed in accordance with the USEPA Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), Good Laboratory Practice (GLP) Standards, Final Rule, 40 CFR Part 160 (2), the facility-specific standard operating procedures (SOPs) at each organization, and the QAPP (1).

4. INTRODUCTION

FEL has been contracted by Battelle Memorial Institute to perform the Amphibian Metamorphosis (Frog) Assay under EPA Test Guidelines OPPTS 890.1100 (3) using 2-ethyhexyl 4-hydroxybenzoate [2-EHHB] (test substance) as directed by USEPA Task Order (TO) 14 under USEPA/Battelle Memorial Institute contract EP-W-11-063. This study will be conducted in accordance with the USEPA Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), Good Laboratory Practice (GLP) Standards, Final Rule, 40 CFR Part 160 (2), and under the FEL Quality Assurance Management Plan (QAMP) (4), relevant facility standard operating procedures (SOPs), and the following Study Protocol No. BATT01-3, prepared for FEL Study No. BATT01-00388.

5. OBJECTIVE

An amphibian metamorphosis assay will be performed in which Nieuwkoop and Faber (NF) (5) stage 51 *Xenopus laevis* larvae are exposed to different concentrations of the test substance for 21-days. In contrast to that specified in EPA Test Guidelines OPPTS 890.1100 (3), which require testing of three independent concentrations of test substance, the general experimental design will entail exposing tadpoles to four (4) different concentrations of the test chemical (n = 4 replicates per concentration) and dilution water control (n = 4 replicates). Larval density at test initiation will be 20 tadpoles per test tank (i.e., replicate for all treatment groups). The treatment tanks will be randomly assigned to a position in the exposure system in order to account for possible variations in temperature and light intensity. The primary endpoints will be hind limb length, body length (snout to vent [SVL]), developmental stage, wet weight, thyroid histology, and daily mortality.

6. TESTING FACILITY

The in-life portion of the study, to include the pre-exposure and exposure phases of the amphibian metamorphosis assay, will be performed at Fort Environmental Laboratories, Inc., 515 South Duncan Street, Stillwater, Oklahoma, USA, 74074. The Study Director, Dr. Douglas Fort, will serve as the study contact for this facility and may be reached at 405.624.6771 or djfort@fortlabs.com.

7. TESTING SITES

The 2-EHHB chemical analysis portion of the study will be performed at ABC Laboratories, Inc., Chemical Services Department, 7200 East ABC Lane, Columbia, Missouri, USA 65202. Dr. Tom Leak, PI of the planned analyses, will serve as study contact for ABC Laboratories and may be reached at 573.777.6050 or leakt@abclabs.com.

Thyroid histopathology will be performed at Experimental Pathology Laboratories, Inc. (EPL), 45600 Terminal Drive, Sterling, Virginia, USA, 20166. Dr. Jeffrey Wolf, D.V.M., DACVP, will serve as the PI and project contact for EPL. Dr. Wolf may be reached at 703.471.7060, etx. 242, or jwolf@epl-inc.com.

8. ANIMAL WELFARE ACT COMPLIANCE

This study will comply with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR). The Sponsor should make particular note of the following:

- The Sponsor signature on this protocol documents for the Study Director the Sponsor's assurance that the study described in this protocol does not unnecessarily duplicate previous experiments.

- Whenever possible, procedures used in this study have been designed to avoid or minimize discomfort, distress or pain to animals. All methods are described in this study protocol or in written laboratory standard operating procedures.

- By design, this study may kill and/or result in the pain and distress of test organisms. Euthanasia of test organisms before completion of the test would interfere with study objectives. Upon completion of the test all distressed amphibians will be painlessly euthanized in a timely manner.

- Methods of euthanasia used during this study will be in conformance with the above referenced regulation and are consistent with EPA Test Guidelines OPPTS 890.1100 (3).

9. STUDY SCHEDULE

The experimental study is proposed to start in January 2016 after Sponsor approval of the Final Study Protocol. The actual in-life test duration will include a range-finding study, a 12-17-day pre-exposure phase, and a 21-day exposure phase. The experimental study termination is anticipated to occur within 14 days after completion of the histopathological analyses.

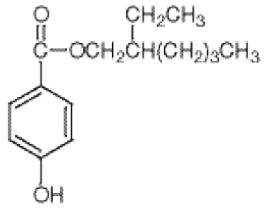
10. EXPERIMENTAL DESIGN

10.1. TEST SUBSTANCE

2-EHHB (TCI America, Portland, OR, lot number H0506, expiration date and re-test date not provided, 98.0% (w/w) pure [w/w] per Certificate of Analysis produced by TCI America) was received from TCI America. Physicochemical information is provided below:

Name: 2-ethyhexyl 4-hydroxybenzoate
IUPAC Name: 2-ethyhexyl 4-hydroxybenzoate

Structure:



Phase/Color:	liquid, colorless, pale yellow
Specific Gravity:	1.04 g/mL at 25°C
Solubility (water):	6.0 mg/L at 25°C
Boiling Point:	270.0°C
Synonyms:	2-ethylhexylparaben, 4-hydroxybenzoic acid 2-ethylhexyl ester, 4-hydroxybenzoic acid octyl ester, octyl 4-hydroxybenzoate, octylparaben
CAS number:	5153-25-3
Molecular formula:	$C_{15}H_{22}O_3$
Molecular weight:	250.3 g/mole

The test substance will be stored in the chemical repository at room temperature (RT) while not in use as recommended by the Certificate of Analysis supplied by TCI America. The definitive test concentrations to be used were determined by a range-finding study designed to identify the concentration of test chemical that causes lethality and morbidity (6). The range-finding study was conducted separately (FEL Study No. BATT01-00385) and was not required to be GLP-compliant. Based on range-finding, the maximum tolerable concentration (MTC) (1) was determined to be 100 µg/L. The test concentration series will be 1x, 0.33x, 0.11x, and 0.04x, where x is the MTC value. Therefore, the test concentrations will be 100.0, 33.0, 10.9, and 3.6 µg/L.

Table 1. Proposed In-life Phase Schedule

Study Phase	Study Day	Study Activity
Range-Finding	---	• Range Finding (5,6)
Main Study Pre-Exposure	PE -1	• Breed *X. laevis* • Calibrate diluter (salt water)
	PE 0	• Sort / count test embryos • Prepare test substance stock solution • Collect / store test substance stock • Start diluter equilibration with test substance
	PE 2	• Collect test solution samples / ship test substance stock and test solutions to ABC Labs
	PE 6	• Breed back-up *X. laevis*
	PE 7	• Sort / count back-up test embryos
	PE 12-16	• *X. laevis* larvae should be at NF stage 51
Main Study Exposure	E 0	• Introduce test larvae to exposure system • Collect test solution samples / ship test substance stock and test solutions to ABC Labs
	E 7	• Collect test solution samples / ship test substance stock and test solutions to ABC Labs • Collect / fix tissue specimens required for day 7 endpoints
	E 14	• Collect test solution samples / ship test substance stock and test solutions to ABC Labs
	E 21	• Test takedown • Collect test solution samples / ship test substance stock and test solutions to ABC Labs • Collect/fix tissue specimens for all test termination endpoints

10.2. Dilution and Laboratory Control Water

FEL will use dechlorinated (charcoal-filtered) tap water as the dilution water for this study. The dechlorinated laboratory water will be prepared by passing tap water through a 4-filter system; a multimedia filter to remove suspended solids in the feed water; a 10" pre-treatment filter (5 µm) to remove any additional solids; a 3.6 ft^3 activated virgin carbon treatment filter to remove chlorine, ammonia, and higher molecular weight organics; and a 5 µm polishing filter to remove any carbon particles from the carbon treatment phase. The dechlorinated tap water will also serve as the laboratory control water. Water quality characteristics of the laboratory water are monitored bimonthly for pH, dissolved oxygen (DO), conductivity, hardness, alkalinity, ammonia, residual oxidants; and at least annually for iodide (I-), polyaromatic hydrocarbons (PAHs), pesticides, and metals. The culture water was most recently analyzed for pesticides, PAHs, and metals on January 22, 2015 and all water quality measurements cited above met the U.S. EPA criteria for aquatic toxicity test culture/dilution water. The next scheduled facility water analysis is scheduled for January, 2016. Results of these analyses will be reported in pertinent protocol amendments thereafter, and the latest results will be included in the draft and final reports for FEL study BATT01-00388. Any departures from recommended values will be promptly brought to the attention of the Study Sponsor/Monitor.

Sufficient iodine (I-) needs to be available to the larvae through a combination of aqueous and dietary sources for the thyroid gland to synthesize thyroid hormones to support normal metamorphosis. If the I- concentration in the culture water is relatively consistent (coefficient of variance [CV] $\leq$ 20%), measurement of aqueous I- concentrations from the culture water can be measured at least once per year and reported with the study data. Based on previous work (1), the amphibian metamorphosis assay has been demonstrated to work well when test water I- concentrations ranged between 0.5 and 10 µg/L. The culture water at FEL was analyzed most recently on September 17, 2015 and contained 8.8 (±0.2) µg/L I-, which falls within the acceptable range, thus no supplementation will be necessary unless the I- level falls below 0.5 µg/L.

10.3. Test System

The test species will be the South African Clawed Frog (*Xenopus laevis)*, from which NF stage 51 larvae will be used in the metamorphosis assay (3). *Xenopus laevis* is a well-studied laboratory animal that exhibits ease of use in the laboratory, rapid development, and simplicity of observation due to transparency during embryo-larval development (7). In addition, *Xenopus laevis* is routinely cultured in laboratories worldwide and is easily obtainable through commercial suppliers. Reproduction can be easily induced in this species throughout the year using human chorionic gonadotropin (hCG) injections and the resultant larvae can be routinely reared to selected developmental stages in large numbers to permit the use of stage-specific test protocols. It is also the test organism required in the OPPTS 890.1100 test guideline (3).

10.3.1 Origin, Handling, and Feeding

The *X. laevis* larvae used for this study will be obtained from an in-house culture (originally purchased from *Xenopus* I, Dexter, MI) where adults will be injected with human

chorionic gonadotropin (hCG) to induce reproduction. Details for adult frog care and breeding, specific to FEL, but consistent with OPPTS 890.1100 test guideline (3), are found in SOPs 8.2 (8) and 8.3 (9), respectively. Fertilized egg collection will be performed as described in ASTM E1439-98 (7) and OPPTS 890.1100 Amphibian Metamorphosis (Frog) test guideline (3). All tadpoles that are used as test organisms will be derived from the same clutch (spawn). In order to guarantee at least 400 stage 51 acceptable larvae at test setup, clutch sizes of ~1,500 embryos are recommended. In addition, 2-3 clutches will be collected to evaluate the quality of the spawns and determine which produce the highest quality larvae for the initiation of the study. Embryos will be held at 22ºC ± 1º for 4 days to allow for hatching and development to NF stage 45/46, at which time they will be randomly divided into groups of approximately 200 and maintained in tanks containing 50 L of dilution water with a population density of 4 larvae/L at a constant flow rate (50 mL/minute) and water temperature (22º ± 1ºC) until they reach developmental NF stage 51.

Tadpoles will be fed Sera Micron® (Sera GmbH, Heinsberg, Germany) throughout the pre-exposure period (after NF stage 45/46) and during the entire test period of 21 days. Sera Micron®, a commercially available tadpole food that has been shown to support proper growth and development of *X. laevis* tadpoles, is a fine particulate that stays suspended in the water column for a long period of time. Therefore, the total daily amount of food will be divided into smaller portions and fed twice daily, except on weekends. Initially, 300 mg Sera Micron® per tank will be fed twice per day (total = 600 mg / d) for the first 4 days of exposure. During the course of the study, the total daily food ration will be increased according to the ration specified in Table 2. Feeding frequency will be twice per day on Monday through Friday, and once per day at twice the weekday volume on weekends. Sera Micron® will be fed as a stock solution (density of 60 mg/mL dilution water). The Sera Micron® stock solution will be freshly prepared every other day. Any contaminants that can reasonably be expected to be present in the Sera Micron® will not affect the scientific integrity of the study.

Table 2. Sera Micron® Feeding Rate for Tadpoles during In-Life Phase of AMA using Flow-Through Conditions

Study Day	Food Ration (mg Sera Micron®/animal/day)
0 - 4	30
5 - 7	40
8 - 10	50
11 - 14	70
15 - 21	80

10.4. EXPOSURE SYSTEM

The route of exposure will be aqueous, which is the most appropriate method for aquatic organisms and readily water soluble test materials. A flow-through diluter system (Benoit Mini-Diluter; ECT, Superior, WI) will be used in the performance of the amphibian metamorphosis assay exposure. The system will have water-contact components of glass (aquaria), stainless steel (diluter housing and water bath), and Teflon® (tubing responsible for test material delivery). Exposure tanks will be glass aquaria (with approximate measurements of 22.5 x 14.0 x 16.5 cm deep) equipped with standpipes that result in an actual tank volume of 4.0 L and minimum water depth of 10 to 15 cm. Each dilution cell within the headbox of the diluter will be labelled with the color-coded laboratory tape with the test concentration. The replicate splitter cell and aquaria delivery tubing will be labeled with the same color tape as the appropriate dilution cell and the replicate for each test concentration. Each aquarium will be labelled with identical tape color and specifically denote test concentration and replicate. Test system calibration will be checked prior to study start, weekly during the study, and at test termination. The system will be capable of supporting up to 5 exposure concentrations and a control, with up to 4 replicates per treatment. The flow rate to each tank will be 25 mL/min which provides a complete volume replacement every 2.7 h. Fluorescent lighting will be used to provide a photoperiod of 12 hr light and 12 hr dark at an intensity that ranges from 600 to 2,000 lux (lumens/m2) at the water surface. Water temperature will be maintained at 22° ± 1°C, pH maintained between 6.5 to 8.5, and the dissolved oxygen (DO) concentration > 3.5 mg/L (> 40% of the air saturation) in each test tank.

Temperature will be measured daily; and pH, DO, and light intensity (lux) will be measured three times per week. Total hardness and alkalinity will be measured in the control and one replicate of the highest concentration once per week. The solubility, purity, and stability (volatility and degradation rate) of the test substance will be provided to FEL prior to in-life test initiation by the Sponsor. Based on this information, FEL will determine the frequency at which fresh stock solutions will be prepared. Test solution from each replicate tank at each concentration will be sampled for chemical analysis during the equilibration phase, on day 0, once per week during in-life study, and on in-life study day 21 at test termination. Thus, during the in-life study 4 sets of samples will be analyzed. A longer calibration phase will be used in the event of technical problems associated with the system equilibration phase of the study. In addition, stock solutions will be analyzed when they are changed, especially if the duration of the stock solution volume does not encompass the routine sampling. In the case of chemicals which cannot be detected at some or all of the concentrations used in the test, stock solutions will be measured and system flow rates will be recorded and used to calculate nominal concentrations.

10.5. TEST SUBSTANCE ANALYSES - ABC LABORATORIES TEST SITE

Test substance analysis of the diluter test solutions and stock solutions will be performed by ABC Laboratories (Columbia, MO). The analytical measurement method for test substance determined by the chemical laboratory in conjunction with the Study Director and Study Monitor will be performed in accordance with procedures and SOPs in place at ABC Laboratories and in accordance with method validation performed at ABC Laboratories. Following completion of the analytical method validation study (10), the protocol will be amended with specific methodological information pertaining to test substance analysis. Complete details of the

analysis of test substance in stock solutions and test solutions will be included in the study records and final study report.

10.6. EQUIPMENT

The following equipment will be needed:

- Mini-diluter system;
- Glass aquaria;
- Aquarium heaters (adjustable to 22°C ± 1°);
- Thermometer;
- Binocular dissection microscope;
- Digital camera with at least 4 mega pixel resolution and micro function;
- Image digitizing software;
- Petri dish (100 x 15 mm) or transparent plastic chamber of comparable size;
- Analytical balance capable of measuring to 3 decimal places (mg);
- Dissolved oxygen meter;
- pH meter;
- Light intensity meter capable of measuring in lux units;
- Miscellaneous glassware (beakers, volumetric flasks, Erlenmeyer flasks, graduated cylinders, etc.);
- Adjustable pipetters (10 to 5,000 µL) or assorted pipettes of equivalent sizes;
- Top stirrer, and
- Water baths

10.7. TEST ANIMAL SELECTION

When a sufficient number of the pre-exposure population reach developmental stage 51 (14 to 17 d post-hatch), larvae will be transferred to a pooling tank containing dilution water. Larvae requiring >17 d to reach NF stage 51 will not be used in the in-life test. If >50% of the larvae in a given clutch require >17 d to reach NF stage 51, an alternate clutch will be used. All larvae used in the in-life study will be from the same clutch of offspring. Individual larvae will be randomly removed from the pooling tank by scooping with a small strainer. Animals will be carefully handled during this transfer in order to minimize handling stress and to avoid any injury.

The developmental stage of the animals will then be determined by using a binocular dissection microscope. The primary developmental landmark for selecting stage 51 organisms is hind limb morphology (3). The morphological characteristics of the hind limbs will be examined under the microscope. The morphological appearance of the hind limbs at stage 51 differs markedly from the limb morphology at stages 50 and 52, making it possible to correctly distinguish the different stages of the larvae.

Animals that meet the stage criteria will be transferred to a holding tank containing 100% dilution water. The selected larvae will be randomly distributed to exposure treatment tanks (including the control) containing 4.0 L of treatment solution until each tank contains 20 larvae

(5 larvae/L density). Each treatment tank will then be inspected for animals with abnormal appearance (e.g., injuries, abnormal swimming behavior, etc.). Overtly unhealthy looking tadpoles will be removed from the treatment tanks and replaced with larvae newly selected from the holding tank. Treatment tanks will be labeled with the study, treatment, and replicate identification at a minimum. The treatment tanks will be randomly assigned to a position in the exposure system in order to account for possible variations in temperature and light intensity.

10.8. Test Method

The randomly selected NF stage 51 larvae will be exposed to 4 test concentrations and a dilution water control. Each test concentration and control will be evaluated in quadruplicate, with 20 organisms per replicate. Once larvae have been placed in the exposure system, mortality observations will be made daily and any dead larvae will be immediately removed. On d 7, body length (SVL), developmental stage, hind limb length, and wet weight will be determined on larvae randomly selected (5/replicate), euthanized, and preserved for possible histology. The test will be terminated on d 21, at which time all test animals will be staged (NF), measured (cm), weighed (g), and visually observed for dysmorphology. Euthanized larvae will be randomly selected (5/replicate) and preserved for possible histology. Critical test parameters and experimental conditions for the in-life study are presented in Table 3.

10.9. Data Collection and Biological Endpoints

Test data and daily observations will be recorded in the study records. Study records will include study tracking sheets, test information sheets, study calendars identifying major events, study logs for recording detailed observations and comments, daily mortality and developmental stage data sheets, and test termination data sheets. The primary endpoints of the metamorphosis assay will be mortality, developmental stage (NF), hind limb length, snout-vent length (SVL), wet body weight, and thyroid histology. Gross morphology (physical appearance at test takedown) will be a secondary endpoint. During the 21-day exposure phase, determination of selected endpoints will be performed on day 7 and day 21. Table 4 provides an overview of the measurement endpoints and the corresponding observation time points.

10.9.1. Mortality

All test tanks will be checked daily for dead tadpoles and the numbers will be recorded for each tank. Dead animals will be removed from the test tank as soon as observed. Mortality rates exceeding 10% may indicate inappropriate test conditions or toxic effects of the test chemical.

10.9.2. Developmental Stage

The developmental stage of *X. laevis* tadpoles will be determined by using the staging criteria of Nieuwkoop and Faber (5). Developmental stage data will be used to determine if development is accelerated, asynchronous, delayed or unaffected. Acceleration or delay of development is determined by making a comparison between the median stage achieved by the control and treated groups. Asynchronous development will be reported when the tissues examined are not malformed or abnormal, but the relative timing of the morphogenesis or development of different tissues is disrupted within a single tadpole. Developmental stage data will be collected at day 7 and in-life test termination (SD 21).

10.9.3. Hind Limb Length

Hind limb development is typically used qualitatively in the determination of developmental stage but will be used in this study as a quantitative endpoint. All length measurements (millimeters) will be based on digital photographs of the surviving organisms from each treatment. For consistency, hind limb length will be measured on the left hind limb. Hind limb length will be evaluated both at day 7 and at in-life test termination (SD 21).

10.9.4. Body Length (SVL)

SVL will be the first of two endpoints used to assess tadpole growth. SVL (millimeters) will be used to help establish generalized toxicity of the test substance. All length measurements will be based on digital photographs of the surviving organisms from each treatment. SVL will be evaluated both at day 7 and at in-life test termination (SD 21).

10.9.5. Wet Body Weight

Determinations of wet body weight will be used to assess possible effects of test substance on the growth rate of tadpoles relative to the control group. Wet weight measurements will be performed on organisms euthanized for collection of day 7 endpoints and on surviving organisms on in-life study day 21 at test termination.

10.9.6. Thyroid Gland Histology

While developmental stage and hind limb length are important endpoints to evaluate exposure-related changes in metamorphic development, developmental delay cannot, by itself, be considered a diagnostic indicator of anti-thyroidal activity. Some changes can only be observed based on routine histopathological analysis. Details on collection of samples for histopathology are presented later in this protocol.

10.9.7. Additional Observations

All cases of abnormal behavior (e.g. uncoordinated swimming, hyperventilation, atypical quiescence, non-feeding, etc.) and grossly visible malformations will be recorded in the study records and included in the final study report.

10.10. DAY 0 TEST INITIATION AND SAMPLE COLLECTION

On study d 0, healthy and normal looking tadpoles of the stock population will be pooled in a single vessel containing an appropriate volume of dilution water. For developmental stage determination, tadpoles will be individually removed from the pooling tank using a small net or strainer and transferred to a transparent measurement chamber containing dilution water. No anesthesia will be used. Animals will be carefully handled during this transfer to minimize handling stress and to avoid injury. The developmental stage of the animals will determined using a binocular dissection microscope.

Tadpoles that meet the stage criteria described above in this protocol will be held in a tank of clean culture water until the staging process is completed. Once the staging is completed, the larvae are randomly distributed to exposure treatment tanks until each tank contains 20 larvae. Each treatment tank is then inspected for animals with abnormal appearance (e.g.,

injuries, abnormal swimming behavior, etc.). Overtly unhealthy tadpoles will be removed from the treatment tanks and replaced with larvae newly selected from the pooling tank. Five randomly selected stage 51 pre-exposed tadpoles will be humanely euthanized in 150 to 200 mg/L buffered MS-222 and preserved to verify stage upon in-life test setup.

10.11. Day 7 Measurements and Sample Collection

On day 7 of the study, 5 randomly chosen tadpoles per replicate will be removed from each test tank and humanely euthanized in 150 to 200 mg/L MS-222, appropriately buffered with sodium bicarbonate to achieve pH 7. Tadpoles will be rinsed in water and blotted dry, followed by body weight determination to the nearest mg. Hind limb length and SVL, along with developmental stage (using a binocular dissection microscope), will be determined for each tadpole.

10.12. Day 21 Measurements

At test termination (day 21), the remaining tadpoles will be removed from the test tanks and humanely euthanized in 150 to 200 mg/L MS-222, appropriately buffered with sodium bicarbonate to achieve pH 7. Tadpoles will be rinsed in water and blotted dry, followed by body weight determination to the nearest mg. Developmental stage, hind limb length, wet body weight, and SVL will be measured for each tadpole.

All larvae will then be placed in Davidson's fixative for 48 to 72 hours as whole body samples for histological assessments. Larvae will then be rinsed in dechlorinated tap water and preserved in 10% (w/v) neutral buffered formalin (NBF). For histopathology, a total of 5 tadpoles will be sampled from each replicate tank. Since follicular cell height is stage dependent, the most appropriate sampling approach for histological analyses will be to use stage-matched individuals, when possible. Animals selected for histopathology (n=5 from each replicate) will be matched to the median stage of the controls (pooled replicates) whenever possible. If replicate tanks with more than five larvae at the appropriate stage exist, then 5 larvae will be randomly selected. If replicate tanks with fewer than five larvae at the appropriate stage exist, randomly selected individuals from the next lower or upper developmental stage will be sampled to reach a total sample size of five larvae per replicate. The decision to sample additional larvae from either the next lower or upper developmental stage will be made based on an overall evaluation of the stage distribution in the control and chemical treatments. If the test substance induces retardation of development, additional larvae will be sampled from the next lower stage. Alternatively, if the chemical treatment is associated with an acceleration of development, then additional larvae will be sampled from the next upper stage.

If severe alteration of tadpole development associated with test exposure is observed, no overlap of the stage distribution in the chemical treatments with the calculated control median developmental stage may occur. In this case, the selection process will be modified by using a stage different from the control median stage to achieve a stage-matched sampling of larvae for thyroid histopathology. Furthermore, if stages are indeterminate (i.e., asynchrony), then 5 tadpoles from each replicate will be randomly chosen for histological analysis. The rationale underlying sampling of any larvae that are not at a stage equivalent to the control median developmental stage will be reported in the study records and final report.

Table 3. Experimental Conditions for In-Life Study

Test substance		2-ethyhexyl 4-hydroxybenzoate	
Test System (species)		*Xenopus laevis* Larvae	
Initial Larval Stage		NF Stage 51	
Exposure Period		21 d	
Larvae Selection Criteria		Developmental Stage and Optional Total Length	
Test Chemical Concentration (µg/L)		0.0 (control), 3.6, 10.9, 33.0, and 100.0	
Exposure System		Flow-Through Mini-Diluter	
Exposure Route		Abiotic Exposure via Culture Media	
Flow-Rate		25 mL/min	
Primary Endpoints / Determination Days		Mortality	Daily
		Developmental Stage	Days 7 and 21
		Hind Limb Length	Days 7 and 21
		Snout-Vent Length	Days 7 and d 21
		Wet Body Weight	Days 7 and d 21
		Thyroid Histology	Day 21
Additional Observations		Morphology/Behavior	Day 7 and Day 21
Dilution Water / Laboratory Control		Dechlorinated Tap Water (charcoal-filtered)	
Larval Density		20 Larvae / Test Vessel (5 / L)	
Test Solution / Test Vessel		4 L (10-15 cm water height)	
Replication		4 Replicates / Test Concentration and Control	
Acceptable Mortality Rate in Controls		≤10%	
Thyroid Fixation	Number Fixed	5 / Replicate (randomly selected, stage matched)	
	Region	Head	
	Fixation Fluid	Davidson's Fixative	
Feeding	Food	Sera Micron®	
	Frequency / Amount	Twice daily / see Table 2	
Lighting	Photoperiod	12 h Light : 12 h dark	
	Intensity	600 to 2,000 lux (Measured at Water Surface)	
Water Temperature		22° ± 1°C	
pH		6.5 – 8.5	
Dissolved Oxygen (DO) Concentration		>3.5 mg/L (>40% Air Saturation)	
Analytical Chemistry Sample Schedule		Equilibration phase and 4 Events (d 0, d 21, and 2 events between d 0 and d 21)	

Table 4. Observation Time Points for Primary Endpoints

Endpoints:	Daily	Day 7	Day 21
Primary[2]:			
Mortality	●		
Developmental Stage		●	●
Hind Limb Length		●	●
Snout-Vent Length		●	●
Wet Body Weight		●	●
Thyroid Gland Histology			●[3]
Secondary:			
Gross Morphology		●	●

10.13. THYROID HISTOPATHOLOGY - EPL TEST SITE

EPL, under the direction of the Sponsor, will perform the tissue preparation, histology, and histopathological interpretation in accordance with appropriate facility guidance documents (SOPs) and the relevant guidance documents on histopathology for the AMA (3,11). Following the conclusion of exposure (SD 21), FEL will send 5 stage-matched NBF preserved larvae per replicate (20 per treatment or control) to EPL via overnight courier for histopathological processing and analyses.

10.13.1. Histopathological Procedure

Histological evaluation of the thyroid will include, but not be limited to: thyroid gland hypertrophy/atrophy, follicular cell hypertrophy, follicular cell hyperplasia, and as additional qualitative criteria: follicular lumen area, colloid quality and follicular cell height/shape. Severity grading (4 grades) will be reported in accordance with Wolf (11). Overt and significant changes in apical endpoints indicating developmental acceleration or asynchrony may preclude the necessity to perform histopathological analysis of the thyroid glands. However, absence of overt morphological changes or evidence of developmental delay will warrant histological analyses. Complete details of the histopathological analyses will be provided in the study records and final report

10.14. DATA ANALYSIS AND STATISTICS

All data from in-life portions of the study will be tabulated in data entry spreadsheet templates (DEST) by FEL with the exception of histopathological results. Histopathological results will be tabulated in spreadsheets by EPL and provided to FEL. Data including histopathological analyses will then be summarized in the final report prepared by FEL. Statistical analyses of the data will be performed by Battelle and will be consistent with OPPTS 890.1100 test guideline (3), the TO 14 QAPP (1), and generally follow procedures described in

[2] Statistical evaluation will be considered for each of the primary endpoints.
[3] Thyroid tissues taken from a subset of 5 animals per treatment tank, but only 10 animals / concentration will be analyzed initially.

the document Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application (12). For all continuous quantitative endpoints (HLL, SVL, wet weight) that follow a monotonic concentration-response, the Jonckheere-Terpstra test will be applied in step-down manner to establish significant treatment effects. For continuous endpoints that are not consistent with a monotonic concentration-response, the data will be evaluated for normality (Shapiro-Wilk's test) and homogeneity (Levene's test). If a data set is found to have a non-normal distribution or a heterogeneous distribution of variance; a normalizing, variance stabilizing transformation will be used. If data sets are normally distributed with homogeneous variance following transformation, the data set will be evaluated using Dunnett's test. If the data sets are normally distributed with heterogeneous variance following data transformation, the Tamhane-Dunnett, (T3 test) or the Mann-Whitney-Wilcoxon U test will be used to evaluate the data. Where no normalizing transformation can be found, the Mann-Whitney-Wilcoxon U test using a Bonferroni-Holm adjustment to the p-values will be used to evaluate the data sets.

Although significant mortality should not be observed based on the test concentrations selected, a Cochran-Armitage test will be applied if the data set has a consistent concentration-response. Alternatively, non-monotonic mortality data sets will be evaluated using Fisher's Exact test with a Bonferroni-Holm adjustment. A significant treatment effect for developmental stage will be determined on the replicate median values using the Jonckheere-Terpstra or Mann-Whitney U test. In the event median values cannot be determined, replicate mean stage values will be used and evaluated using Dunnett's test. Concentration-response monotonicity will be assessed visually from the replicate and treatment medians or means. The statistical significance of all tests indicated will be assessed at $p = 0.05$.

10.14.1. Use of Compromised Treatment Levels

Several factors will be considered when determining whether a replicate or entire treatment demonstrates overt toxicity and should be removed from analysis. Overt toxicity will be defined as >2 mortalities in any replicate that can only be explained by toxicity rather than technical error. Other signs of overt toxicity include hemorrhage, abnormal behaviors, abnormal swimming patterns, anorexia, and any other clinical signs of disease. For sub-lethal signs of toxicity, qualitative evaluations may be necessary, and will be made in reference to the appropriate control group.

10.14.2. Evaluating Treatments at or above Developmental Stage (NF Stage) 60

After stage 60, tadpoles show a reduction in size and weight due to tissue resorption, re-organization and reduction of absolute water content. Therefore, measurements of wet weight and SVL cannot appropriately be used in statistical analyses for differences in growth rates. In this circumstance, wet weight and length data from organisms >NF stage 60 will be censored from the data sets and cannot be used in analyses of replicate means or replicate medians. Two different approaches will be considered in the analysis of these growth-related parameters.

First, only tadpoles with developmental stages lower or equal to NF stage 60 will be included in the statistical analyses of wet weight and/or SVL. In order to use this approach, ≤20% of the test organisms within a given treatment or the control can be removed from the data set. If an increased number of tadpoles show development beyond stage 60 (≥20%) a given

treatment or control, then a two-factor ANOVA with a nested variance structure will be used on all test organisms to assess growth effects due to chemical treatments while taking into account the effect of late stage development on growth.

10.15. Performance Criteria and Test Validity

General performance criteria are provided in Table 5 (3).

Table 5. General Test Performance Criteria

Criterion	Acceptable Limits
Test concentrations	≤20 % CV of measure test concentration
Control mortality	≤ 10 % in any replicate of the control
Minimum median control developmental stage at test termination	57
Range of control developmental stages	≤ 4 for the 10^{th} and 90^{th} percentile
DO	≥ 40 % of air saturation
pH	6.5 – 8.5
Water temperature	22 ± 1°C with inter-replicate variability ≤ 0.5°C
Test concentrations without overt toxicity (excluding control)	≥ 2
Replicate performance	≤ 2 replicates amongst test can be compromised

The following performance criteria will be generally used to assess test validity:

- Test supporting no thyroid activity exerted by test substance
  - For any given treatment including controls, mortality will be ≤ 10 %. For a given replicate, no more than 3 tadpoles will die; or the replicate is considered compromised.
  - At least 2 test concentrations with all four uncompromised replicates will be available for formal analysis.
  - At least 2 test concentrations without overt toxicity will be available for analysis.
- Test supporting potential thyroid activity
  - Mortality of no more than two tadpoles/replicate in the control will occur.

11. SAMPLE HANDLING AND CUSTODY

All samples received, generated during the course of testing, and submitted to ABC Laboratories (10) and EPL (11) in this study will be accompanied with an appropriately signed chain of custody and handled in accordance with facility SOPs (13,14). Samples will be entered

into a sample check-in logbook and assigned a unique sample tracking number. Each sample will also be properly labeled with its assigned sample tracking number. Sets of test solution samples collected by FEL as described in section 10.5 and preserved as described by ABC Laboratories (10) will be shipped to ABC Laboratories by commercial carrier. Whole body tissue samples collected at the conclusion of the in-life phase will be shipped to EPL via commercial carrier. Samples, when not in use, will be properly preserved and stored, based on sample matrix.

12. REPORTING

FEL will provide progress reports to the Study Monitor throughout the actual study, as required. At study conclusion, a Final Report will be provided to the Sponsor by FEL. The report will include sections on introduction, materials and methods, results, and discussion and conclusions. More specifically, the report will include the following information:

- **Test substance:**
  - Test substance: Will include name and CAS number.
  - Characterization of the test substance: Will include physical-chemical properties; information on stability and biodegradability.
  - Chemical observations and data: Will include method and frequency of preparation of stock solutions, nominal and measured concentrations of the test chemical, and in some cases, non-parent chemical, as appropriate.
- **Test System:**
  - Organism: Will include scientific name, age, supplier, pre-treatment (if used)
- **Test conditions:**
  - Test method: Will include range finding design and definitive test design, delivery process (flow-through), aeration, test system loading
  - Operational parameters records: These parameters will consist of observations pertaining to the functioning of the test system and the supporting environment and infrastructure. Records will include: ambient temperature, test temperature, photoperiod, status of critical components of the exposure system (*e.g.* pumps), flow rates, water levels, stock bottle changes, and feeding records. General water quality parameters will include: pH, DO, conductivity, total iodine, alkalinity, and hardness.
  - Analytical methods: Analytical methods used will be included in the report
  - Deviations from the test method: This information will include any information or narrative descriptions of deviations from the test method.
- **Results:**
  - Range-finding results
  - Biological observations and data: Information will include daily observations of mortality, food consumption, abnormal swimming behavior, lethargy, loss of equilibrium, malformations, lesions, etc.

Observations and data collected at predetermined intervals include: developmental stage, hind limb length, SVL, and wet weight.

- Analytical results: Results of analytical testing and evaluation of analytical results in relation to the relevant test acceptance criteria will be included in the report.
- Statistical analytical techniques and justification of techniques used: Results of the statistical analysis preferably in tabular form, methods for determining whether outliers exist, and justification for not using outliers.
- Histological data: These include narrative descriptions, as well as graded severity and incidence scores of specific observations, as detailed in the histopathology guidance document.
- Ad hoc observations: These observations should include narrative descriptions of the study that do not fit into the previously described categories.

- **Discussion of the results**

Appendices containing raw data, statistical reports, analytical reports, histology reports etc. will be included in the final report. Support laboratories will prepare draft reports summarizing the testing performed at their facilities for review by FEL and the Study Monitor. A draft of the Final Report, including draft reports from the supporting laboratories, will be sent to the Study Monitor for review. After Study Monitor review, the support laboratories will provide copies of their final reports to FEL for inclusion in the Final Report of the study. The Final Report of the study will be sent to the Sponsor after Study Monitor approval of the draft report.

13. STUDY AMENDMENTS AND DEVIATIONS

Permanent changes to the final Study Protocol will require written amendments. Any amendments will be reviewed to determine the potential impact on the study. The amendment will be attached to the Study Protocol and become an active component of the study. Any deviations from the protocol (temporary changes due to unforeseen problems) will be recorded in the study records, dated, and initialed by the Study Director. Deviations will also be addressed in the Final Report of the study.

14. RECORD MAINTENANCE AND ARCHIVAL

Test facility-related records (personnel training, equipment calibration and maintenance, storage temperature records, etc.) (15) will be retained at the Test Facility. No records will be disposed of without the authorization of the Sponsor. The records will be organized and include an index.

Certified exact copies of the original raw data, derived data, QA reports, study guidance documents, correspondence, and draft and final reports will be electronically maintained at the In-life test facility in accordance with facility SOPs (15) until study finalization. All original raw data and the original Final Study Report will be kept in designated file cabinets located in a secured file room at the Test Facility. After final approval of all reports and conclusion of the study, all electronic files will be transferred to compact discs (CDs) and verified as exact copies

of the original. Copies of the electronic disc and the Final Study Report will be sent to the Sponsor. Immediately following finalization of the final report, all original handwritten raw data, original raw data files, the original Final Study Report, protocol and protocol amendments associated with the study will be maintained in the archive at FEL until shipped to the location below archived (15,16). In addition, all EPL-generated histology data records will be shipped to the Sponsor for archiving. Original raw analytical data and original analytical reports from ABC Laboratories will be sent to the Sponsor for archiving. The archive location will be:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2696
Attn: Vincent J. Brown, Ph.D.
614-424-5928
brownv@battelle.org

15. SPECIMENS ARCHIVAL

The preserved test specimens will be labeled and stored at FEL until study finalization in accordance with facility SOPs (17). Following study finalization, specimens remaining at FEL will be shipped to a location designated by the Sponsor or representative, in consultation with the USEPA Task Order Contracting Officer's Representative. Material produced during the histopathological analyses (paraffin blocks and slides) will be stored at EPL until study finalization. After study finalization, these materials will be shipped to a location designated by the sponsor or representative in consultation with the USEPA.

16. TEST SUBSTANCE WASTE DISPOSAL

Disposal of waste material generated by the study will be performed in accordance with those requirements provided in the Material Safety Data Sheets (MSDS) and facility SOPs (18). The test substance will either be returned to the Sponsor or disposed of in accordance with those requirements provided in the Material Safety Data Sheets (MSDS) and facility SOPs.

17. REFERENCES

1. QAPP, Endocrine Disruptor Screening Program (EDSP) Tier 1 and Tier 2 *In Vivo* Testing of Selected Chemicals for Potential Endocrine Effects in Non-Mammals. EPA Contract No. EP-W-11-063 Task Order 14, version 1.2, August 28, 2015.

2. USEPA, Federal Insecticide, Fungicide, Rodenticide Act (FIFRA). Good Laboratory Practice Standards, Title 40 Code of Federal Regulations Part 160 (effective October 16, 1989).

3. OPPTS. 890.1100 Amphibian Metamorphosis (Frog), United States Environmental Protection Agency, Washington DC, EPA 740-C-09-002, October 2009.

4. FEL Quality Assurance Management Plan (QAMP), Fort Environmental Laboratories, Stillwater, OK, July 23, 2010 (current version).

5. Nieuwkoop, P.D. and Faber, J. Normal Tables of *Xenopus laevis* (Daudin). Garland Publishing, London, 1994.

6. SOP 8.14. Test Dose/Concentration Determination and Range-Finding, Fort Environmental Laboratories, 2015.

7. Standard Guide for Conducting the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX). American Society for Testing and Materials (ASTM), E 1439-98, 1998 (Reapproved 2004).

8. SOP 8.2, Adult frog care and maintenance, Fort Environmental Laboratories, 2000.

9. SOP 8.3, *Xenopus* breeding, Fort Environmental Laboratories, 2001.

10. ABC Laboratories. (In Preparation). Validated Analytical Chemistry Test Method for 2-Ethyhexyl 4-Hydroxybenzoate in Aquatic Assays for EPA EDSP. Under subcontract with Battelle for U.S. EPA Prime Contract No. EP-W-11-063, Task Order 14, 2015.

11. Wolf, J. (12 Aug 2015). Draft Procedures for EDSP Studies. EPL (Experimental Pathology Laboratories, Inc.) internal document.

12. OECD. Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. Environmental Health and Safety Publications. Series on Testing and Assessment, No. 54, Paris, France, 2006.

13. SOP 6.1.2, Sample Tracking and Handling, Fort Environmental Laboratories, 2010.

14. SOP 6.4, Receipt, Storage, and Distribution of Test Substances, Fort Environmental Laboratories, 2010.

15. SOP 4.10, Study File Document Collection and Organization, Fort Environmental Laboratories, 2001.

16. SOP 4.11, Maintenance of Study Archives, Fort Environmental Laboratories, 2001.

17. SOP 12.1, Archiving of Test Specimens, Fort Environmental Laboratories, 2003.

18. SOP 6.2, Waste Collection, Storage, and Disposal, Fort Environmental Laboratories, 2001.

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FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-ethylhexyl 4-hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

Amendment Number: 01 | **Document ID Number:** BATT01-3 (00388)

Submitted By: Douglas J. Fort | **Date:** 3/18/2016

Amendment Relating To: BATT01-00388

[X] Protocol **[ ] Study Plan** **[ ] QAPP** **[ ] QAMP** **[ ] SOP**

[ ] Other (describe):

Original Specifications:

Page 11, Section 10.5, Test Substance Analyses - ABC Laboratories Test Site

"Following completion of the analytical method validation study (10), the protocol will be amended with specific methodological information pertaining to test substance analysis."

Changed To:

Add analytical method validation studies (ABC Laboratories). See attached.

Reason for Change: Provide method of chemical analysis.

Approval:

Study Director: _____ Date: 4/4/2016

Sponsor Representative: Vincent J. Brown Date: 4/4/2016

FEL

Analytical Test Method for 2-ethylhexyl 4-hydroxybenzoate (2-EHHB) in Freshwater

Test samples will be analyzed for the concentration of 2-EHHB using liquid chromatography with a mass spectrometer detector (LC-MS/MS) in a multiple reaction monitoring mode (MRM). Analysis was accomplished based on methods developed and validated at ABC Laboratories.

At each sampling point, an appropriate volume of sample (e.g., 5 mL) will be collected and placed into a test culture tube or equivalent container. Samples will be first diluted with 5 mL of Methanol at a 1:1 ratio. Samples will be further diluted, if necessary, with 50:50 MeOH: HPLC-grade Water to produce sample concentrations that fall within the calibration curve. At least two quality control (QC) fortification spikes will be prepared in a similar manner at concentrations that bracket the expected high and low test substance treatment concentrations. The samples will be capped and shaken/vortexed to mix prior to being vialed for analysis by LC-MS/MS. The peak responses of 2-EHHB were integrated and quantitated using Analyst 1.6.2 software by AB SCIEX.

Instrument: UPLC system: Shimadzu Nexera
MS Spectrometer: AB Sciex API-5000 or instrument with similar or greater sensitivity

Column: Phenomenex Kinetex XB-C18 50mm x 2.1mm, 1.7µm

Ionization: TurboIon Spray

Mobile Phase A: 0.01% Formic Acid in Water (aq)

Mobile Phase B: 0.01% Formic Acid in Methanol

Gradient:

Time (min)	A%	B%
0.00	40	60
3.00	10	90
4.00	10	90
4.01	40	60
5.00	40	60

Flow Rate: 0.500 mL/min

Injection Volume: 1 µL

Mass Spec. Scan Mode: MRM

Polarity: Negative

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Compound	MRM transitions		
	Q1 (Da)	Q3 (Da)	Dwell Time (msec)
2-EHHB	249.0	92.0	100
2-EHHB a	249.0	136.0	200

Note: Instrument conditions may be changed to optimize chromatography.

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-ethylhexyl 4-hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

Amendment Number: 02 | **Document ID Number:** BATT01-3 (00388)

Submitted By: Douglas J. Fort | **Date:** 4/29/2016

Amendment Relating To: BATT01-00388

[X] Protocol **[ ] Study Plan** **[ ] QAPP** **[ ] QAMP** **[ ] SOP**

[ ] Other (describe):

Original Specifications: Page 9, Section 10.2, Dilution and Laboratory Control Water

"The culture water was most recently analyzed for pesticides, PAHs, and metals on January 22, 2015 and all water quality measurements cited above met the U.S. EPA criteria for aquatic toxicity test culture/dilution water. The next scheduled facility water analysis is scheduled for January, 2016. Results of these analyses will be reported in pertinent protocol amendments thereafter, and the latest results will be included in the draft and final reports for FEL study BATT01-00388. Any departures from recommended values will be promptly brought to the attention of the Study Sponsor/Monitor.

Sufficient iodine (I^-) needs to be available to the larvae through a combination of aqueous and dietary sources for the thyroid gland to synthesize thyroid hormones to support normal metamorphosis. If the I^- concentration in the culture water is relatively consistent (coefficient of variance [CV] ≤ 20%), measurement of aqueous I^- concentrations from the culture water can be measured at least once per year and reported with the study data. Based on previous work (1), the amphibian metamorphosis assay has been demonstrated to work well when test water I^- concentrations ranged between 0.5 and 10 µg/L. The culture water at FEL was analyzed most recently on September 17, 2015 and contained 8.8 (±0.2) µg/L I^-, which falls within the acceptable range, thus no supplementation will be necessary unless the I- level falls below 0.5 µg/L.

Changed To: Results of dilution and laboratory control water analysis for pesticides, PAHs, and metals are attached. In addition, I- analyses will be performed on dilution/laboratory control water on Study Day (SD) 0 and SD 21.

Reason for Change: Provide results of annual facility water quality analysis. Provide addition I- data during the conduct of study 00388.

Approval:

Study Director: _____ Date: 5/11/2016

Sponsor Representative: _____ Date: 5/11/2016

Page 1 of 2

FEL

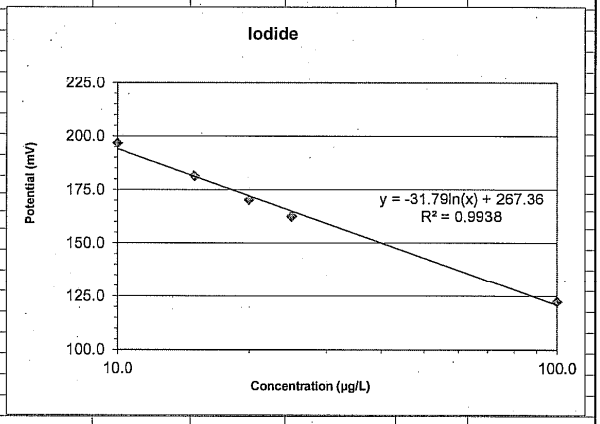
Attachment: Results of Dilution / Laboratory Control Water Analyses

FEL

FEL

FEL01-00000							
Iodide							
Test Article/Protocol:	ISE						
Kit:	ISE Probe						
Date:	2/4/2016						
Standard:	100 µg/L						
Sample Type:	Water						

Concentration (µg/L)	Standards mV Reading
0.0	0.0
10.0	196.8
15.0	181.5
20.0	170.3
25.0	162.5
100.0	122.5



FEL01-00000		Date:	2/4/2016		MDL = 5 µg/L		
Iodide		Standard:	100 µg/L				
Test Article/Protocol:	ISE	Sample Type:	Water				

Treatment	Replicate	Sample ID	df	mV Reading	Calc Conc (µg/L)		Mean (µg/L)
100 µg/L Standard	1	100	1	120.7	100.826		
	2		1	121.0	99.879		
	3		1	120.3	102.103		
	4		1	120.6	101.144		**101.0**
						SEM	**0.091**
Culture Room	1		1	197.5	9.003		
	2		1	196.4	9.320		
	3		1	199.1	8.561		
	4		1	199.3	8.507		**8.8**
						SEM	**0.129**
Exposure Room	1		1	198.5	8.724		
	2		1	199.2	8.534		
	3		1	198.9	8.615		
	4		1	198.6	8.697		**8.6**
						SEM	**0.029**
DI	1		1	644.0	0.000		
Analyst / Date:				2/4/2016			

Red River

Environmental Laboratory and Consulting Company

Analytical Laboratory - Environmental Consulting - Permit Application and Compliance

Certificate of Analysis

ODEQ ID # 9953

To: DOUG FORT
FORT ENVIRONMENTAL LABORATORIES
515 SOUTH DUNCAN STREET
STILLWATER, OK 74074

Project #:
Project Name:

Date Received: 1/19/2016
Report Date: 2/1/2016

Lab Number	Sample Identification	Date Sampled	Analysis Date	Time	Analyzed By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201600236	001 DeCl2 WATER	1/18/2016	1/20/2016	9:30	PB	TPH DRO Extraction		Start				EPA_3510	32401
		1/18/2016	2/1/2016	11:30	MY	TPH-DRO	U	BDL	mg/l	0.1	0.1	OK8000/81	32482
													32437
201600237	002 DeCl2 WATER					TPH-G-W:							32437
		1/18/2016	1/22/2016	17:56	MY	TPH-GRO	U	BDL	mg/l	0.02	0.02	EPA_624	32437
		1/18/2016	1/22/2016		MY	TFT (Surr)		119	%Rec	70	70	EPA_624	32437
		1/18/2016	1/22/2016		MY	BFB (Surr)		102	%Rec	70	70	EPA_624	32437
													32422
201600238	003 DeCl2 WATER	1/18/2016	1/25/2016	9:59	PB/S	Pesticide Extraction		Start				EPA_3510	32422
													32471
201600238	003 DeCl2 WATER					PEST-OC-608:							32471
		1/18/2016	1/28/2016	15:15	MY	Alpha-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Beta-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Gamma-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Delta-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Heptachlor	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Aldrin	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Heptachlor Epoxide	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	g-Chlordane	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Endosulfan I	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	a-Chlordane	U	BDL	ug/l	0.05	0.05	EPA_608	32471

Susie Southwell

Laboratory Authorized Signature

6510 S. Western Ave., Suite 207, Oklahoma City, OK 73139
Phone: (405)-232-1966 or 1-800-USA-KNOW Fax: 405-235-8234 www.RedRiverELCC.com

Red River

Environmental Laboratory and Consulting Company

Analytical Laboratory - Environmental Consulting - Permit Application and Compliance

Certificate of Analysis

ODEQ ID # 9953

To: DOUG FORT
FORT ENVIRONMENTAL LABORATORIES
515 SOUTH DUNCAN STREET
STILLWATER, OK 74074

Project #:
Project Name:

Date Received: 1/19/2016
Report Date: 2/1/2016

Lab Number	Sample Identification	Date Sampled	Analysis Date	Analyzed Time	By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201600238	003 DeCl2 WATER	1/18/2016	1/28/2016		MY	Dieldrin	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	4,4-DDE	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Endrin	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Endosulfan II	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	4,4-DDD	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Endrin Aldehyde	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Endosulfan Sulfate	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	4,4-DDT	U	BDL	ug/l	0.1	0.1	EPA_608	32471
		1/18/2016	1/28/2016		MY	Endrin Ketone	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Methoxyclor	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	Toxaphene	U	BDL	ug/l	0.05	0.05	EPA_608	32471
		1/18/2016	1/28/2016		MY	TCMX (Surr)		49	%Rec	30	30	EPA_608	32471
		1/18/2016	1/28/2016		MY	1,2-dcbp (Surr)		71	%Rec	65	65	EPA_608	32471
													32461
201600239	004 DeCl2 WATER	1/18/2016	1/27/2016	13:10	JL	Arsenic	U	BDL	mg/l	0.01	0.01	200.7/6010	32461
		1/18/2016	1/27/2016	13:10	JL	Cadmium	U	BDL	mg/l	0.001	0.001	200.7/6010	32463
		1/18/2016	1/27/2016	13:10	JL	Chromium	U	BDL	mg/l	0.002	0.002	200.7/6010	32459
		1/18/2016	1/27/2016	13:10	JL	Copper	U	BDL	mg/l	0.001	0.001	200.7/6010	32455
		1/18/2016	1/27/2016	13:10	JL	Iron	U	BDL	mg/l	0.009	0.009	200.7/6010	32458
		1/18/2016	1/27/2016	13:10	JL	Lead	U	BDL	mg/l	0.005	0.005	200.7/6010	32460
		1/18/2016	1/27/2016	14:37	SV	Mercury	U	BDL	mg/l	.0002	0.0002	EPA_245.2	32466
		1/18/2016	1/27/2016	13:10	JL	Nickel	U	BDL	mg/l	0.006	0.006	200.7/6010	32457

Susie Southwell

Laboratory Authorized Signature

6510 S. Western Ave., Suite 207, Oklahoma City, OK 73139
Phone: (405)-232-1966 or 1-800-USA-KNOW Fax: 405-235-8234 www.RedRiverELCC.com

Red River

Environmental Laboratory and Consulting Company

Analytical Laboratory - Environmental Consulting - Permit Application and Compliance

Certificate of Analysis

ODEQ ID # 9953

To: DOUG FORT
FORT ENVIRONMENTAL LABORATORIES
515 SOUTH DUNCAN STREET
STILLWATER, OK 74074

Project #:
Project Name:

Date Received: 1/19/2016
Report Date: 2/1/2016

Lab Number	Sample Identification	Date Sampled	Analysis Date	Analyzed Time	By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201600239	004 DeCl2 WATER	1/18/2016	1/27/2016	13:10	JL	Zinc	U	BDL	mg/l	0.002	0.002	200.7/6010	32456
201600240	005 DeCl2 WATER 1 OF 2	1/18/2016	1/19/2016	15:28	PB	Semi-Volatile Extraction		Start				EPA_3510	32389
													32415
201600240	005 DeCl2 WATER 1 OF 2					PAH-625-W:							32415
		1/18/2016	1/21/2016	19:12	MY	Naphthalene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	2-Methylnaphthalene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	2-Chloronaphthalene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Acenaphthylene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Acenaphthene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Fluorene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Phenanthrene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Anthracene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Fluoranthene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Pyrene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Benzo(a)anthracene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Chrysene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Benzo(b)fluoranthene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Benzo(k)fluoranthene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Benzo(a)pyrene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Indeno(1,2,3-Cd)pyrene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	Dibenz(a,h)anthracene	U	BDL	ug/l	5	5	EPA_625	32415

Susie Southwell

Laboratory Authorized Signature

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Red River

Environmental Laboratory and Consulting Company

Analytical Laboratory - Environmental Consulting - Permit Application and Compliance

Certificate of Analysis

ODEQ ID # 9953

To: DOUG FORT
FORT ENVIRONMENTAL LABORATORIES
515 SOUTH DUNCAN STREET
STILLWATER, OK 74074

Project #:
Project Name:

Date Received: 1/19/2016
Report Date: 2/1/2016

Lab Number	Sample Identification	Date Sampled	Analysis Date	Time	Analyzed By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201600240	005 DeCl2 WATER 1 OF 2	1/18/2016	1/21/2016		MY	Benzo(g,h,i)perylene	U	BDL	ug/l	5	5	EPA_625	32415
		1/18/2016	1/21/2016		MY	NB-d5 (Surr)		69	%Rec	25	25	EPA_625	32415
		1/18/2016	1/21/2016		MY	2-FBP (Surr)		61	%Rec	12	12	EPA_625	32415
		1/18/2016	1/21/2016		MY	2,4,6-TBP (Surr)		107	%Rec	14	14	EPA_625	32415

Note:

RL = Reporting Limit. SQL* = Sample Quantitation Level.
B = Analyte was detected in both the sample and associated blank.
OL2 = Subcontracted to ODEQ Lab #7211.
M = Matrix effect present
BDL = Analyte was analyzed for but not detected above RL.
J = Analyte was detected above the RL but below the PQL.
Q = Surrogate recovery fell outside acceptance limits.
U = Analyte was analyed for but not detected above RL

* When a sample contains a high concentration of either a target or non-target compound(s) or interference, it must be diluted. SQL = Dilution factor x MDL. Samples are disposed of 20 days after the sample is reported.

Susie Southwell

Laboratory Authorized Signature

6510 S. Western Ave., Suite 207, Oklahoma City, OK 73139
Phone: (405)-232-1966 or 1-800-USA-KNOW Fax: 405-235-8234 www.RedRiverELCC.com

FEL

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DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethyhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*	
Amendment Number: 03	**Document ID Number**: BATT01-3 (00388)
Submitted By: Douglas J. Fort	**Date**: 6/6/2016
Amendment Relating To: BATT01-00388 [X] Protocol [ ] Study Plan [ ] QAPP [ ] QAMP [ ] SOP [ ] Other (describe):	
Original Specifications: Protocol Page 1, Title Page and throughout document, 2-Ethyhexyl 4-Hydroxybenzoate	
Changed To: 2-Ethylhexyl 4-Hydroxybenzoate	
Reason for Change: Corrected typographical error in compound name.	

Approval:

Study Director: _____ Date: 6/6/2016

Sponsor Representative: _____ Date: 6 JULY 2016

Page 1 of 1

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethyhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

Amendment Number: 04 | **Document ID Number**: BATT01-3 (00388)

Submitted By: Douglas J. Fort | **Date**: 1/11/2017

Amendment Relating To: BATT01-00388

[X] Protocol [ ] Study Plan [ ] QAPP [ ] QAMP [ ] SOP

[ ] Other (describe):

Original Specifications:

1. Page 5, Section 2, Good Laboratory Practice

2. Page 22, Section 15, Specimen Archival

"Following study finalization, specimens remaining at FEL will be shipped to a location designated by the Sponsor or representative, in consultation with the USEPA Task Order Contracting Officer's Representative. Material produced during the histopathological analyses (paraffin blocks and slides) will be stored at EPL until study finalization. After study finalization, these materials will be shipped to a location designated by the sponsor or representative in consultation with the USEPA."

Changed To:

1. Add the following to the GLP exception list:
   - Wet specimens and tissues imbedded in paraffin blocks remaining after study finalization will be destroyed at their respective labs rather than submitted to archiving.

2. Per Sponsor mandated exception, following study finalization, specimens remaining at FEL, and embedded tissues or specimens maintained by EPL will be disposed of in accordance with QMP, QAPP, and respective facility SOPs. All slides produced during the histopathological analyses will be stored at EPL until study finalization. After study finalization, all slides will be shipped to the sponsor at the address below.

   Sharlene R. Matten, Ph.D.
   Senior Biologist, US EPA
   Exposure Assessment Coordination and Policy Division
   Office of Science Coordination and Policy
   1200 Pennsylvania Ave., N.W., Mail Code 7203M
   Washington, D.C. 20460
   Tel: 202-564-0130

   e-mail: matten.sharlene@epa.gov

FEL

Reason for Change: Provides specific archive location for slides produced for study BATT01-00388 and disposal instructions following study finalization for specimens remaining at FEL, and embedded tissues or specimens maintained at EPL.

Approval:

Study Director: _____ Date: 1/23/2017

Sponsor Representative: _____ Date: 11 JAN 2017

FEL

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethyhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

Amendment Number: 05 | **Document ID Number:** BATT01-3 (00388)

Submitted By: Douglas J. Fort | **Date:** 2/7/2017

Amendment Relating To: BATT01-00388

[X] Protocol **[ ] Study Plan** **[ ] QAPP** **[ ] QAMP** **[ ] SOP**

[ ] Other (describe):

Original Specifications:

1. Page 18-19, Section 10.14. Data Analysis and Statistics

"... If the data sets are normally distributed with heterogeneous variance following data transformation, the Tamhane-Dunnett, (T3 test) or the Mann-Whitney-Wilcoxon U test will be used to evaluate the data. Where no normalizing transformation can be found, the Mann-Whitney-Wilcoxon U test using a Bonferroni-Holm adjustment to the p-values will be used to evaluate the data sets."

Changed To:

1. "... If the data sets are normally distributed with heterogeneous variance following data transformation, the Tamhane-Dunnett, (T3 test) or the Mann-Whitney-Wilcoxon U test with a Bonferroni-Holm adjustment to the p-values will be used to evaluate the data. Where no normalizing transformation can be found, the Mann-Whitney-Wilcoxon U test using a Bonferroni-Holm adjustment to the p-values will be used to evaluate the data sets."

Reason for Change:

Rectifies inconsistency between OPPTS 890:1100 (3) which does not recommend a Mann-Whitney-Wilcoxon U test with a Bonferroni-Holm adjustment to the p-values and OECD Series on Testing and Assessment, No. 54 (12) which recommends the use of the Bonferroni-Holm adjustment to the p-values. Based on guidance from statisticians at Battelle Memorial Institute, this change in analysis is recommended.

Approval:

Study Director: _____ Date: 2/7/2017

Sponsor Representative: _____ Date: 7 FEB 2017

Page 1 of 1

FEL

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethyhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

Amendment Number: 06 | **Document ID Number**: BATT01-3 (00388)

Submitted By: Douglas J. Fort | **Date**: 2/16/2017

Amendment Relating To: BATT01-00388

[X] Protocol **[ ] Study Plan** **[ ] QAPP** **[ ] QAMP** **[ ] SOP**

[ ] Other (describe):

Original Specifications:

1. Page 17, Section 10.13. Thyroid Histopathology, EPL Test Site.
   "EPL, under direction of the sponsor, will perform the tissue preparation, histology, and histopathological interpretation in accordance with appropriate facility guidance documents (SOPs) and the relevant guidance documents on histopathology for the AMA (3,11)."

2. Page 17, Section 10.13.1, Histopathological Procedure

   "Severity grading (4 grades) will be reported in accordance with Wolf (11)."

3. Page 19, Section 11., Sample Handling and Custody

   "All samples received, generated during the course of testing, and submitted to ABC Laboratories (10) and EPL (11) in this study will be accompanied with an appropriately signed chain of custody and handled in accordance with facility SOPs (13,14)."

4. Page 23, Section 17. References

   "11. Wolf, J. (12 Aug 2015). Draft Procedures for EDSP Studies. EPL (Experimental Pathology Laboratories, Inc.) internal document."

Changed To:

1. EPL, under direction of the sponsor, will perform the tissue preparation and histology in accordance with appropriate facility guidance documents (SOPs) and the relevant guidance documents on histology for the AMA (3,19). In accordance with USEPA and OECD guidelines (3, 11), the paraffin blocks will not be sealed as per Wolf, 2015 (19)."

2. "Severity grading will be reported in accordance with USEPA and OECD guidelines (3,11)."

3. "All samples received, generated during the course of testing, and submitted to EAG Laboratories (Columbia, MO) and EPL in this study will be accompanied with an appropriately signed chain of custody and handled in accordance with facility SOPs."

4.	11. OECD (2007). Guidance Document on Amphibian Thyroid Histology. Environmental Health and Safety Publications. Series on Testing and Assessment. No. 82. Paris, France.
5.	19. Wolf, J. (12 Aug 2015). Draft Procedures for EDSP Studies. EPL (Experimental Pathology Laboratories, Inc.) internal document.

Reason for Change:

Replaced Wolf, J. (12 Aug 2015) with OECD (2007) as reference 11. These changes provide consistent specifications for severity grading processes consistent with standard AMA guidance (3,11).

Approval:

Study Director: _____ Date: 2/16/2017

Sponsor Representative: Vincent J. Brown (388) Date: 16 FEB 2017

BATT01-00388
Protocol Amendment 07

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

Amendment Number: 07 | **Document ID Number**: BATT01-3 (00388)

Submitted By: Douglas J. Fort | **Date**: 8/9/2017

Amendment Relating To: BATT01-00388

[X] Protocol **[ ] Study Plan** **[ ] QAPP** **[ ] QAMP** **[ ] SOP**

[ ] Other (describe):

Original Specifications:

1. Page 1, Title Page

PI Support Site: (Analytical Chemistry)
ABC Laboratories
7200 East ABC Lane
Columbia, MO 65202

2. Page 6, Section 7, Testing Sites

The 2-EHHB chemical analysis portion of the study will be performed at ABC Laboratories, Inc., Chemical Services Department, 7200 East ABC Lane, Columbia, Missouri, USA 65202. Dr. Tom Leak, PI of the planned analyses, will serve as study contact for ABC Laboratories and may be reached at 573.777.6050 or leakt@abclabs.com.

3. Remainder of protocol

ABC Laboratories

Changed To:

1. Page 1, Title Page

Analytical Bio-Chemistry Laboratories, Inc.
a wholly owned subsidiary of EAG, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202

2. Page 6, Section 7, Testing Sites

The 2-EHHB chemical analysis portion of the study will be performed at EAG Laboratories, Inc., Chemical Services Department, 7200 East ABC Lane, Columbia, Missouri, USA 65202. Dr. Tom Leak, PI of the planned analyses, will serve as study contact for EAG Laboratories and may be reached at 573.777.6050 or tleak@eag.com.

BATT01-00388

FEL

Protocol Amendment 07

4. Remainder of protocol

EAG Laboratories

Reason for Change:

Acquisition of ABC Laboratories by EAG, Inc. resulting in change in name of PI laboratory and PI email address.

Approval:

Study Director: _____ Date: 8/10/2017

Sponsor Representative: _____ Date: 10 AUG 2017

BATT01-00388
Protocol Amendment 08

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

Amendment Number: 08 | **Document ID Number**: BATT01-3 (00388)

Submitted By: Douglas J. Fort | **Date**: 9/1/2017

Amendment Relating To: BATT01-00388

[X] Protocol **[ ] Study Plan** **[ ] QAPP** **[ ] QAMP** **[ ] SOP**

[ ] Other (describe):

Original Specifications:

1. Page 1, Title Page

Sponsor: U.S. Environmental Protection Agency
1200 Pennsylvania Ave., NW
Washington DC 20460C

2. Amendment 7 Changed To: Page 6, Section 7, Testing Sites

The 2-EHHB chemical analysis portion of the study will be performed at EAG Laboratories, Inc., Chemical Services Department, 7200 East ABC Lane, Columbia, Missouri, USA 65202. Dr. Tom Leak, PI of the planned analyses, will serve as study contact for EAG Laboratories and may be reached at 573.777.6050 or tleak@eag.com.

Changed To:

1. Page 1, Title Page

Sponsor: U.S. Environmental Protection Agency
1200 Pennsylvania Ave., NW
Washington DC 20460

2. Amendment 7 Changed To: Page 6, Section 7, Testing Sites

The 2-EHHB chemical analysis portion of the study will be performed at EAG Laboratories, Chemical Services Department, 7200 East ABC Lane, Columbia, Missouri, USA 65202. Dr. Tom Leak, PI of the planned analyses, will serve as study contact for EAG Laboratories and may be reached at 573.777.6050 or tleak@eag.com.

BATT01-00388 FEL

Protocol Amendment 08

Reason for Change:

Correct typographical errors in sponsor zip code and in EAG company name.

Approval:

Study Director: _____ Date: 11/27/2017

Sponsor Representative: _____ Date: Oct 10, 2017

BATT01-00388
Protocol Amendment 09

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

Amendment Number: 09 | **Document ID Number:** BATT01-3 (00388)

Submitted By: Douglas J. Fort | **Date:** 2/12/2018

Amendment Relating To: BATT01-00388

[X] Protocol **[ ] Study Plan** **[ ] QAPP** **[ ] QAMP** **[ ] SOP**

[ ] Other (describe):

Original Specifications:

1. Page 7, Section 10.1 Test Substance

2-EHHB (TCI America, Portland, OR, lot number H0506, expiration date and re-test date not provided, 98.0% (w/w) pure [w/w] per Certificate of Analysis produced by TCI America) was received from TCI America.

Changed To:

1. Page 7, Section 10.1 Test Substance

2-EHHB (TCI America, Portland, OR, lot number 7CZZO, expiration date and re-test date not provided, 99.3% (w/w) pure [w/w] per Certificate of Analysis produced by TCI America) was received from TCI America.

Reason for Change:

Correct error in the lot number and purity of test substance.

Approval:

Study Director: _____ Date: 2/13/2018

Sponsor Representative: Vincent J. Brown Date: 13 FEB 2018

Page 1 of 1

FEL

FEL

DOCUMENT DEVIATION FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*	
Deviation Number: 01	**Document ID Number**: BATT01-3
Submitted By: Douglas J. Fort	**Date**: 1/11/2017
Deviation Relating To: BATT01-00388 **[X] Protocol [ ] Study Plan [ ] QAPP [ ] QMP [ ] SOP** **[ ] Other (describe):**	
Original Specifications: Page 17, Table 4, footnote 3 "Thyroid tissues taken from a subset of 5 animals per treatment tank, but only 10 animals / concentration will be analyzed initially."	
Deviation: – EPL Test Site All 20 animals / concentration were analyzed.	
Reason/Impact: It was always intended that all 20 animals per concentration be analyzed for thyroid histopathology. This deviation will have no impact on the study.	
Schedule for Completion of Corrective Action: Immediate.	

Approval:

Study Director: _____ Date: 1/12/2017

Sponsor: _____ Date: 12 JAN 2017

FEL

DOCUMENT DEVIATION FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

Deviation Number: 02 | **Document ID Number**: BATT01-3

Submitted By: Douglas J. Fort | **Date**: 2/17/2017

Deviation Relating To: BATT01-00388

[X] Protocol [ ] Study Plan [ ] QAPP [ ] QMP [ ] SOP

[ ] Other (describe):

Original Specifications:

1. Page 18-19, Section 10.14. Data Analysis and Statistics and Protocol BATT01-3 Amendment 5

   "… If the data sets are normally distributed with heterogeneous variance following data transformation, the Tamhane-Dunnett, (T3 test) or the Mann-Whitney-Wilcoxon U test will be used to evaluate the data. Where no normalizing transformation can be found, the Mann-Whitney-Wilcoxon U test using a Bonferroni-Holm adjustment to the p-values will be used to evaluate the data sets."

2. Page 22-23, Section 17. References and Protocol BATT01-3 Amendment 6

   19. Wolf, J. (12 Aug 2015). Draft Procedures for EDSP Studies. EPL (Experimental Pathology Laboratories, Inc.) internal document.

Deviation:

1. "… If the data sets are normally distributed with heterogeneous variance following data transformation, the Tamhane-Dunnett, (T3 test) or the Mann-Whitney-Wilcoxon U test with a Bonferroni-Holm adjustment to the p-values will be used to evaluate the data. Where no normalizing transformation can be found, the Mann-Whitney-Wilcoxon U test using a Bonferroni-Holm adjustment to the p-values will be used to evaluate the data sets. A test termed RSCABS (Rao-Scott Cochran Armitage by Slices) that uses a step-down Rao-Scott adjusted Cochran-Armitage trend test on each level of severity in a histopathology response will be used to evaluate histopathology data (20)."

2. Add 20. Green J.W., Springer T.A., Saulnier A.N., Swintek J. 2014. Statistical analysis of histopathology endpoints. Environmental Toxicology and Chemistry. 33(5):1108-1116.

Reason/Impact:

Adds RSCABS statistical analyses of histopathology data and appropriate reference for analysis. Provides a more current approach to evaluating histopathology results. No impact on study anticipated.

Schedule for Completion of Corrective Action: Immediate.

FEL

FEL

Approval:

Study Director: [signature] Date: 2/20/2017

Sponsor: [signature] Date: 20 FEB 2017

(388)

Appendix B

CERTIFICATE OF ANALYSIS

Certificate of Analysis

Oct 16, 2015 (JST)

TOKYO CHEMICAL INDUSTRY CO., LTD.
4-10-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023 Japan

Chemical Name: 2-Ethylhexyl 4-Hydroxybenzoate		
Product Number: H0506 CAS: 5153-25-3	Lot: 7CZZO	

Tests	Results	Specifications
Purity(HPLC)	99.3 area%	min. 98.0 area%
Purity(Neutralization titration)	99.8 %	min. 98.0 %
Specific gravity (20/20)	1.0382	1.0360 to 1.0390
Refractive index n20/D	1.5210	1.5190 to 1.5220

TCI Lot numbers are 4-5 characters in length.
Characters listed after the first 4-5 characters are control numbers for internal purpose only.

Customer service:

TCI AMERICA
Tel: +1-800-423-8616 / +1-503-283-1681
Fax: +1-888-520-1075 / +1-503-283-1987
E-mail: Sales-US@TCIchemicals.com

Appendix C

EAG LABORATORIES (COLUMBIA, MO) ANALYTICAL REPORT

ANALYTICAL DATA REPORT
Revision No. 1

Study Title

21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

EAG Study Number: 83231
FEL Protocol Number: BATT01-3
FEL Study Number: BATT01-00388

Regulations

USEPA, FIFRA
Good Laboratory Practice (GLP) Standards, Final Rule (40 CFR Part 160, 1989)
and US EPA OPPTS 890.1100, Amphibian Metamorphosis Assay

In-Life Testing Facility

Fort Environmental Laboratories, Inc.
515 South Duncan Street
Stillwater, Oklahoma 74074

Analytical Testing Facility

EAG LABORATORIES™

Analytical Bio-Chemistry Laboratories, Inc.
a wholly owned subsidiary of EAG, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202

Sponsor

U.S. Environmental Protection Agency
1200 Pennsylvania Ave., NW
Washington, DC 20460

FEL Protocol Number: BATT01-3 Revision No. 1 FEL Study Number: BATT01-00388

STATEMENT OF GLP COMPLIANCE

Compound: 2-Ethylhexyl 4-Hydroxybenzoate

Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

The study described in this report, with the following exceptions, was conducted in compliance with the following Good Laboratory Practice Standards:

EPA, FIFRA, Good Laboratory Practice (GLP) Regulations as set forth in Title 40, Part 160 of the Code of Federal Regulations of the United States of America

1) The test substance was not characterized in accordance with the stated Good Laboratory Practices.
2) A method validation and stability test for 2-Ethylhexyl 4-Hydroxybenzoate were performed before the protocol was signed. There was no impact on the study due to this, because the method validation and stability studies were routine laboratory procedures that did not need to be strictly defined, and because the protocol does not address this work.

These were the only exceptions to the stated GLP principles and did not adversely affect the study integrity or the interpretation of the results generated from this study.

The original raw data and the study plan were provided to Battelle Memorial Institute with the final report. Copies of all data in support of this report were retained at EAG along with facility records and a copy of the final report and the study plan.

17 May 18
Tom Leak, Ph.D. Date
Principal Investigator
Analytical Bio-Chemistry Laboratories, Inc.

17 May 18
John Aufderheide Date
Director, Operations
Analytical Bio-Chemistry Laboratories, Inc.

QUALITY ASSURANCE STATEMENT

EAG's Quality Assurance Unit (QAU) reviewed Study No. 83231 entitled, "21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*", for U.S. Environmental Protection Agency/Battelle Memorial Institute. The following inspections/audits were conducted on this study:

Date of Study Based Inspection	Phase Inspected	Date Reported to the Principal Investigator / Study Director	Date Reported to Principal Investigator Management / Study Director Management
13 - 15 July 2016	Raw Data and Draft Report	15 July 2016 / 16 March 2018	01 September 2016 / 16 March 2018
09 March 2018	Final Analytical Phase Report	12 March 2018 / 16 March 2018	16 March 2018 / 16 March 2018
16 May 2018	Analytical Phase Report Revision No. 1	17 May 2018 / 17 May 2018	17 May 2018 / 17 May 2018

These audits indicate that the report is an accurate reflection of the study as it was conducted by EAG.

Jeri Hofen — 17May2018

Jeri Hofen
Manager, Quality Assurance
Analytical Bio-Chemistry Laboratories, Inc.

Date

APPROVAL

The following is to be signed by appropriate personnel:

Principal Investigator:

Name (signed): _____ Date: 17May18
Name (typed): Tom Leak, Ph.D.
Principal Scientist
Analytical Bio-Chemistry Laboratories, Inc.

Management:

Name (signed): _____ Date: 17 May 18
Name (typed): John Auderheide
Director, Operations
Analytical Bio-Chemistry Laboratories, Inc.

STUDY PERSONNEL

Tom Leak, Ph.D.	Principal Scientist/ Principal Investigator
Lindsey Anderson	Assistant Scientist I
Wesley Fain	Senior Scientist
Gerald Nothdurft	Principal Technician
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REASON FOR REPORT REVISION

Report Revision No. 1

This report has been revised as follows:

- Spelling of the test substance corrected from "ethyhexyl" to ethylhexyl" throughout the report.

1.0 INTRODUCTION

All amphibian metamorphosis assay (AMA) diluter and stock solution samples obtained from Fort Environmental Labs, Stillwater, Oklahoma (FEL) during this study (FEL Protocol No. BATT01-3) were analyzed for concentrations of 2-ethylhexyl 4-hydroxybenzoate at EAG. Samples were analyzed using a validated LC-MS/MS method covering the concentration range of 2.00 to 100 ng/mL.

A summary of the analytical evaluation and method used for 2-ethylhexyl 4-hydroxybenzoate is provided in this report. The precision and accuracy of the method are described, based on the method validation data as well as the data for the quality control samples analyzed with the study samples. The concentration of 2-ethylhexyl 4-hydroxybenzoate from each sample is also summarized.

2.0 MATERIALS AND METHOD

2.1 Test Substance

The test substance, 2-Ethylhexyl 4-Hydroxybenzoate (EHHB), was received from FEL on 29 October 2015 and given EAG Reference No. MM-13551-00001. A copy of the chain-of-custody form accompanied the test substance. The certificate of analysis is shown in Appendix 1. The certificate of analysis is summarized below:

Name:	2-Ethylhexyl 4-Hydroxybenzoate
Lot No.:	7CZZO
Purity (%):	99.3
Storage:	Stored at Room Temperature
CAS No.:	5153-25-3
Do Not Use Beyond Date:	29 October 2016

2.2 Analytical Method

Twenty milliliters of methanol were added to each sample (20 mL) at FEL. Further dilutions were made, if necessary, at EAG using 50:50 methanol:water. Sample analysis was performed using a liquid chromatography system with tandem mass spectrometry (LC-MS/MS) in accordance with procedures and SOPs in place at EAG and in accordance with method validation performed at EAG. Complete details of the method used and analysis of test substance in samples submitted from the study are provided in sections 2.3 and 3 below.

2.3 Study Samples

2.3.1 Sample Source, Storage, and Receipt Dates

A total of 215 study samples were received from FEL from 15 January 2016 to 03 March 2016, prepared for analysis, stored refrigerated and typically analyzed within 24 hours of preparation. Table 1 lists sample collection dates, sample processing dates, and analysis dates.

2.3.2 Sample Preparation and Analysis

All diluter and stock solution samples were processed and analyzed following the methods described in Section 2.2 and below. All method validation and stability samples were also analyzed in this manner.

The following instrument and instrument parameters were used in the analyses of the AMA study water samples for EHHB:

Liquid Chromatograph:	Shimadzu Nexara X2 UHPLC
Detector:	ABSciex API-6500 Q-Trap Mass Spectrometer
Column:	Phenomenex Kinetex XB-C18 50mm × 2.1mm, 1.7µm
Mobile Phase A:	0.01% Formic Acid (aq)
Mobile Phase B:	0.01% Formic Acid in Methanol
Gradient:	

Time (min)	%A	%B
0.00	40	60
3.00	10	90
4.00	10	90
4.01	40	60
5.00	40	60

Flow Rate:	0.500 mL/minute
Injection Volume:	1 - 5 µL
Column Temp:	40 °C
Mass Spec. Scan Type:	MRM
Polarity:	Negative
Curtain Gas (N_2):	40 psi
Collision Gas (N_2):	Medium
Source Temperature:	500 °C
Gas 1:	60 psi
Gas 2:	40 psi
Ion Spray Voltage:	-4,500 V
Declustering Potential:	-80 V
Entrance Potential:	-10 V
Collision Exit Potential:	-10 V

	Ions			
Compound	Q1 Mass (Da)	Q3 Mass (Da)	Dwell Time (msec)	Collision Energy (V)
2-Ethylhexyl 4-Hydroxybenzoate	249.00	92.00	100	-28
2-Ethylhexyl 4-Hydroxybenzoate a	249.00	136.00	100	-34

2.4 Calculations

Calculation of 2-ethylhexyl 4-hydroxybenzoate concentrations in test samples was performed by the external standard analysis function of Analyst 1.6.2 software. The concentration 2-ethylhexyl 4-hydroxybenzoate from each sample was determined directly from the standard curve by the equation (note, by definition, ng/mL = µg/L, corrected for purity):

$$\frac{\begin{pmatrix}\text{ng/mL from}\\ \text{standard curve}\end{pmatrix}\begin{pmatrix}\text{analysis volume}\\ \text{in mL}\end{pmatrix}}{\text{sample volume in mL}} = \text{ng/mL}$$

Example calculation for low spike at study initiation (#15, ID 83231-15):

The standard curve equation is of the form: $y = mx + b$

where:

y = peak area units
m = slope
x = ng/mL 2-ethylhexyl 4-hydroxybenzoate
b = y-intercept of the standard curve

Standard Curve: y = 39,448.98x + 1,660.14
Sample Peak Area: = 44,086

Substituting the sample response in peak area units into the following equation and solving for x gave the concentration of 2-ethylhexyl 4-hydroxybenzoate:

x = (44,086 – 1,660.14) / (39,448.98)
x = 1.07547 ng/mL

The concentration value (x), determined from a standard curve using the linear regression function of an Excel spreadsheet, was then multiplied by the analysis volume (10 mL) and then divided by the sample volume (5 mL), resulting in a measured concentration of 2.15 ng/mL, equivalent to 2.15 µg/L.

Recovery of low spike sample at initiation:

$$\frac{2.15\ \mu g/L}{2.00\ \mu g/L} \times 100 = 108\%$$

The minimum quantifiable limit (MQL) for 2-ethylhexyl 4-hydroxybenzoate was calculated as follows:

$$MQL = \frac{(\text{Lowest standard concentration}) \times (\text{Volume for analysis})}{\text{Volume of sample}}$$

Example calculation for minimum quantifiable limit (MQL) for study initiation chromatographic run:

Lowest standard concentration: 0.104 ng/mL
Volume for analysis: 10 mL
Volume of sample: 5 mL

Therefore, for study initiation chromatographic run:

$$MQL = \frac{(0.104\ ng/mL) \times (10\ mL)}{5\ mL} = 0.208\ ng/mL$$

3.0 RESULTS AND DISCUSSIONS

Analytical sets were typically run with seven calibration standards interspersed throughout the run. The acceptance criterion for a reportable analytical set was a correlation coefficient of greater than or equal to 0.995. Each set also included QC fortifications at each of two nominal concentrations, which bracketed the nominal treatment concentrations from the protocol. Blank water and methanol injections (provided along with AMA study water samples) were analyzed to assess matrix interference. No significant interference peaks were observed in the blank samples. A summary of the data runs is included in Table 1.

3.1 Calibration Curves

The calibration standards for sample analysis were prepared on 17 December 2015 and 02 March 2016. The concentration range for 2-ethylhexyl 4-hydroxybenzoate was 0.104 to 52.0 ng/mL in 50:50 methanol:water.

A representative calibration curve is provided in Figure 1.

3.2 Method Validation

A method validation for the recovery 2-ethylhexyl 4-hydroxybenzoate in freshwater was performed on 04 December 2015. Nine 5-mL volumes of freshwater were collected in culture tubes. Three samples (low spikes) were fortified with 0.250 mL of a 40.0 ng/mL solution for a

nominal concentration of 2.00 ng/mL. Three samples (high spikes) were fortified with 0.250 mL of a 2.00 mg/mL solution for a nominal concentration of 100 ng/mL. Three samples consisted of freshwater only (matrix blank). The method validation samples were processed and analyzed following the methods described in Section 2.2 and 2.3.2.

Measured concentrations of 2-ethylhexyl 4-hydroxybenzoate from samples during the method validation in freshwater ranged from 90 to 92% of nominal at a concentration of 2.00 ng/mL and from 115 to 116% of nominal at a concentration of 100 ng/mL. The analytical method was demonstrated to be valid for quantifying 2-ethylhexyl 4-hydroxybenzoate in freshwater (Table 2).

These results indicate the method is acceptable for the recovery of 2-ethylhexyl 4-hydroxybenzoate in AMA study water samples.

3.3 Accuracy and Precision

The QC samples were prepared with each set from a 2-ethylhexyl 4-hydroxybenzoate spiking solution prepared on 03 December 2015. The QC sample concentrations ranged from 2.00 to 100 ng/mL, and were prepared as described above.

The results from the analysis of the QC fortifications are presented in Table 3.

3.4 Stability Determination

Stability determinations were conducted from 17 to 20 December 2015 for refrigerated samples and from 22 to 26 December 2015 for room temperature samples. Nine 5-mL volumes of freshwater were collected in culture tubes. Three samples (low spikes) were fortified with 0.250 mL of a 40.0 ng/mL solution for a nominal concentration of 2.00 ng/mL. Three samples (high spikes) were fortified with 0.250 mL of a 2.00 mg/mL solution for a nominal concentration of 100 ng/mL. Three samples consisted of freshwater only (matrix blank). The stability determination samples were processed and analyzed at 0-hour and after 1, 2, and 3 days of refrigerated or room temperature storage following the methods described in Section 2.2 and 2.3.2.

Measured concentrations 2-ethylhexyl 4-hydroxybenzoate in freshwater during the refrigerated stability determination are presented in Table 4. Mean measured 2-ethylhexyl 4-hydroxybenzoate concentrations from the 2.00 ng/mL nominal concentration samples were 106, 104, and 105% of the 0-hour values after 1, 2, or 3 days of storage, respectively. Mean measured 2-ethylhexyl 4-hydroxybenzoate concentrations from the 100 ng/mL nominal concentration samples were 99% of the 0-hour values after 1, 2, or 3 days of storage, respectively. These results did not indicate a decline in 2-ethylhexyl 4-hydroxybenzoate in freshwater after 3 days of refrigerated storage.

Measured concentrations 2-ethylhexyl 4-hydroxybenzoate in freshwater during the room temperature stability determination are presented in Table 5. Mean measured 2-ethylhexyl 4-hydroxybenzoate concentrations from the 2.00 ng/mL nominal concentration samples were 105, 46, and 77% of the 0-hour values after 1, 2, or 3 days of storage, respectively. Mean

measured 2-ethylhexyl 4-hydroxybenzoate concentrations from the 100 ng/mL nominal concentration samples were 99, 92, and 84% of the 0-hour values after 1, 2, or 3 days of storage, respectively. These results did not indicate a decline in 2-ethylhexyl 4-hydroxybenzoate in freshwater after 3 days of room temperature storage.

In some cases, samples were held longer before analysis than the duration of the stability studies, specifically for duplicate samples. In these cases, sample stability can be inferred from calibration standard stability. The stability of calibration standards is demonstrated by a consistent linear response during the period of use for the standards (at least two months), as well as consistently acceptable recoveries of QC samples measured against the standards. Since the calibration standards were prepared in the same diluent as the duplicate samples and stored similarly, the duplicate samples can be expected to be stable.

3.5 Amphibian Metamorphosis Assay Study Water Sample Concentrations

The calculated concentrations of 2-ethylhexyl 4-hydroxybenzoate in amphibian metamorphosis study water samples during the pre-exposure period are provided in Table 6. Table 7 shows the measured concentrations of 2-ethylhexyl 4-hydroxybenzoate in amphibian metamorphosis study water samples.

4.0 CONCLUSIONS

The percent of nominal concentration recovered from the 2.00, and 100 ng/mL 2-ethylhexyl 4-hydroxybenzoate QC spikes in reagent water ranged from 97 to 118%, respectively. In one instance (2.00 ng/mL in set 01262016D), spike recovery was 180%; it was considered an anomalous event and therefore was not considered to have had an impact of the validity of the data from the set.

These results indicate the method is acceptable for the recovery of 2-ethylhexyl 4-hydroxybenzoate in amphibian metamorphosis study water samples.

5.0 ARCHIVING

At the Sponsor's discretion, study samples will be disposed upon finalization of the report.

Electronic raw data, e.g., data acquired from automated data collection systems are maintained at EAG. Upon completion of the study, study-specific original paper raw data and appropriate representations of electronic raw data will be submitted along with the final report to the Sponsor for archiving. The archive location will be:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2696
Attn: Vincent J. Brown, Ph.D.
614-424-5928
brownv@battelle.org

A copy of the final report, copies of raw data from the study, and all original electronic raw data and facility records (for example, equipment, logbooks, and temperature records) will be kept on file in EAG archives.

6.0 PROTOCOL DEVIATIONS

None.

Table 1 Data Summary of Amphibian Metamorphosis Study Water Sample Analysis

LC-MS/MS File Name	Sample Identification	Sample Collection Date	Sample Processing Date	LC-MS/MS Analysis Date	Deviation from Method	Run Accepted or Rejected
01152016Z	Stock_10Jan16 (002)	10 January 2016	15 January 2016	15 January 2016	NA	Accepted [a]
	Stock_12Jan16 (004)	12 January 2016				
01262016D	0.0 µg/L (006)	21 January 2016	26 January 2016	26 January 2016	NA	Accepted [a]
	3.6 µg/L (007)					
	10.9 µg/L (008)					
	33.0 µg/L (009)					
	100 µg/L (010)					
	DI Blank (011)					
01272016D	Stock_21Jan16 (005)	21 January 2016	27 January 2016	27 January 2016	NA	Accepted
	Stock_26Jan16 (012)	26 January 2016				
01282016A	Stock #6 (013)	27 January 2016	28 January 2016	28 January 2016	NA	Accepted
	0.0 µg/L (014)					
	3.6 µg/L (015)					
	10.9 µg/L (016)					
	33.0 µg/L (017)					
	100 µg/L (018)					
	DI Blank (019)					
	Methanol (020)					

[a] Stock Sample 002 was re-diluted in duplicate and injected in 01192016A for re-analysis. Samples 006, 007, 008, 009, 010, and 011 were re-diluted in duplicate and injected in 01272016B for re-analysis. Re-analysis results supported the original results, therefore original results reported.

Table 1 Data Summary of Amphibian Metamorphosis Study Water Sample Analysis (continued)

LC-MS/MS File Name	Sample Identification	Sample Collection Date	Sample Processing Date	LC-MS/MS Analysis Date	Deviation from Method	Run Accepted or Rejected
01282016B	ABC H_2O – A (3) ABC H_2O – B (4) ABC H_2O – C (5) OK H_2O – A (6) OK H_2O – B (7) OK H_2O – C (8)	28 January 2016	28 January 2016	28 January 2016	NA	Accepted
02012016B	Stock #7 (021) 0.0 µg/L (022) 3.6 µg/L (023) 10.9 µg/L (024) 33.0 µg/L (025) 100 µg/L (026) DI Blank (027)	29 January 2016	01 February 2016	01 February 2016	NA	Accepted
02032016A	Stock #8 (028) Stock #9 (029) Blank (030) 0.0 µg/L (031)	02 February 2016	03 February 2016	04 February 2016	NA	Accepted

Table 1 Data Summary of Amphibian Metamorphosis Study Water Sample Analysis (continued)

LC-MS/MS File Name	Sample Identification	Sample Collection Date	Sample Processing Date	LC-MS/MS Analysis Date	Deviation from Method	Run Accepted or Rejected
02092016C	Blank (032)	05 February 2016	09 February 2016	09 February 2016	NA	Accepted
	Stock #10 (033)					
	0.0 µg/L A (034)					
	0.0 µg/L B (035)					
	0.0 µg/L C (036)					
	0.0 µg/L D (037)					
	3.6 µg/L A (038)					
	3.6 µg/L B (039)					
	3.6 µg/L C (040)					
	3.6 µg/L D (041)					
	10.9 µg/L A (042)					
	10.9 µg/L B (043)					
	10.9 µg/L C (044)					
	10.9 µg/L D (045)					
	33.0 µg/L A (046)					
	33.0 µg/L B (047)					
	33.0 µg/L C (048)					
	33.0 µg/L D (049)					
	100 µg/L A (050)					
	100 µg/L B (051)					
	100 µg/L C (052)					
	100 µg/L D (053)					

Table 1 Data Summary of Amphibian Metamorphosis Study Water Sample Analysis (continued)

LC-MS/MS File Name	Sample Identification	Sample Collection Date	Sample Processing Date	LC-MS/MS Analysis Date	Deviation from Method	Run Accepted or Rejected
02152016 B and 02182016A	Blank DUPLICATE (032)	05 February 2016	15 February 2016	15 February 2016	NA	Accepted
	Stock #10 DUPLICATE (033)					
	0.0 µg/L A DUPLICATE (034)					
	0.0 µg/L B DUPLICATE (035)					
	0.0 µg/L C DUPLICATE (036)					
	0.0 µg/L D DUPLICATE (037)					
	3.6 µg/L A DUPLICATE (038)					
	3.6 µg/L B DUPLICATE (039)					
	3.6 µg/L C DUPLICATE (040)					
	3.6 µg/L D DUPLICATE (041)					
	10.9 µg/L A DUPLICATE (042)					
	10.9 µg/L B DUPLICATE (043)					
	10.9 µg/L C DUPLICATE (044)					
	10.9 µg/L D DUPLICATE (045)					
	33.0 µg/L A DUPLICATE (046)					
	33.0 µg/L B DUPLICATE (047)					
	33.0 µg/L C DUPLICATE (048)					
	33.0 µg/L D DUPLICATE (049)					
	100 µg/L A DUPLICATE (050)					
	100 µg/L B DUPLICATE (051)					
	100 µg/L C DUPLICATE (052)					
	100 µg/L D DUPLICATE (053)					

Table 1 Data Summary of Amphibian Metamorphosis Study Water Sample Analysis (continued)

LC-MS/MS File Name	Sample Identification	Sample Collection Date	Sample Processing Date	LC-MS/MS Analysis Date	Deviation from Method	Run Accepted or Rejected
02152016C	Blank (161) Stock #11 (182) 0.0 µg/L A (162) 0.0 µg/L B (163) 0.0 µg/L C (164) 0.0 µg/L D (165) 3.6 µg/L A (166) 3.6 µg/L B (167) 3.6 µg/L C (168) 3.6 µg/L D (169) 10.9 µg/L A (170) 10.9 µg/L B (171) 10.9 µg/L C (172) 10.9 µg/L D (173) 33.0 µg/L A (174) 33.0 µg/L B (175) 33.0 µg/L C (176) 33.0 µg/L D (177) 100 µg/L A (178) 100 µg/L B (179) 100 µg/L C (180) 100 µg/L D (181)	12 February 2016	15 February 2016	15 February 2016	NA	Accepted

Table 1 Data Summary of Amphibian Metamorphosis Study Water Sample Analysis (continued)

LC-MS/MS File Name	Sample Identification	Sample Collection Date	Sample Processing Date	LC-MS/MS Analysis Date	Deviation from Method	Run Accepted or Rejected
02222016A	Blank (185)	19 February 2016	22 February 2016	22 February 2016	NA	Accepted
	Stock #12 (186)					
	0.0 µg/L A (187)					
	0.0 µg/L B (188)					
	0.0 µg/L C (189)					
	0.0 µg/L D (190)					
	3.6 µg/L A (191)					
	3.6 µg/L B (192)					
	3.6 µg/L C (193)					
	3.6 µg/L D (194)					
	10.9 µg/L A (195)					
	10.9 µg/L B (196)					
	10.9 µg/L C (197)					
	10.9 µg/L D (198)					
	33.0 µg/L A (199)					
	33.0 µg/L B (200)					
	33.0 µg/L C (201)					
	33.0 µg/L D (202)					
	100 µg/L A (203)					
	100 µg/L B (204)					
	100 µg/L C (205)					
	100 µg/L D (206)					

Table 1 Data Summary of Amphibian Metamorphosis Study Water Sample Analysis (continued)

LC-MS/MS File Name	Sample Identification	Sample Collection Date	Sample Processing Date	LC-MS/MS Analysis Date	Deviation from Method	Run Accepted or Rejected
02292016A	3.6 µg/L C DUPLICATE (168)	12 February 2016	29 February 2016	29 February 2016	NA	Accepted
	100 µg/L A DUPLICATE (203)	19 February 2016				
	100 µg/L B DUPLICATE (204)					
	100 µg/L C DUPLICATE (205)					
	100 µg/L D DUPLICATE (206)					
	Blank (510)					
	Stock #13 (531)					
	0.0 µg/L A (511)					
	0.0 µg/L B (512)					
	0.0 µg/L C (513)					
	0.0 µg/L D (514)					
	3.6 µg/L A (515)					
	3.6 µg/L B (516)					
	3.6 µg/L C (517)					
	3.6 µg/L D (518)					
	10.9 µg/L A (519)	26 February 2016				
	10.9 µg/L B (520)					
	10.9 µg/L C (521)					
	10.9 µg/L D (522)					
	33.0 µg/L A (523)					
	33.0 µg/L B (524)					
	33.0 µg/L C (525)					
	33.0 µg/L D (526)					
	100 µg/L A (527)					
	100 µg/L B (528)					
	100 µg/L C (529)					
	100 µg/L D (530)					

Table 1 Data Summary of Amphibian Metamorphosis Study Water Sample Analysis (continued)

LC-MS/MS File Name	Sample Identification	Sample Collection Date	Sample Processing Date	LC-MS/MS Analysis Date	Deviation from Method	Run Accepted or Rejected
03032016D	0.0 µg/L A DUPLICATE (511)	26 February 2016	03 March 2016	03 March 2016	NA	Accepted
	0.0 µg/L B DUPLICATE (512)					
	0.0 µg/L C DUPLICATE (513)					
	0.0 µg/L D DUPLICATE (514)					
	3.6 µg/L A DUPLICATE (515)					
	3.6 µg/L B DUPLICATE (516)					
	3.6 µg/L C DUPLICATE (517)					
	3.6 µg/L D DUPLICATE (518)					
	10.9 µg/L A DUPLICATE (519)					
	10.9 µg/L B DUPLICATE (520)					
	10.9 µg/L C DUPLICATE (521)					
	10.9 µg/L D DUPLICATE (522)					
	33.0 µg/L A DUPLICATE (523)					
	33.0 µg/L B DUPLICATE (524)					
	33.0 µg/L C DUPLICATE (525)					
	33.0 µg/L D DUPLICATE (526)					
	100 µg/L A DUPLICATE (527)					
	100 µg/L B DUPLICATE (528)					
	100 µg/L C DUPLICATE (529)					
	100 µg/L D DUPLICATE (530)					

Table 2. Measured Concentrations of 2-Ethylhexyl 4-hydroxybenzoate During the Method Validation in Freshwater

Sample Identification	Nominal Concentration (ng/mL)		Measured Concentration (ng/mL)	Percent of Nominal (%)
Control A	0		<MQL [a]	--
Control B	0		<MQL [a]	--
Control C	0		<MQL [a]	--
Low Spike A	2.00		1.80	90
Low Spike B	2.00		1.83	92
Low Spike C	2.00		1.83	92
		Mean	1.82	91
High Spike A	100		115	115
High Spike B	100		116	116
High Spike C	100		115	115
		Mean	115	115

[a] MQL = 0.998 ng/mL

Table 3. QC Data of 2-Ethylhexyl 4-hydroxybenzoate Measured in Amphibian Metamorphosis Assay Study Water Samples

	Low Spike		High Spike	
	2.00 ng/mL		100 ng/mL	
LC-MS/MS Filename:	Measured Concentration (ng/mL)	% Recovery	Measured Concentration (ng/mL)	% Recovery
01262016D	3.49 [a]	175	109	109
01282016A	2.26	113	96.8	97
01282016B	2.14	107	102	102
02012016B	2.18	109	106	106
02062016C	2.15	108	111	111
02152016B & 02182016A	2.29 [a]	115	115	115
02152016C	2.26	113	111	111
02222016A	2.35	118	113	113
02292016A	2.36	118	107	107
03032016D	2.28	114	112	112

[a] Sample re-diluted in duplicate and re-analyzed. Average of original and duplicate re-analyses reported.

Table 4. Measured Concentrations of 2-Ethylhexyl 4-hydroxybenzoate During the Refrigerated Stability Determination in Freshwater

			0 Hour		Day 1		Day 2		Day 3	
Sample Number	Sample Identification	Nominal Concentration (ng/mL)	Measured Conc. (ng/mL)	% of Nominal	Measured Conc. (ng/mL)	% of 0-Hour	Measured Conc. (ng/mL)	% of 0-Hour	Measured Conc. (ng/mL)	% of 0-Hour
83147-ST1	Control - A	0	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA
83147-ST2	Control - B	0	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA
83147-ST3	Control - C	0	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA
83147-ST4	Low Spike - A	2.00	1.92	96%	1.99	104	1.90	99	1.92	100
83147-ST5	Low Spike - B	2.00	1.79	90%	1.91	107	1.88	105	2.01	112
83147-ST6	Low Spike - C	2.00	1.91	96%	2.03	106	2.04	107	1.96	103
		Mean:	1.87	94%		106		104		105
83147-ST7	High Spike - A	100	116	116%	113	97	112	97	113	97
83147-ST8	High Spike - B	100	111	111%	111	100	112	101	109	98
83147-ST9	High Spike - C	100	111	111%	112	101	110	99	111	100
		Mean:	113	113%		99		99		99

[a] MQL = 1.04 ng/mL

Table 5. Measured Concentrations of 2-Ethylhexyl 4-hydroxybenzoate During the Room Temperature Stability Determination in Freshwater

			0 Hour		Day 1		Day 2		Day 3	
Sample Number	Sample Identification	Nominal Concentration (ng/mL)	Measured Conc. (ng/mL)	% of Nominal	Measured Conc. (ng/mL)	% of 0-Hour	Measured Conc. (ng/mL)	% of 0-Hour	Measured Conc. (ng/mL)	% of 0-Hour
83147-ST1	Control - A	0	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA
83147-ST2	Control - B	0	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA
83147-ST3	Control - C	0	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA	< MQL [a]	NA
83147-ST4	Low Spike - A	2.00	1.92	96%	1.93	101	1.05	55	1.60	83
83147-ST5	Low Spike - B	2.00	1.79	90%	1.94	108	0.886	49	1.56	87
83147-ST6	Low Spike - C	2.00	1.91	96%	2.04	107	0.624	33	1.15	60
		Mean:	1.87	94%		105		46		77
83147-ST7	High Spike - A	100	116	116%	112	97	107	92	95.2	82
83147-ST8	High Spike - B	100	111	111%	112	101	103	93	96.0	86
83147-ST9	High Spike - C	100	111	111%	112	101	99.6	90	92.1	83
		Mean:	113	113%		99		92		84

[a] MQL = 1.04 ng/mL

Table 6 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate (ng/mL) in Amphibian Metamorphosis Study Pre-Exposure Samples

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
002	Stock_10Jan16	NA	102 mg/L [b, c]
004	Stock_12Jan16	NA	11.2 mg/L [b]
006	0.0 µg/L	0.00	59.3 [c]
007	3.6 µg/L	3.60	89.0 [c]
008	10.9 µg/L	10.9	137 [c]
009	33.0 µg/L	33.0	271 [c]
010	100 µg/L	100	693 [c]
011	DI Blank	0.00	2.92 [c]
005	Stock_21Jan16	10.0 mg/L	92.2 mg/L [b]
012	Stock_26Jan16	10.0 mg/L	70.8 mg/L [b]
013	Stock #6	10.0 mg/L	78.6 mg/L [b]
014	0.0 µg/L	0.00	0.396
015	3.6 µg/L	3.60	24.2
016	10.9 µg/L	10.9	55.0
017	33.0 µg/L	33.0	163
018	100 µg/L	100	617
019	DI Blank	0.00	0.308
020	Methanol	0.00	0.432
3	ABC H_2O – A	0.0	<MQL [d]
4	ABC H_2O – B	0.0	<MQL [d]
5	ABC H_2O – C	0.0	<MQL [d]
6	OK H_2O - A	0.0	<MQL [d]
7	OK H_2O - B	0.0	<MQL [d]
8	OK H_2O – C	0.0	<MQL [d]

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL)

[b] Measured Conc. (mg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL) / 1000

[c] Sample re-diluted in duplicate and re-analyzed, average of original and duplicate re-analyses reported.

[d] MQL = 0.208 µg/L

NA = Not applicable

Table 6 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate (ng/mL) in Amphibian Metamorphosis Study Pre-Exposure Samples (continued)

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
021	Stock #7	10.0 mg/L	25.6 mg/L [b]
022	0.0 µg/L	0.00	0.244
023	3.6 µg/L	3.60	7.43
024	10.9 µg/L	10.9	17.9
025	33.0 µg/L	33.0	67.4
026	100 µg/L	100	210
027	DI Blank	0.00	0.296
028	Stock #8	10.0 mg/L	50.0 mg/L [b]
029	Stock #9	10.0 mg/L	89.0 mg/L [b]
030	Blank	0.00	<MQL [c]
031	0.0 µg/L	0.00	<MQL [c]

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL)

[b] Measured Conc. (mg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL) / 1000

[c] MQL = 0.208 µg/L

NA = Not applicable

Table 7 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate (ng/mL) in Amphibian Metamorphosis Study Exposure Samples

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
Study Day 0 (Initiation)			
032	Blank	0	<MQL [b]
033	Stock #10	70.0 mg/L	18.2 mg/L [c]
034	0.0 µg/L A	0.00	<MQL [b]
035	0.0 µg/L B	0.00	<MQL [b]
036	0.0 µg/L C	0.00	<MQL [b]
037	0.0 µg/L D	0.00	<MQL [b]
038	3.6 µg/L A	3.60	1.61
039	3.6 µg/L B	3.60	1.64
040	3.6 µg/L C	3.60	1.59
041	3.6 µg/L D	3.60	1.68
042	10.9 µg/L A	10.9	3.07
043	10.9 µg/L B	10.9	3.41
044	10.9 µg/L C	10.9	3.13
045	10.9 µg/L D	10.9	3.35
046	33.0 µg/L A	33.0	8.17
047	33.0 µg/L B	33.0	7.97
048	33.0 µg/L C	33.0	7.84
049	33.0 µg/L D	33.0	8.04
050	100 µg/L A	100	22.9
051	100 µg/L B	100	24.1
052	100 µg/L C	100	25.3
053	100 µg/L D	100	26.2

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL)

[b] MQL = 0.208 µg/L

[c] Measured Conc. (mg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL) / 1000

NA = Not applicable

Table 7 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate (ng/mL) in Amphibian Metamorphosis Study Exposure Samples (continued)

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
Study Day 0 (Initiation) (Duplicates)			
032	Blank DUPLICATE	0	<MQL [b]
033	Stock #10 DUPLICATE	20.0 mg/L	28.7 mg/L [c]
034	0.0 µg/L A DUPLICATE	0.00	<MQL [b]
035	0.0 µg/L B DUPLICATE	0.00	<MQL [b]
036	0.0 µg/L C DUPLICATE	0.00	<MQL [b]
037	0.0 µg/L D DUPLICATE	0.00	0.315
038	3.6 µg/L A DUPLICATE	3.60	5.64
039	3.6 µg/L B DUPLICATE	3.60	5.52
040	3.6 µg/L C DUPLICATE	3.60	5.60
041	3.6 µg/L D DUPLICATE	3.60	5.85
042	10.9 µg/L A DUPLICATE	10.9	15.0
043	10.9 µg/L B DUPLICATE	10.9	13.9
044	10.9 µg/L C DUPLICATE	10.9	15.9
045	10.9 µg/L D DUPLICATE	10.9	16.7
046	33.0 µg/L A DUPLICATE	33.0	37.5
047	33.0 µg/L B DUPLICATE	33.0	36.7
048	33.0 µg/L C DUPLICATE	33.0	42.7
049	33.0 µg/L D DUPLICATE	33.0	37.5
050	100 µg/L A DUPLICATE	100	97.0
051	100 µg/L B DUPLICATE	100	94.3
052	100 µg/L C DUPLICATE	100	131
053	100 µg/L D DUPLICATE	100	107

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL)

[b] MQL = 0.208 µg/L

[c] Measured Conc. (mg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL) / 1000

NA = Not applicable

Table 7 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate (ng/mL) in Amphibian Metamorphosis Study Exposure Samples (continued)

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
Study Day 7			
161	Blank	0	<MQL [b]
162	0.0 µg/L A	0.00	<MQL [b]
163	0.0 µg/L B	0.00	<MQL [b]
164	0.0 µg/L C	0.00	<MQL [b]
165	0.0 µg/L D	0.00	<MQL [b]
166	3.6 µg/L A	3.60	4.47
167	3.6 µg/L B	3.60	7.28
168	3.6 µg/L C	3.60	11.2
169	3.6 µg/L D	3.60	5.94
170	10.9 µg/L A	10.9	13.3
171	10.9 µg/L B	10.9	14.7
172	10.9 µg/L C	10.9	12.6
173	10.9 µg/L D	10.9	13.5
174	33.0 µg/L A	33.0	35.4
175	33.0 µg/L B	33.0	30.5
176	33.0 µg/L C	33.0	31.0
177	33.0 µg/L D	33.0	30.5
178	100 µg/L A	100	83.3
179	100 µg/L B	100	86.5
180	100 µg/L C	100	87.1
181	100 µg/L D	100	78.6
182	Stock #11	20.0 mg/L	135 mg/L [c]
Study Day 7 (Duplicate)			
168	3.6 µg/L C DUPLICATE	3.60	0.887

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL)

[b] MQL = 0.208 µg/L

[c] Measured Conc. (mg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL) / 1000

NA = Not applicable

Table 7 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate (ng/mL) in Amphibian Metamorphosis Study Exposure Samples (continued)

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
Study Day 14			
185	Blank	0	<MQL [b]
186	Stock #12	20.0 mg/L	11.8 mg/L [c]
187	0.0 µg/L A	0.00	<MQL [b]
188	0.0 µg/L B	0.00	<MQL [b]
189	0.0 µg/L C	0.00	<MQL [b]
190	0.0 µg/L D	0.00	<MQL [b]
191	3.6 µg/L A	3.60	5.08
192	3.6 µg/L B	3.60	8.05
193	3.6 µg/L C	3.60	5.86
194	3.6 µg/L D	3.60	6.50
195	10.9 µg/L A	10.9	14.9
196	10.9 µg/L B	10.9	10.9
197	10.9 µg/L C	10.9	12.9
198	10.9 µg/L D	10.9	13.6
199	33.0 µg/L A	33.0	26.8
200	33.0 µg/L B	33.0	28.9
201	33.0 µg/L C	33.0	31.6
202	33.0 µg/L D	33.0	31.2
203	100 µg/L A	100	65.5
204	100 µg/L B	100	84.4
205	100 µg/L C	100	87.5
206	100 µg/L D	100	80.1
Study Day 14 (Duplicates)			
203	100 µg/L A DUPLICATE	100	37.1
204	100 µg/L B DUPLICATE	100	32.1
205	100 µg/L C DUPLICATE	100	37.0
206	100 µg/L D DUPLICATE	100	33.9

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL)

[b] MQL = 0.208 µg/L

[c] Measured Conc. (mg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL) / 1000

NA = Not applicable

Table 7 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate (ng/mL) in Amphibian Metamorphosis Study Exposure Samples (continued)

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
Study Day 21			
510	Blank	0	<MQL [b]
531	Stock #13	20.0 mg/L	6.58 mg/L [c]
511	0.0 µg/L A	0.00	<MQL [b]
512	0.0 µg/L B	0.00	<MQL [b]
513	0.0 µg/L C	0.00	<MQL [b]
514	0.0 µg/L D	0.00	<MQL [b]
515	3.6 µg/L A	3.60	1.26
516	3.6 µg/L B	3.60	1.19
517	3.6 µg/L C	3.60	1.12
518	3.6 µg/L D	3.60	1.33
519	10.9 µg/L A	10.9	3.95
520	10.9 µg/L B	10.9	3.82
521	10.9 µg/L C	10.9	4.11
522	10.9 µg/L D	10.9	3.87
523	33.0 µg/L A	33.0	12.5
524	33.0 µg/L B	33.0	16.6
525	33.0 µg/L C	33.0	11.4
526	33.0 µg/L D	33.0	15.7
527	100 µg/L A	100	44.3
528	100 µg/L B	100	42.1
529	100 µg/L C	100	43.2
530	100 µg/L D	100	40.2

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL)

[b] MQL = 0.208 µg/L

[c] Measured Conc. (mg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL) / 1000

NA = Not applicable

Table 7 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate (ng/mL) in Amphibian Metamorphosis Study Exposure Samples (continued)

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
Study Day 21 (Duplicates)			
511	0.0 µg/L A DUPLICATE	0.00	<MQL [b]
512	0.0 µg/L B DUPLICATE	0.00	<MQL [b]
513	0.0 µg/L C DUPLICATE	0.00	<MQL [b]
514	0.0 µg/L D DUPLICATE	0.00	<MQL [b]
515	3.6 µg/L A DUPLICATE	3.60	3.58
516	3.6 µg/L B DUPLICATE	3.60	3.42
517	3.6 µg/L C DUPLICATE	3.60	3.42
518	3.6 µg/L D DUPLICATE	3.60	3.52
519	10.9 µg/L A DUPLICATE	10.9	6.42
520	10.9 µg/L B DUPLICATE	10.9	6.91
521	10.9 µg/L C DUPLICATE	10.9	6.36
522	10.9 µg/L D DUPLICATE	10.9	6.03
523	33.0 µg/L A DUPLICATE	33.0	16.7
524	33.0 µg/L B DUPLICATE	33.0	16.2
525	33.0 µg/L C DUPLICATE	33.0	18.0
526	33.0 µg/L D DUPLICATE	33.0	15.8
527	100 µg/L A DUPLICATE	100	38.7
528	100 µg/L B DUPLICATE	100	40.0
529	100 µg/L C DUPLICATE	100	39.1
530	100 µg/L D DUPLICATE	100	38.2

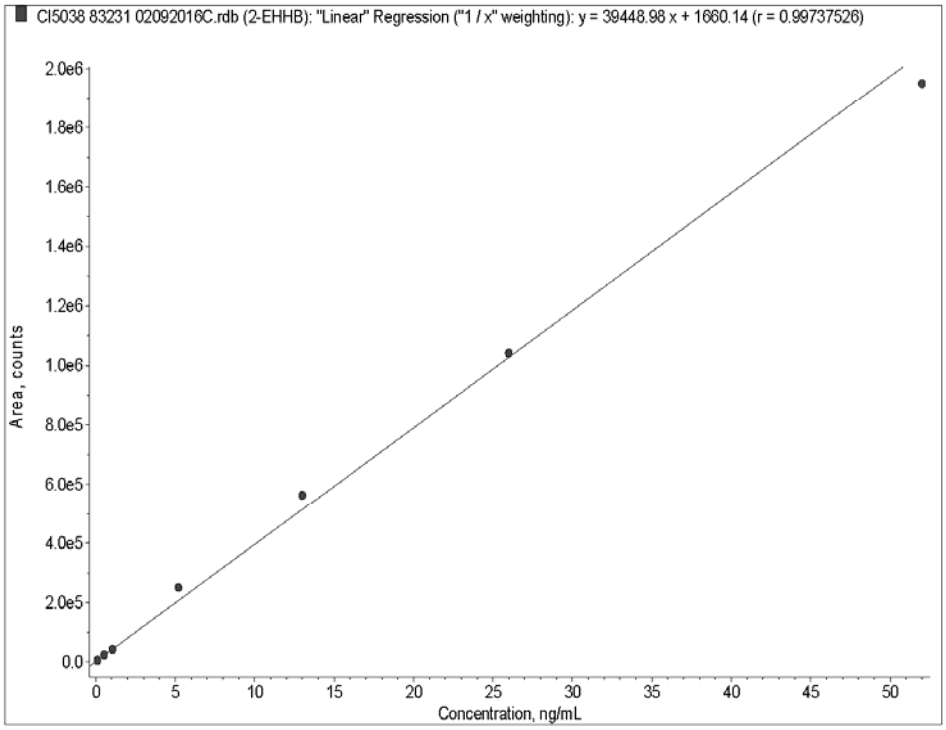
[a] Measured Conc. (µg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL)

[b] MQL = 0.208 µg/L

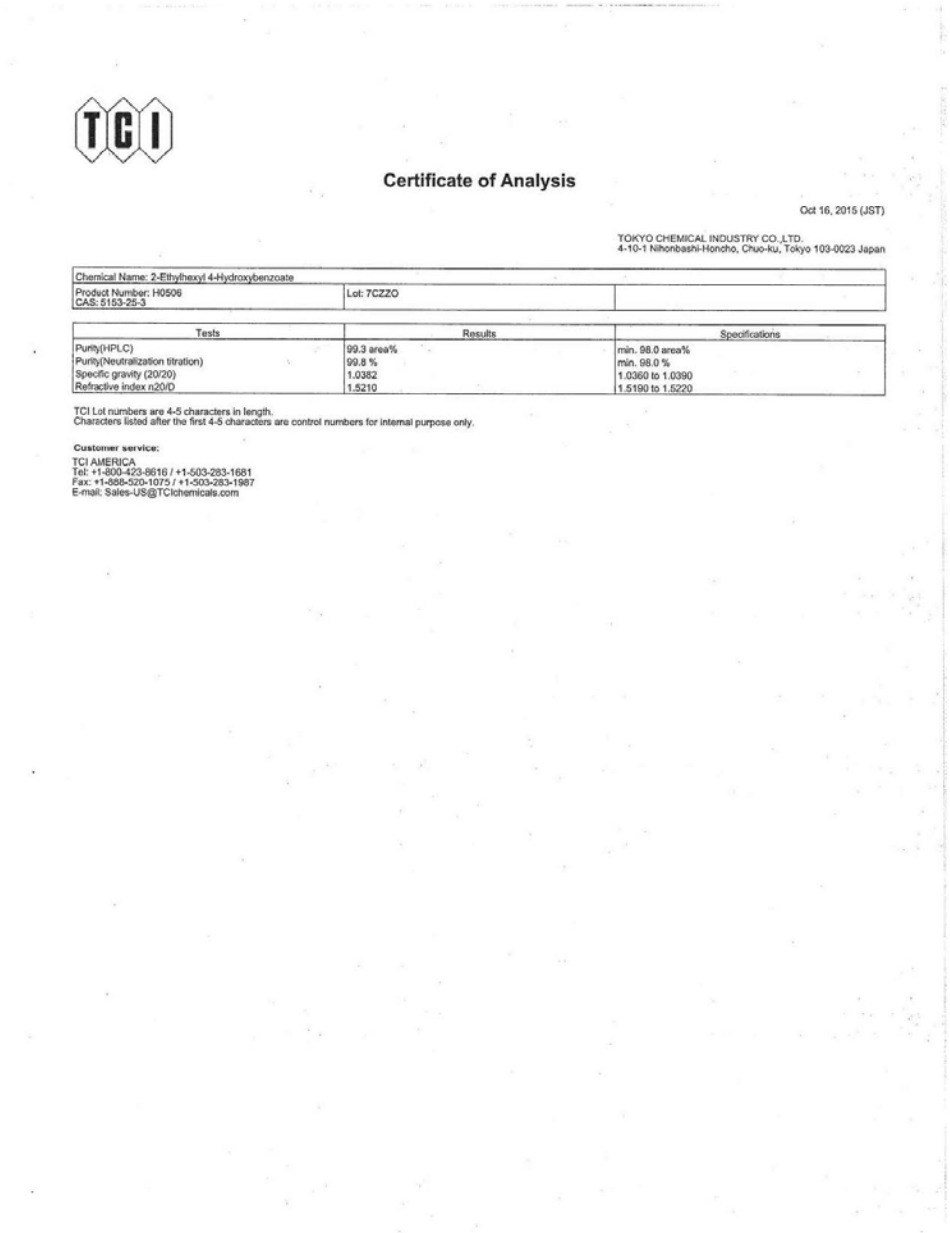
[c] Measured Conc. (mg/L) = Measured Conc. from Curve (ng/mL) × Analysis Volume (mL) / Sample Volume (mL) / 1000

NA = Not applicable

Figure 1 Typical Calibration Curve for 2-Ethylhexyl 4-Hydroxybenzoate from the Amphibian Metamorphosis Study Water Sample Analysis



APPENDIX 1 CERTIFICATE OF ANALYSIS



Certificate of Analysis

Oct 16, 2015 (JST)

TOKYO CHEMICAL INDUSTRY CO.,LTD.
4-10-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023 Japan

Chemical Name: 2-Ethylhexyl 4-Hydroxybenzoate		
Product Number: H0506 CAS: 5153-25-3	Lot: 7CZZO	

Tests	Results	Specifications
Purity(HPLC)	99.3 area%	min. 98.0 area%
Purity(Neutralization titration)	99.8 %	min. 98.0 %
Specific gravity (20/20)	1.0382	1.0360 to 1.0390
Refractive index n20/D	1.5210	1.5190 to 1.5220

TCI Lot numbers are 4-5 characters in length.
Characters listed after the first 4-5 characters are control numbers for internal purpose only.

Customer service:

TCI AMERICA
Tel: +1-800-423-8616 / +1-503-283-1681
Fax: +1-888-520-1075 / +1-503-283-1987
E-mail: Sales-US@TCIchemicals.com

APPENDIX 2 EXPOSURE PHASE RAW DATA SHEETS

Note: It is acknowledged that the analytical chemistry principal investigator (Tom Leak) signed raw data from the chemistry phase as study director; this is the result of an internal process error at EAG and all of Tom Leak's signatures should be taken as those of the analytical chemistry principal investigator, not study director.

ABC Study Number: 83231
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Initiation
Data file name: CI5038 83231 02092016C

Sample Number	Sample Identification	Nominal Concentration (ng/mL)	Measured Conc. from Curve (ng/mL)	Sample Volume (mL)	Anaylsis Volume (mL)	Measured Concentration ① (ng/mL)	% of Nominal	
83231-032	Blank	0	< low std	5	10	< MQL [a]	NA	
83231-033	Stock #10	70.0 mg/L	5.69253	5	16000	18.2	26%	②
83231-034	0.0 µg/L A	0.00	< low std	5	10	< MQL [a]	NA	
83231-035	0.0 µg/L B	0.00	< low std	5	10	< MQL [a]	NA	
83231-036	0.0 µg/L C	0.00	< low std	5	10	< MQL [a]	NA	
83231-037	0.0 µg/L D	0.00	< low std	5	10	< MQL [a]	NA	
83231-038	3.6 µg/L A	3.60	0.80514	5	10	1.61	45%	
83231-039	3.6 µg/L B	3.60	0.82061	5	10	1.64	46%	
83231-040	3.6 µg/L C	3.60	0.79421	5	10	1.59	44%	
83231-041	3.6 µg/L D	3.60	0.83825	5	10	1.68	47%	
83231-042	10.9 µg/L A	10.9	0.30721	5	50	3.07	28%	
83231-043	10.9 µg/L B	10.9	0.34075	5	50	3.41	31%	
83231-044	10.9 µg/L C	10.9	0.31324	5	50	3.13	29%	
83231-045	10.9 µg/L D	10.9	0.33549	5	50	3.35	31%	
83231-046	33.0 µg/L A	33.0	0.40826	5	100	8.17	25%	
83231-047	33.0 µg/L B	33.0	0.39864	5	100	7.97	24%	
83231-048	33.0 µg/L C	33.0	0.39201	5	100	7.84	24%	
83231-049	33.0 µg/L D	33.0	0.40179	5	100	8.04	24%	
83231-050	100 µg/L A	100	0.57238	5	200	22.9	23%	
83231-051	100 µg/L B	100	0.60260	5	200	24.1	24%	
83231-052	100 µg/L C	100	0.63360	5	200	25.3	25%	
83231-053	100 µg/L D	100	0.65517	5	200	26.2	26%	
83231-15	Low Spike	2.00	1.07547	5	10	2.15	108%	
83231-16	High Spike	100	2.76915	5	200	111	111%	

[a] MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.104\ \text{ng/mL} \times 10\ \text{mL}}{5\ \text{mL}} = 0.208\ \text{ng/mL}$$

① Measured Concentration (ng/mL) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL)
② Measured Concentration (mg/L) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL) / 1000

Prepared by: Daniel R. O'Connor Date: 04 March 16
Reviewed by: Randall A. Robbins Date: March 21, 2016
Study Director: [signature] Date: [illegible]

File Location: X:\Shared\Client\Battelle\Study Folders\83231_EDSP\Data\83231_Results.xls

ABC Study Number: 83231
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Initiation Duplicate
Data file name: CI5038 83231 02152016B and CI5038 83231 02182016A

Sample Number	Sample Identification	Nominal Concentration (ng/mL)	Measured Conc. from Curve (ng/mL)	Sample Volume (mL)	Analysis Volume (mL)	Measured Concentration ① (ng/mL)	% of Nominal	
83231-032	Blank DUPLICATE	0	No Peak	5	10	< MQL *	NA	
83231-033	Stock #10 DUPLICATE	20.0 mg/L	8.96222	5	16000	28.7	144%	②
83231-034	0.0 µg/L A DUPLICATE	0.00	< low std	5	10	< MQL *	NA	
83231-035	0.0 µg/L B DUPLICATE	0.00	< low std	5	10	< MQL *	NA	
83231-036	0.0 µg/L C DUPLICATE	0.00	< low std	5	10	< MQL *	NA	
83231-037	0.0 µg/L D DUPLICATE	0.00	0.15733	5	10	0.315	NA	
83231-038	3.6 µg/L A DUPLICATE	3.60	2.82090	5	10	5.64	157%	
83231-039	3.6 µg/L B DUPLICATE	3.60	2.76170	5	10	5.52	153%	
83231-040	3.6 µg/L C DUPLICATE	3.60	2.79567	5	10	5.60	156%	
83231-041	3.6 µg/L D DUPLICATE	3.60	2.92294	5	10	5.85	163%	
83231-042	10.9 µg/L A DUPLICATE	10.9	1.49638	5	50	15.0	138%	
83231-043	10.9 µg/L B DUPLICATE	10.9	1.38828	5	50	13.9	128%	
83231-044	10.9 µg/L C DUPLICATE	10.9	1.58727	5	50	15.9	146%	
83231-045	10.9 µg/L D DUPLICATE	10.9	1.66938	5	50	16.7	153%	
83231-046	33.0 µg/L A DUPLICATE	33.0	1.87412	5	100	37.5	114%	
83231-047	33.0 µg/L B DUPLICATE	33.0	1.83884	5	100	36.7	111%	
83231-048	33.0 µg/L C DUPLICATE	33.0	2.13670	5	100	42.7	129%	
83231-049	33.0 µg/L D DUPLICATE	33.0	1.87586	5	100	37.5	114%	
83231-050	100 µg/L A DUPLICATE	100	2.42551	5	200	97.0	97%	
83231-051	100 µg/L B DUPLICATE	100	2.35634	5	200	94.3	94%	
83231-052	100 µg/L C DUPLICATE	100	3.27214	5	200	131	131%	
83231-053	100 µg/L D DUPLICATE	100	2.68507	5	200	107	107%	
83231-17	Low Spike	2.00	1.22412	5	10	2.45	123%	③
83231-17	Low Spike R-1	2.00	1.10947	5	10	2.21	111%	
83231-17	Low Spike R-2	2.00	1.10986	5	10	2.22	111%	
					mean =	2.29	115%	④
83231-18	High Spike	100	2.88012	5	200	115	115%	

* MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.104 \text{ ng/mL} \times 10 \text{ mL}}{5 \text{ mL}} = 0.208 \text{ ng/mL}$$

① Measured Concentration (ng/mL) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL)
② Measured Concentration (mg/L) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL) / 1000
③ Sample was re-diluted and re-analyzed in duplicate. Results are in data set CI5038 83231 02182016A.
④ The average of the original and re-dilution data will be reported.

Prepared by: Date: 07 Mar 16
Reviewed by: Date:
Study Director: Date:

File Location: X:\Shared\Client\Battelle\Study Folders\83231_EDSP\Data\83231_Results.xls

ABC Study Number: 83231
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Week 1
Data file name: CI5038 83231 02152016C

Sample Number	Sample Identification	Nominal Concentration (ng/mL)	Measured Conc. from Curve (ng/mL)	Sample Volume (mL)	Anaylsis Volume (mL)	Measured Concentration ① (ng/mL)	% of Nominal	
83231-161	Blank	0	No Peak	5	10	< MQL [4]	NA	
83231-162	Stock #11	20.0 mg/L	42.30250	5	16000	135	675%	②
83231-162	0.0 µg/L A	0.00	< low std	5	10	< MQL [4]	NA	
83231-163	0.0 µg/L B	0.00	< low std	5	10	< MQL [4]	NA	
83231-164	0.0 µg/L C	0.00	< low std	5	10	< MQL [4]	NA	
83231-165	0.0 µg/L D	0.00	< low std	5	10	< MQL [4]	NA	
83231-166	3.6 µg/L A	3.60	2.23584	5	10	4.47	124%	
83231-167	3.6 µg/L B	3.60	3.64025	5	10	7.28	202%	
83231-168	3.6 µg/L C	3.60	5.60314	5	10	11.2	311%	
83231-169	3.6 µg/L D	3.60	2.96931	5	10	5.94	165%	
83231-170	10.9 µg/L A	10.9	1.32567	5	50	13.3	122%	
83231-171	10.9 µg/L B	10.9	1.46598	5	50	14.7	135%	
83231-172	10.9 µg/L C	10.9	1.25956	5	50	12.6	116%	
83231-173	10.9 µg/L D	10.9	1.34599	5	50	13.5	124%	
83231-174	33.0 µg/L A	33.0	1.76380	5	100	35.4	107%	
83231-175	33.0 µg/L B	33.0	1.52452	5	100	30.5	92%	
83231-176	33.0 µg/L C	33.0	1.54969	5	100	31.0	94%	
83231-177	33.0 µg/L D	33.0	1.52712	5	100	30.5	92%	
83231-178	100 µg/L A	100	2.08169	5	200	83.3	83%	
83231-179	100 µg/L B	100	2.16295	5	200	86.5	87%	
83231-180	100 µg/L C	100	2.17776	5	200	87.1	87%	
83231-181	100 µg/L D	100	1.96386	5	200	78.6	79%	
83231-19	Low Spike	2.00	1.13030	5	10	2.26	113%	
83231-20	High Spike	100	2.77660	5	200	111	111%	

[3] MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.104\ \text{ng/mL} \times 10\ \text{mL}}{5\ \text{mL}} = 0.208\ \text{ng/mL}$$

① Measured Concentration (ng/mL) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL)
② Measured Concentrstion (mg/L) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL) / 1000

Prepared by: Date:
Reviewed by: Date: March 24 2016
Study Director: Date:

File Location: X:\Shared\Client\Battelle\Study Folders\83231_EDSP\Data\83231_Results.xls

ABC Study Number: 83231
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Week 2
Data file name: CI5038 83231 02222016A

Sample Number	Sample Identification	Nominal Concentration (ng/mL)	Measured Conc. from Curve (ng/mL)	Sample Volume (mL)	Analysis Volume (mL)	Measured Concentration ① (ng/mL)	% of Nominal	
83231-185	Blank	0	No Peak	5	10	< MQL *	NA	
83231-186	Stock #12	20.0 mg/L	3.67890	5	16000	11.8	59%	②
83231-187	0.0 µg/L A	0.00	< low std	5	10	< MQL *	NA	
83231-188	0.0 µg/L B	0.00	< low std	5	10	< MQL *	NA	
83231-189	0.0 µg/L C	0.00	< low std	5	10	< MQL *	NA	
83231-190	0.0 µg/L D	0.00	< low std	5	10	< MQL *	NA	
83231-191	3.6 µg/L A	3.60	2.54023	5	10	5.08	141%	
83231-192	3.6 µg/L B	3.60	4.02603	5	10	8.05	224%	
83231-193	3.6 µg/L C	3.60	2.92771	5	10	5.86	163%	
83231-194	3.6 µg/L D	3.60	3.25110	5	10	6.50	181%	
83231-195	10.9 µg/L A	10.9	1.48941	5	50	14.9	137%	
83231-196	10.9 µg/L B	10.9	1.09252	5	50	10.9	100%	
83231-197	10.9 µg/L C	10.9	1.29073	5	50	12.9	118%	
83231-198	10.9 µg/L D	10.9	1.35842	5	50	13.5	125%	
83231-199	33.0 µg/L A	33.0	1.34233	5	100	26.8	81%	
83231-200	33.0 µg/L B	33.0	1.44451	5	100	28.9	88%	
83231-201	33.0 µg/L C	33.0	1.58028	5	100	31.6	96%	
83231-202	33.0 µg/L D	33.0	1.55760	5	100	31.2	95%	
83231-203	100 µg/L A	100	1.63649	5	200	65.5	66%	
83231-204	100 µg/L B	100	2.10896	5	200	84.4	84%	
83231-205	100 µg/L C	100	2.18710	5	200	87.5	88%	
83231-206	100 µg/L D	100	2.00266	5	200	80.1	80%	
83231-21	Low Spike	2.00	1.17325	5	10	2.35	118%	
83231-22	High Spike	100	2.62006	5	200	113	113%	

* MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.104\ \text{ng/mL} \times 10\ \text{mL}}{5\ \text{mL}} = 0.208\ \text{ng/mL}$$

① Measured Concentration (ng/mL) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL)
② Measured Concentration (mg/L) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL) / 1000

Prepared by: Date:
Reviewed by: Date:
Study Director: Date:

File Location: X:\Shared\Client\Battelle\Study Folders\83231_EDSP\Data\83231_Results.xls

ABC Study Number: 83231
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Week 3
Data file name: CI5038 83231 02292016A

Sample Number	Sample Identification	Nominal Concentration (ng/mL)	Measured Conc. from Curve (ng/mL)	Sample Volume (mL)	Analysis Volume (mL)	Measured Concentration ① (ng/mL)	% of Nominal	
83231-510	Blank	0	No Peak	5	10	< MQL [a]	NA	
83231-531	Stock #13	20.0 mg/L	2.05712	5	16000	6.58	33%	②
83231-511	0.0 µg/L A	0.00	No Peak	5	10	< MQL [a]	NA	
83231-512	0.0 µg/L B	0.00	No Peak	5	10	< MQL [a]	NA	
83231-513	0.0 µg/L C	0.00	No Peak	5	10	< MQL [a]	NA	
83231-514	0.0 µg/L D	0.00	No Peak	5	10	< MQL [a]	NA	
83231-515	3.6 µg/L A	3.60	0.63011	5	10	1.26	35%	
83231-516	3.6 µg/L B	3.60	0.59286	5	10	1.19	33%	
83231-517	3.6 µg/L C	3.60	0.58163	5	10	1.12	31%	
83231-518	3.6 µg/L D	3.60	0.66309	5	10	1.33	37%	
83231-519	10.9 µg/L A	10.9	0.39482	5	50	3.95	36%	
83231-520	10.9 µg/L B	10.9	0.38192	5	50	3.82	35%	
83231-521	10.9 µg/L C	10.9	0.41082	5	50	4.11	38%	
83231-522	10.9 µg/L D	10.9	0.38668	5	50	3.87	36%	
83231-523	33.0 µg/L A	33.0	0.62434	5	100	12.5	38%	
83231-524	33.0 µg/L B	33.0	0.82247	5	100	16.5	50%	
83231-525	33.0 µg/L C	33.0	0.56987	5	100	11.4	35%	
83231-526	33.0 µg/L D	33.0	0.78405	5	100	15.7	48%	
83231-527	100 µg/L A	100	1.10756	5	200	44.3	44%	
83231-528	100 µg/L B	100	1.05214	5	200	42.1	42%	
83231-529	100 µg/L C	100	1.08108	5	200	43.2	43%	
83231-530	100 µg/L D	100	1.00563	5	200	40.2	40%	
83231-23	Low Spike	2.00	1.17976	5	10	2.36	118%	
83231-24	High Spike	100	2.68102	5	200	107	107%	
83231-168	3.6 µg/L C DUPLICATE	3.60	0.44355	5	10	0.887	25%	
83231-203	100 µg/L A DUPLICATE	100	0.92727	5	200	37.1	37%	
83231-204	100 µg/L B DUPLICATE	100	0.80176	5	200	32.1	32%	
83231-205	100 µg/L C DUPLICATE	100	0.92498	5	200	37.0	37%	
83231-206	100 µg/L D DUPLICATE	100	0.84541	5	200	33.9	34%	

[a] MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.104\ \text{ng/mL} \times 10\ \text{mL}}{5\ \text{mL}} = 0.208\ \text{ng/mL}$$

① Measured Concentration (ng/mL) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL)
② Measured Concentration (mg/L) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL) / 1000

Prepared by: Date:
Reviewed by: Date:
Study Director: Date:

File Location: X:\Shared\Client\Battelle\Study Folders\83231_EDSP\Data\83231_Results.xls

ABC Study Number: 83231
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Week 3 DUPLICATE
Data file name: CI5038 83231 03032016D

Sample Number	Sample Identification	Nominal Concentration (ng/mL)	Measured Conc. from Curve (ng/mL)	Sample Volume (mL)	Analysis Volume (mL)	Measured Concentration ① (ng/mL)	% of Nominal
83231-511	0.0 µg/L A DUPLICATE	0.00	No Peak	5	10	< MQL [a]	NA
83231-512	0.0 µg/L B DUPLICATE	0.00	No Peak	5	10	< MQL [a]	NA
83231-513	0.0 µg/L C DUPLICATE	0.00	No Peak	5	10	< MQL [a]	NA
83231-514	0.0 µg/L D DUPLICATE	0.00	No Peak	5	10	< MQL [a]	NA
83231-515	3.6 µg/L A DUPLICATE	3.60	1.78860	5	10	3.58	99%
83231-516	3.6 µg/L B DUPLICATE	3.60	1.71040	5	10	3.42	95%
83231-517	3.6 µg/L C DUPLICATE	3.60	1.71164	5	10	3.42	95%
83231-518	3.6 µg/L D DUPLICATE	3.60	1.75821	5	10	3.52	98%
83231-519	10.9 µg/L A DUPLICATE	10.9	0.64164	5	50	6.42	59%
83231-520	10.9 µg/L B DUPLICATE	10.9	0.69061	5	50	6.91	63%
83231-521	10.9 µg/L C DUPLICATE	10.9	0.63552	5	50	6.36	58%
83231-522	10.9 µg/L D DUPLICATE	10.9	0.60349	5	50	6.03	55%
83231-523	33.0 µg/L A DUPLICATE	33.0	0.83644	5	100	16.7	51%
83231-524	33.0 µg/L B DUPLICATE	33.0	0.81167	5	100	16.2	49%
83231-525	33.0 µg/L C DUPLICATE	33.0	0.90236	5	100	18.0	55%
83231-526	33.0 µg/L D DUPLICATE	33.0	0.78923	5	100	15.8	48%
83231-527	100 µg/L A DUPLICATE	100	0.96789	5	200	38.7	39%
83231-528	100 µg/L B DUPLICATE	100	1.00032	5	200	40.0	40%
83231-529	100 µg/L C DUPLICATE	100	0.97769	5	200	39.1	39%
83231-530	100 µg/L D DUPLICATE	100	0.95470	5	200	38.2	38%
83231-25	Low Spike	2.00	1.14108	5	10	2.28	114%
83231-26	High Spike	100	2.79926	5	200	112	112%

[a] MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.104\ \text{ng/mL} \times 10\ \text{mL}}{5\ \text{mL}} = 0.208\ \text{ng/mL}$$

① Measured Concentration (ng/mL) = Measured Conc. From Curve (ng/mL) x Analysis Volume (mL) / Sample Volume (mL)

Prepared by: Derrick R. C Cam Date: 04 March 2011
Reviewed by: Daniel A. Nothwehr Date: March 2, 2011
Study Director: [signature] Date: June 2, 11

File Location: X:\Shared\Client\Battelle\Study Folders\83231_EDSP\Data\83231_Results.xls

Appendix D
EPL PATHOLOGY REPORT

Experimental Pathology Laboratories, Inc.

FORT ENVIRONMENTAL LABORATORIES, INC.
STUDY NUMBER BATT01-00388
EPL PROJECT NUMBER 237-072

21-D AMPHIBIAN METAMORPHOSIS ASSAY (AMA) OF 2-ETHYLHEXYL 4-HYDROXYBENZOATE WITH AFRICAN CLAWED FROG, *XENOPUS LAEVIS*

PATHOLOGY REPORT

Submitted by:

Experimental Pathology Laboratories, Inc.

Street Address:	Mailing Address:
45600 Terminal Drive	P.O. Box 169
Sterling, VA 20166	Sterling, VA 20167-0169

(703) 471-7060

Original submitted to:

Test Facility
Fort Environmental Laboratories, Inc.
Stillwater, OK 74074

Copy submitted to:

Sponsor Representative
Battelle Memorial Institute
Columbus, OH 43201

March 19, 2018

FINAL REPORT

Experimental Pathology Laboratories, Inc.

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PATHOLOGY NARRATIVE

Experimental Pathology Laboratories, Inc.

FORT ENVIRONMENTAL LABORATORIES, INC.
STUDY NUMBER BATT01-00388
EPL PROJECT NUMBER 237-072

21-D AMPHIBIAN METAMORPHOSIS ASSAY (AMA) OF 2-ETHYLHEXYL 4-HYDROXYBENZOATE WITH AFRICAN CLAWED FROG, *XENOPUS LAEVIS*

PATHOLOGY NARRATIVE

INTRODUCTION

An amphibian metamorphosis assay was performed in which Nieuwkoop and Faber (NF; Nieuwkoop and Faber, 1994) Stage 51 *Xenopus laevis* larvae were exposed to different concentrations of the test substance (2-ethylhexyl 4-hydroxybenzoate; 2-EHHB) for 21-days. In contrast to that specified in EPA Test Guidelines OPPTS 890.1100 (US EPA, 2009) which require testing of three independent concentrations of test substance, the general experimental design entailed exposing tadpoles to four (4) different concentrations of the test chemical (n = 4 replicates per concentration) and dilution water control (n = 4 replicates). Larval density at test initiation were 20 tadpoles per test tank (i.e., replicate for all treatment groups). Larvae selected were stage matched to the greatest extent possible based on stage distribution, and from those larvae that were identified, specimens were randomly selected. The treatment tanks were randomly assigned to a position in the exposure system in order to account for possible variations in temperature and light intensity. The primary endpoints were hind limb length, body length (snout to vent [SVL]), developmental stage, wet weight, thyroid histology, and daily mortality. The experimental design is presented in Table 1.

Table 1. Experimental Design

2-EHHB Treatment Group (µg/L)	Number of Replicates	Number of Frogs Examined per Replicate	Number of Frogs Examined per Treatment Group
0.0 (control)	4	5	20
3.6	4	5	20
10.9	4	5	20[a]
33.0	4	5	20
100.0	4	5	20
		TOTAL	100

[a] Thyroid gland tissue was not recovered from one frog of this group

METHODS

Whole body samples were submitted to Experimental Pathology Laboratories, Inc. (EPL®), Sterling, Virginia for histologic processing and pathologic evaluation. At EPL, the heads were dissected from the carcasses and processed for paraffin embedding on an automated processer using routine methods. A separate decalcification step was not deemed necessary and was not performed. The processed specimens were embedded so that the cut surface of the posterior margin (neck side) of each sample was microtomed first. For each block, excess paraffin was trimmed away until at least one of the thyroid glands was reached (approximately 500 microns into the tissue). Step sections, each 4-5 microns thick, were then obtained at 50 micron intervals until the maximum diameter of at least one gland had been reached, at which point a section was retained. Two additional step sections were then microtomed at 50 micron intervals, and all three sections were placed on a single glass slide. The sections were stained with hematoxylin and eosin, and mounted with a glass coverslip using an appropriate permanent mounting medium. If, upon inspection of the completed sections, inadequate numbers of follicles were represented, and it was apparent that further thyroid tissue remained in the block, additional step sections were cut at 50 micron intervals until the appropriate sections were captured, or until it was determined that additional recuts would not yield the required tissue. Following microtomy, all blocks were sealed with paraffin.

The pathologist evaluated all sections using brightfield microscopy, and was aware of the treatment group status of individual animals during the examinations. Histopathologic findings were scored for severity according to the following grading system: X = not remarkable (inconspicuous to minimal or <20% of tissue affected), Grade 1 = mild (~30-50% of tissue affected), Grade 2 = moderate (~60-80% of tissue affected), and Grade 3 = severe (>80% of tissue affected), in accordance with previously reported criteria (OECD, 2007; Grim et al., 2009). Results were recorded into a proprietary electronic data recording system, and were subsequently converted into spreadsheet format. Individual animal results were tabulated in the Histopathology Incidence Tables (HIT) and summarized in the Summary Incidence Table (SIT).

RESULTS

There were no histopathologic findings related to 2-EHHB exposure in this study (Table 2). The two findings recorded in this study were follicular cell hypertrophy and follicular cell hyperplasia. The former was characterized by a relative increase in the proportion of follicular epithelial cells that exhibited increased cell height (i.e., columnar shape relative to cuboidal), and the latter by a proportional increase in stratification, crowding, or papillary infolding of follicular epithelial cells. Because anuran metamorphosis is considered to be a thyroid-dependent process, basal levels of follicular cell hypertrophy and hyperplasia are anticipated findings in control frogs at the developmental stage at which they were sacrificed in the current study (i.e., median NF Stage 58) (Grim et al., 2009).

There were slight, non-dose-responsive increases in the incidence and/or severity of follicular cell hypertrophy (mild to moderate), and in the incidence of follicular cell hyperplasia (mild), in some treated groups compared to controls; however, these relative differences were too insubstantial to conclude that they represented treatment effects.

Thyroid tissue was not recovered from a single frog of the 10.9 µg/L dose group, because the technician inadvertently sectioned through the tissue. The lack of this specimen had no effect on the study outcome.

Table 2. Prevalence and Severity of Histopathologic Findings in the Thyroid Gland

2-EHHB Treatment Group (µg/L)	0.0 (control)	3.6	10.9	33.0	100.0
Median NF Stage [b]	58	58	58	58	58
n	20	20	19 [a]	20	20
Follicular Cell Hyperplasia	1	1	0	3	2
mild	1	1	-	3	2
Follicular Cell Hypertrophy	10	7	13	13	12
mild	10	6	13	12	11
moderate	-	1	-	1	1

[a] Thyroid gland tissue was not recovered from one frog of this group.

[b] Median stage based on scores of frogs received.

DISCUSSION

Anuran metamorphosis is considered to be a thyroid-dependent process; therefore, basal levels of follicular cell hypertrophy and hyperplasia are anticipated findings in control frogs at or around the developmental stages (i.e., metamorphic climax) at which they were sacrificed in the current study (Grim et al., 2009). The stimulus for both follicular cell hypertrophy and hyperplasia is increased circulating levels of thyroid stimulating hormone (TSH) (Tietge et al., 2010), concentrations of which are highest in the *X. laevis* pituitary between Nieuwkoop and Faber (NF) Stages 58 and 62 (Korte et al., 2011). For reasons that are not yet completely clear, the rapid elevation in TSH that is associated with metamorphic climax occurs despite a concomitant rise in circulating thyroid hormones (TH), which would otherwise be expected to suppress pituitary TSH production via the classic hypothalamus-pituitary-thyroid (HPT) negative feedback mechanism (Buckbinder and Brown, 1993; Sternberg et al., 2011). Following metamorphic climax (e.g., NF Stage 66), levels of TSH and TH decrease, at which point the histological appearance of the thyroid glands becomes more quiescent (Grim et al., 2009).

CONCLUSION AND SUMMARY

There were no histopathologic findings related to 2-EHHB exposure in this study.

19 March 2018

JEFFREY C. WOLF, DVM, Diplomate, ACVP — Date
Senior Pathologist

JCW/cb

REFERENCES

Buckbinder L, Brown DD. (1993). Expression of the *Xenopus laevis* prolactin and thyrotropin genes during metamorphosis. Proc Natl Acad Sci U S A, 90:3820-3824.

Grim KC, Wolfe M, Braunbeck T, Iguchi T, Ohta Y, Tooi O, Touart L, Wolf DC, Tietge J. (2009). Thyroid Histopathology Assessments for the Amphibian Metamorphosis Assay to Detect Thyroid-active Substances. Toxicol Pathol, 37:415-424.

Korte JJ, Sternberg RM, Serrano JA, Thoemke KR, Moen SM, Lillegard KE, Hornung MW, Tietge JE, Degitz SJ. (2011). Thyroid-stimulating hormone (TSH): measurement of intracellular, secreted, and circulating hormone in *Xenopus laevis* and *Xenopus tropicalis*. Gen Comp Endocrinol, 171:319-325.

Nieuwkoop PD., Faber J. (1994). Normal Table of *Xenopus laevis* (Daudin). Garland Publishing, Inc. New York and London.

OEDC 2007. Guidance Document on Amphibian Thyroid Histology. Series on Testing and Assessment, Number 82. ENV/JM/MONO(2007)31. October 24, 2007.

Sternberg RM, Thoemke KR, Korte JJ, Moen SM, Olson JM, Korte L, Tietge JE, Degitz SJ Jr. (2011). Control of pituitary thyroid-stimulating hormone synthesis and secretion by thyroid hormones during *Xenopus* metamorphosis. Gen Comp Endocrinol, 173:428-437.

Tietge JE, Butterworth BC, Haselman JT, Holcombe GW, Hornung MW, Korte JJ, Kosian PA, Wolfe M, Degitz SJ. (2010). Early temporal effects of three thyroid hormone synthesis inhibitors in *Xenopus laevis*. Aquat Toxicol, 98:44-50.

US EPA, 2009. OPPTS 890.1100 Amphibian Metamorphosis (Frog), United States Environmental Protection Agency, EPA 740-C-09-002, October 2009.

EPL® Experimental Pathology Laboratories, Inc.

COMPLIANCE STATEMENT

Test Facility	Fort Environmental Laboratories, Inc.	EPL Principal Investigator	Dr. Jeffrey C. Wolf
Study No.	BATT01-00388	EPL Pathologist	Dr. Jeffrey C. Wolf
Species	*Xenopus laevis* (South African clawed frog)	EPL Project Number	237-072
Study Title	21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*		
Test Article	2-Ethylhexyl 4-Hydroxybenzoate (2-EHHB)		

The Histopathology portions of the above-referenced study were conducted in compliance with the Good Laboratory Practice regulations of the Environmental Protection Agency as stipulated by 40 CFR Part 160 (FIFRA); and all applicable amendments.

EPL Principal Investigator

19 March 2018
Date

EPL® Experimental Pathology Laboratories, Inc.

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*

Client Study: BATT01-00388 EPL Principal Investigator: Dr. Jeffrey C. Wolf

EPL Project Number: 237-072 EPL Pathologist: Dr. Jeffrey C. Wolf

The following aspects of this study were inspected by the Quality Assurance Unit of Experimental Pathology Laboratories, Inc. Dates inspections were performed and findings reported to the EPL Principal Investigator and Management are indicated below.

Area Inspected	Dates	
	Inspection	Reporting
EPL Project Sheets	3/24/16; 5/11/16; 3/15/17	3/24/16; 5/11/16; 3/15/17
Project Setup	4/1/16	4/1/16
In-Process - Pathology	5/12/16	5/12/16
Data Review	4/4/16; 5/11/16	4/4/16; 5/11/16
Draft Pathology Report	6/3/16; 3/9,12-14/18	6/3/16; 3/14/18
Final Pathology Report	3/19/18	3/19/18

Date reported to Study Director/Management 3/19/18

Date of last annual facility inspection 12/17

Beth Harwich
EPL Quality Assurance Unit

3/19/18
Date

SUMMARY INCIDENCE TABLE

SUMMARY INCIDENCE TABLE

BATT01-00388
Terminal Sacrifice
Xenopus laevis

	GROUP 0.0 (Control)	GROUP 3.6 µg/L	GROUP 10.9 µg/L	GROUP 33.0 µg/L	GROUP 100.0 µg/L	
THYROID (NO. EXAMINED)	(20)	(20)	(19)	(20)	(20)	
Follicular Cell Hyperplasia	1	1		3	2	
Follicular Cell Hypertrophy	10	7	13	13	12	

HISTOPATHOLOGY INCIDENCE TABLES

HISTOPATHOLOGY INCIDENCE TABLE

BATT01-00388
Terminal Sacrifice
Xenopus laevis

GROUP
0.0 (Control)

ANIMAL	210	212	215	221	223	228	231	232	236	239	247	249	250	252	254	258	259	260	262	264
THYROID	X	X	X			X			X		X	X		X	X		X			
Follicular Cell Hyperplasia																			1	
Follicular Cell Hypertrophy				1	1		1	1		1			1			1		1	1	1

Key: X=Not Remarkable N=No Section 1=mild 2=moderate 3=severe

HISTOPATHOLOGY INCIDENCE TABLE

BATT01-00388
Terminal Sacrifice
Xenopus laevis

	GROUP 3.6 µg/L																			
ANIMAL	271	272	278	279	284	285	293	295	297	299	301	302	304	310	312	317	320	323	324	328
THYROID		X	X			X	X	X	X	X		X	X	X	X		X			X
Follicular Cell Hyperplasia																1				
Follicular Cell Hypertrophy	1			1	1						1					2		1	1	

EPL Experimental Pathology Laboratories, Inc.

II-2

Key: X=Not Remarkable N=No Section 1=mild 2=moderate 3=severe

HISTOPATHOLOGY INCIDENCE TABLE

GROUP
10.9 µg/L

BATT01-00388
Terminal Sacrifice
Xenopus laevis

ANIMAL	330	334	341	343	344	345	347	349	354	356	360	361	362	367	368	376	377	380	381	388
THYROID	N									X			X	X	X			X	X	
Follicular Cell Hyperplasia																				
Follicular Cell Hypertrophy		1	1	1	1	1	1	1	1		1	1				1	1			1

EPL Experimental Pathology Laboratories, Inc.

II-3

Key: X=Not Remarkable N=No Section 1=mild 2=moderate 3=severe

HISTOPATHOLOGY INCIDENCE TABLE

BATT01-00388
Terminal Sacrifice
Xenopus laevis

	GROUP 33.0 µg/L																			
ANIMAL	394	395	399	400	404	406	407	410	412	413	420	425	429	430	433	435	436	437	438	442
THYROID	X		X	X				X	X	X							X			
Follicular Cell Hyperplasia															1	1				1
Follicular Cell Hypertrophy		1			1	1	1				1	1	1	2	1	1		1	1	1

EPL Experimental Pathology Laboratories, Inc.

II-4

Key: X=Not Remarkable N=No Section 1=mild 2=moderate 3=severe

HISTOPATHOLOGY INCIDENCE TABLE

BATT01-00388
Terminal Sacrifice
Xenopus laevis

	GROUP 100.0 µg/L																			
ANIMAL	455	456	459	461	463	465	466	469	470	477	480	482	483	486	493	496	498	500	503	506
THYROID	X		X							X	X	X			X			X		
Follicular Cell Hyperplasia							1						1							
Follicular Cell Hypertrophy		1		1	1	2	1	1	1					1		1	1		1	1

EPL Experimental Pathology Laboratories, Inc.

II-5

Key: X=Not Remarkable N=No Section 1=mild 2=moderate 3=severe

Appendix E

RAW DATA SUMMARY -
DATA ENTRY SPREADSHEET TEMPLATES (DESTs)

DEST BATT01-00388 Total Length (Day 0)

Day 0 Animal ID	Comments
054	NF Stage 51
055	NF Stage 51
056	NF Stage 51
057	NF Stage 51
058	NF Stage 51

Name of Treatment Group	Nominal Treatment Concentration (µg/L)	Replicate ID	Test Day (0 to 21)	Measured Treatment Concentration (µg/L)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (ug/L)	Conductivity (µs/cm)	Iodide (µg/L)	Chlorine (ug/L)	Comments
Facility		1-28	-9		22.8	3.6	8.1	746							Pre-Exposure
Facility		1-27	-9		22.4	3.8	8.1	750							Name of Treatment=**Facility**
Facility		1-26	-9		22.3	3.8	8.1	732							
Facility		1-25	-9		22.2	3.5	8.1	692							Replicate ID= **Clutch No.-Culture Room Tub No.**
Facility		2-23	-9		22.2	4.2	8.1	675							
Facility		2-22	-9		22.1	4.0	8.1	690							
Facility		2-21	-9		22.1	4.4	8.2	610							(Example 1-28 is Clutch #1 and Tub #28)
Facility		1-28	-7		22.6	5.8	8.4	792							
Facility		1-27	-7		22.6	5.6	8.4	734							Clutch #2 used in in-life phase of study
Facility		1-26	-7		22.7	5.0	8.3	722							
Facility		1-25	-7		22.6	5.3	8.3	691							
Facility		2-23	-7		22.4	4.8	8.3	794							
Facility		2-22	-7		22.4	5.6	8.3	748							
Facility		2-21	-7		22.3	5.4	8.3	751							
Facility		3-29	-7		22.9	5.1	8.3	691							
Facility		3-30	-7		22.3	5.1	8.3	671							
Facility		4-24	-7		22.5	5.8	8.4	702							
Facility		1-28	-4		22.8	4.2	8.1	700							
Facility		1-27	-4		22.6	3.5	8.1	625							
Facility		1-26	-4		22.7	4.8	8.1	832							
Facility		1-25	-4		22.8	3.8	8.1	625							
Facility		2-23	-4		22.6	4.0	8.0	731							
Facility		2-22	-4		22.6	4.2	8.0	842							
Facility		2-21	-4		22.5	4.2	8.1	621							
Facility		3-29	-4		22.5	5.0	8.2	821							
Facility		3-30	-4		21.4	5.6	8.2	738							
Facility		4-24	-4		22.5	4.4	8.1	800							
Facility		1-28	-3			6.2									
Facility		1-27	-3			6.0									
Facility		1-26	-3			6.1									
Facility		1-25	-3			6.0									
Facility		2-23	-3			6.2									
Facility		2-22	-3			6.1									
Facility		2-21	-3			6.2									
Facility		3-29	-3			6.0									
Facility		3-30	-3			6.1									
Facility		4-24	-3			5.9									
Facility		1-28	-1		22.2	6.2	7.5	656							
Facility		1-27	-1		22.1	6.3	7.6	626							
Facility		1-26	-1		22.1	6.2	7.6	642							
Facility		1-25	-1		22.3	6.0	7.6	717							
Facility		2-23	-1		22.0	5.8	7.7	675							
Facility		2-22	-1		22.0	6.2	7.7	724							
Facility		2-21	-1		22.0	6.2	7.8	708							
Facility		3-29	-1		22.5	6.0	7.5	628							
Facility		3-30	-1		22.2	6.2	7.6	646							
Facility		4-24	-1		22.0	5.8	7.8	719							
(Ctl) 0.0 µg/L	0.0	A	0	0.104	22.3	7.5	7.8	690	140	68			9.3		I-measured in facility dilution water; **Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment. Based on mean of original (0.104µg/L) and duplicate (0.104µg/L) sample
(Ctl) 0.0 µg/L	0.0	B	0	0.104	22.5	7.8	7.8	764							**Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment. Based on mean of original (0.104µg/L) and duplicate (0.104µg/L) sample
(Ctl) 0.0 µg/L	0.0	C	0	0.104	22.4	8.0	7.8	615							**Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment. Based on mean of original (0.104µg/L) and duplicate (0.104µg/L) sample
(Ctl) 0.0 µg/L	0.0	D	0	0.104	22.4	7.7	7.9	603							**Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment. Week 0 0.0µg/L D - 0.315µg/L identified as outlier as determined by Q3+(IQR* 1.5). Original sample used for analysis of mean measured concentration.
3.6 µg/L	3.6	A	0	3.63	22.7	7.7	7.8	783							**Measured Treatment Concentration** - Based on mean of original (1.61µg/L) and duplicate (5.64µg/L) sample
3.6 µg/L	3.6	B	0	3.58	22.5	7.6	7.9	768							**Measured Treatment Concentration** - Based on mean of original (1.64µg/L) and duplicate (5.52µg/L) sample
3.6 µg/L	3.6	C	0	3.60	22.5	7.8	7.9	715							**Measured Treatment Concentration** - Based on mean of original (1.59µg/L) and duplicate (5.60µg/L) sample
3.6 µg/L	3.6	D	0	3.77	22.6	7.6	7.9	683							**Measured Treatment Concentration** - Based on mean of original (1.68µg/L) and duplicate (5.85µg/L) sample

BATT01-00388 FEL

Name of Treatment Group	Nominal Treatment Concentration (µg/L)	Replicate ID	Test Day (0 to 21)	Measured Treatment Concentration (µg/L)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (ug/L)	Conductivity (µs/cm)	Iodide (µg/L)	Chlorine (ug/L)	Comments
10.9 µg/L	10.9	A	0	9.04	22.3	8.1	7.8	690							**Measured Treatment Concentration** - Based on mean of original (3.07µg/L) and duplicate (15.0µg/L) sample
10.9 µg/L	10.9	B	0	8.66	22.5	7.9	7.7	672							**Measured Treatment Concentration** - Based on mean of original (3.41µg/L) and duplicate (13.9µg/L) sample
10.9 µg/L	10.9	C	0	9.52	22.4	7.7	7.8	615							**Measured Treatment Concentration** - Based on mean of original (3.13µg/L) and duplicate (15.9µg/L) sample
10.9 µg/L	10.9	D	0	10.0	22.4	8.0	7.8	613							**Measured Treatment Concentration** - Based on mean of original (3.35µg/L) and duplicate (16.7µg/L) sample
33 µg/L	33	A	0	22.8	22.6	7.7	7.9	615							**Measured Treatment Concentration** - Based on mean of original (8.17µg/L) and duplicate (37.5µg/L) sample
33 µg/L	33	B	0	22.3	22.5	7.8	7.9	638							**Measured Treatment Concentration** - Based on mean of original (7.97µg/L) and duplicate (36.7µg/L) sample
33 µg/L	33	C	0	25.3	22.5	7.7	7.9	642							**Measured Treatment Concentration** - Based on mean of original (7.84µg/L) and duplicate (42.7µg/L) sample
33 µg/L	33	D	0	22.8	22.7	7.6	7.9	605							**Measured Treatment Concentration** - Based on mean of original (8.04µg/L) and duplicate (37.5µg/L) sample
100 µg/L	100	A	0	60.0	22.4	7.6	7.9	615	140	72					**Measured Treatment Concentration** - Based on mean of original (22.9µg/L) and duplicate (97.0µg/L) sample
100 µg/L	100	B	0	59.2	22.5	7.5	8.0	782							**Measured Treatment Concentration** - Based on mean of original (24.1µg/L) and duplicate (94.3µg/L) sample
100 µg/L	100	C	0	78.2	22.4	7.5	8.0	714							**Measured Treatment Concentration** - Based on mean of original (25.3µg/L) and duplicate (131µg/L) sample
100 µg/L	100	D	0	66.6	22.4	7.6	8.0	637							**Measured Treatment Concentration** - Based on mean of original (26.2µg/L) and duplicate (107µg/L) sample
(Ctl) 0.0 µg/L	0.0	A	1		22.4										
(Ctl) 0.0 µg/L	0.0	B	1		22.4										
(Ctl) 0.0 µg/L	0.0	C	1		22.5										
(Ctl) 0.0 µg/L	0.0	D	1		22.5										
3.6 µg/L	3.6	A	1		22.6										
3.6 µg/L	3.6	B	1		22.5										
3.6 µg/L	3.6	C	1		22.6										
3.6 µg/L	3.6	D	1		22.5										
10.9 µg/L	10.9	A	1		22.5										
10.9 µg/L	10.9	B	1		22.4										
10.9 µg/L	10.9	C	1		22.5										
10.9 µg/L	10.9	D	1		22.5										
33 µg/L	33	A	1		22.5										
33 µg/L	33	B	1		22.6										
33 µg/L	33	C	1		22.6										
33 µg/L	33	D	1		22.6										
100 µg/L	100	A	1		22.5										
100 µg/L	100	B	1		22.4										
100 µg/L	100	C	1		22.5										
100 µg/L	100	D	1		22.4										
(Ctl) 0.0 µg/L	0.0	A	2		22.5										
(Ctl) 0.0 µg/L	0.0	B	2		22.5										
(Ctl) 0.0 µg/L	0.0	C	2		22.4										
(Ctl) 0.0 µg/L	0.0	D	2		22.5										
3.6 µg/L	3.6	A	2		22.5										
3.6 µg/L	3.6	B	2		22.5										
3.6 µg/L	3.6	C	2		22.5										
3.6 µg/L	3.6	D	2		22.6										
10.9 µg/L	10.9	A	2		22.5										
10.9 µg/L	10.9	B	2		22.4										
10.9 µg/L	10.9	C	2		22.5										
10.9 µg/L	10.9	D	2		22.5										
33 µg/L	33	A	2		22.5										
33 µg/L	33	B	2		22.5										
33 µg/L	33	C	2		22.5										
33 µg/L	33	D	2		22.6										
100 µg/L	100	A	2		22.4										
100 µg/L	100	B	2		22.4										
100 µg/L	100	C	2		22.5										
100 µg/L	100	D	2		22.5										
(Ctl) 0.0 µg/L	0.0	A	3		22.4	7.8	8.1	710							
(Ctl) 0.0 µg/L	0.0	B	3		22.5	8.0	8.1	662							
(Ctl) 0.0 µg/L	0.0	C	3		22.5	8.1	7.9	802							
(Ctl) 0.0 µg/L	0.0	D	3		22.5	7.8	7.9	912							
3.6 µg/L	3.6	A	3		22.4	7.6	7.9	693							
3.6 µg/L	3.6	B	3		22.5	6.0	7.8	723							
3.6 µg/L	3.6	C	3		22.5	7.9	7.8	861							

Name of Treatment Group	Nominal Treatment Concentration (µg/L)	Replicate ID	Test Day (0 to 21)	Measured Treatment Concentration (µg/L)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (ug/L)	Conductivity (µs/cm)	Iodide (µg/L)	Chlorine (ug/L)	Comments
3.6 µg/L	3.6	D	3		22.4	6.8	7.8	803							
10.9 µg/L	10.9	A	3		22.4	6.8	7.8	631							
10.9 µg/L	10.9	B	3		22.5	7.5	7.8	725							
10.9 µg/L	10.9	C	3		22.4	6.9	7.7	602							
10.9 µg/L	10.9	D	3		22.4	6.5	7.7	653							
33 µg/L	33	A	3		22.5	7.2	7.7	620							
33 µg/L	33	B	3		22.4	6.5	7.8	693							
33 µg/L	33	C	3		22.6	6.8	7.7	724							
33 µg/L	33	D	3		22.5	7.7	7.8	665							
100 µg/L	100	A	3		22.4	7.4	7.8	692							
100 µg/L	100	B	3		22.5	7.4	7.9	715							
100 µg/L	100	C	3		22.5	7.2	7.8	782							
100 µg/L	100	D	3		22.4	7.3	7.8	614							
(Ctl) 0.0 µg/L	0.0	A	4		22.4										
(Ctl) 0.0 µg/L	0.0	B	4		22.5										
(Ctl) 0.0 µg/L	0.0	C	4		22.5										
(Ctl) 0.0 µg/L	0.0	D	4		22.4										
3.6 µg/L	3.6	A	4		22.5										
3.6 µg/L	3.6	B	4		22.5										
3.6 µg/L	3.6	C	4		22.5										
3.6 µg/L	3.6	D	4		22.4										
10.9 µg/L	10.9	A	4		22.5										
10.9 µg/L	10.9	B	4		22.4										
10.9 µg/L	10.9	C	4		22.5										
10.9 µg/L	10.9	D	4		22.4										
33 µg/L	33	A	4		22.5										
33 µg/L	33	B	4		22.5										
33 µg/L	33	C	4		22.4										
33 µg/L	33	D	4		22.5										
100 µg/L	100	A	4		22.5										
100 µg/L	100	B	4		22.4										
100 µg/L	100	C	4		22.5										
100 µg/L	100	D	4		22.6										
(Ctl) 0.0 µg/L	0.0	A	5		22.3	7.3	7.8	818							
(Ctl) 0.0 µg/L	0.0	B	5		22.3	7.3	7.8	744							
(Ctl) 0.0 µg/L	0.0	C	5		22.4	7.4	7.8	851							
(Ctl) 0.0 µg/L	0.0	D	5		22.4	7.3	7.8	813							
3.6 µg/L	3.6	A	5		22.3	7.1	7.7	912							
3.6 µg/L	3.6	B	5		22.5	7.0	7.6	805							
3.6 µg/L	3.6	C	5		22.4	7.1	7.8	872							
3.6 µg/L	3.6	D	5		22.7	5.4	7.7	854							
10.9 µg/L	10.9	A	5		22.7	6.7	7.7	615							
10.9 µg/L	10.9	B	5		22.4	5.9	7.9	631							
10.9 µg/L	10.9	C	5		22.5	5.4	7.6	782							
10.9 µg/L	10.9	D	5		22.3	5.9	7.8	667							
33 µg/L	33	A	5		22.7	6.3	7.8	683							
33 µg/L	33	B	5		22.7	6.4	7.8	774							
33 µg/L	33	C	5		22.3	6.4	7.7	702							
33 µg/L	33	D	5		22.7	5.9	7.8	670							
100 µg/L	100	A	5		22.3	7.0	7.8	740							
100 µg/L	100	B	5		22.3	6.9	7.8	812							
100 µg/L	100	C	5		22.7	6.5	7.8	682							
100 µg/L	100	D	5		22.4	6.4	7.8	640							
(Ctl) 0.0 µg/L	0.0	A	6		22.5				140	68	<0.06	626		<0.05	Facility dechlorinated
(Ctl) 0.0 µg/L	0.0	B	6		22.4										tap water
(Ctl) 0.0 µg/L	0.0	C	6		22.4										
(Ctl) 0.0 µg/L	0.0	D	6		22.5										
3.6 µg/L	3.6	A	6		22.5										
3.6 µg/L	3.6	B	6		22.5										
3.6 µg/L	3.6	C	6		22.4										
3.6 µg/L	3.6	D	6		22.4										
10.9 µg/L	10.9	A	6		22.5										
10.9 µg/L	10.9	B	6		22.5										
10.9 µg/L	10.9	C	6		22.4										
10.9 µg/L	10.9	D	6		22.4										
33 µg/L	33	A	6		22.5										
33 µg/L	33	B	6		22.4										
33 µg/L	33	C	6		22.4										
33 µg/L	33	D	6		22.5										

Name of Treatment Group	Nominal Treatment Concentration (µg/L)	Replicate ID	Test Day (0 to 21)	Measured Treatment Concentration (µg/L)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (ug/L)	Conductivity (µs/cm)	Iodide (µg/L)	Chlorine (ug/L)	Comments
100 µg/L	100	A	6		22.5										
100 µg/L	100	B	6		22.5										
100 µg/L	100	C	6		22.4										
100 µg/L	100	D	6		22.5										
(Ctl) 0.0 µg/L	0.0	A	7	0.104	22.4	7.4	7.5	754							**Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctl) 0.0 µg/L	0.0	B	7	0.104	22.5	7.3	7.7	632	140	64					**Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctl) 0.0 µg/L	0.0	C	7	0.104	22.4	7.4	7.7	817							**Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctl) 0.0 µg/L	0.0	D	7	0.104	22.4	7.5	7.8	805							**Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
3.6 µg/L	3.6	A	7	4.47	22.5	5.9	7.8	713							
3.6 µg/L	3.6	B	7	7.28	22.5	6.7	7.8	745							
3.6 µg/L	3.6	C	7	6.04	22.4	6.4	7.8	781							**Measured Treatment Concentration** - Based on mean of original (11.2µg/L) and duplicate (0.887µg/L) sample
3.6 µg/L	3.6	D	7	5.94	22.4	5.6	7.8	642							
10.9 µg/L	10.9	A	7	13.3	22.5	6.3	7.7	716							
10.9 µg/L	10.9	B	7	14.7	22.4	6.1	7.8	831							
10.9 µg/L	10.9	C	7	12.6	22.4	7.2	7.7	714							
10.9 µg/L	10.9	D	7	13.5	22.5	6.4	7.6	697							
33 µg/L	33	A	7	35.4	22.4	7.0	7.7	743							
33 µg/L	33	B	7	30.5	22.5	7.2	7.8	739							
33 µg/L	33	C	7	31.0	22.4	6.4	7.8	778							
33 µg/L	33	D	7	30.5	22.4	6.6	7.8	694							
100 µg/L	100	A	7	89.3	22.5	6.7	7.8	813							
100 µg/L	100	B	7	86.5	22.4	6.3	7.8	740	148	68					
100 µg/L	100	C	7	87.1	22.4	6.3	7.8	817							
100 µg/L	100	D	7	78.6	22.5	6.6	7.7	765							
(Ctl) 0.0 µg/L	0.0	A	8		22.4										
(Ctl) 0.0 µg/L	0.0	B	8		22.5										
(Ctl) 0.0 µg/L	0.0	C	8		22.4										
(Ctl) 0.0 µg/L	0.0	D	8		22.5										
3.6 µg/L	3.6	A	8		22.6										
3.6 µg/L	3.6	B	8		22.4										
3.6 µg/L	3.6	C	8		22.5										
3.6 µg/L	3.6	D	8		22.5										
10.9 µg/L	10.9	A	8		22.4										
10.9 µg/L	10.9	B	8		22.5										
10.9 µg/L	10.9	C	8		22.5										
10.9 µg/L	10.9	D	8		22.6										
33 µg/L	33	A	8		22.4										
33 µg/L	33	B	8		22.5										
33 µg/L	33	C	8		22.5										
33 µg/L	33	D	8		22.4										
100 µg/L	100	A	8		22.4										
100 µg/L	100	B	8		22.4										
100 µg/L	100	C	8		22.5										
100 µg/L	100	D	8		22.5										
(Ctl) 0.0 µg/L	0.0	A	9		22.4										
(Ctl) 0.0 µg/L	0.0	B	9		22.4										
(Ctl) 0.0 µg/L	0.0	C	9		22.5										
(Ctl) 0.0 µg/L	0.0	D	9		22.4										
3.6 µg/L	3.6	A	9		22.6										
3.6 µg/L	3.6	B	9		22.4										
3.6 µg/L	3.6	C	9		22.5										
3.6 µg/L	3.6	D	9		22.5										
10.9 µg/L	10.9	A	9		22.5										
10.9 µg/L	10.9	B	9		22.6										
10.9 µg/L	10.9	C	9		22.4										
10.9 µg/L	10.9	D	9		22.4										
33 µg/L	33	A	9		22.4										
33 µg/L	33	B	9		22.5										
33 µg/L	33	C	9		22.6										
33 µg/L	33	D	9		22.5										
100 µg/L	100	A	9		22.4										
100 µg/L	100	B	9		22.4										
100 µg/L	100	C	9		22.5										
100 µg/L	100	D	9		22.5										

BATT01-00388 FEL

Name of Treatment Group	Nominal Treatment Concentration (µg/L)	Replicate ID	Test Day (0 to 21)	Measured Treatment Concentration (µg/L)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (ug/L)	Conductivity (µs/cm)	Iodide (µg/L)	Chlorine (ug/L)	Comments
(Ctl) 0.0 µg/L	0.0	A	10		22.3	7.7	8.0	692							
(Ctl) 0.0 µg/L	0.0	B	10		22.8	7.8	7.9	713							
(Ctl) 0.0 µg/L	0.0	C	10		22.3	7.7	7.8	851							
(Ctl) 0.0 µg/L	0.0	D	10		22.4	7.8	7.8	739							
3.6 µg/L	3.6	A	10		22.8	7.3	7.8	654							
3.6 µg/L	3.6	B	10		22.3	5.7	7.8	743							
3.6 µg/L	3.6	C	10		22.5	7.6	7.8	807							
3.6 µg/L	3.6	D	10		22.3	5.8	7.7	802							
10.9 µg/L	10.9	A	10		22.7	5.9	7.8	751							
10.9 µg/L	10.9	B	10		22.5	6.5	7.7	769							
10.9 µg/L	10.9	C	10		22.3	6.3	7.8	894							
10.9 µg/L	10.9	D	10		22.3	7.6	7.7	642							
33 µg/L	33	A	10		22.5	6.1	7.7	615							
33 µg/L	33	B	10		22.7	6.5	7.7	778							
33 µg/L	33	C	10		22.3	6.9	7.7	638							
33 µg/L	33	D	10		22.5	7.0	7.7	832							
100 µg/L	100	A	10		22.4	6.6	7.7	654							
100 µg/L	100	B	10		22.3	7.0	7.8	739							
100 µg/L	100	C	10		22.4	6.9	7.7	641							
100 µg/L	100	D	10		22.5	7.0	7.6	761							
(Ctl) 0.0 µg/L	0.0	A	11		22.4										
(Ctl) 0.0 µg/L	0.0	B	11		22.5										
(Ctl) 0.0 µg/L	0.0	C	11		22.7										
(Ctl) 0.0 µg/L	0.0	D	11		22.4										
3.6 µg/L	3.6	A	11		22.6										
3.6 µg/L	3.6	B	11		22.3										
3.6 µg/L	3.6	C	11		22.4										
3.6 µg/L	3.6	D	11		22.3										
10.9 µg/L	10.9	A	11		22.4										
10.9 µg/L	10.9	B	11		22.6										
10.9 µg/L	10.9	C	11		22.5										
10.9 µg/L	10.9	D	11		22.5										
33 µg/L	33	A	11		22.5										
33 µg/L	33	B	11		22.7										
33 µg/L	33	C	11		22.4										
33 µg/L	33	D	11		22.4										
100 µg/L	100	A	11		22.3										
100 µg/L	100	B	11		22.5										
100 µg/L	100	C	11		22.3										
100 µg/L	100	D	11		22.4										
(Ctl) 0.0 µg/L	0.0	A	12		22.3	8.3	7.2	632							
(Ctl) 0.0 µg/L	0.0	B	12		22.3	7.9	7.3	741							
(Ctl) 0.0 µg/L	0.0	C	12		22.7	7.8	7.3	715							
(Ctl) 0.0 µg/L	0.0	D	12		22.7	7.8	7.3	631							
3.6 µg/L	3.6	A	12		22.7	7.4	7.4	715							
3.6 µg/L	3.6	B	12		22.6	7.5	7.7	749							
3.6 µg/L	3.6	C	12		22.6	7.3	7.7	762							
3.6 µg/L	3.6	D	12		22.5	7.0	7.8	731							
10.9 µg/L	10.9	A	12		22.7	7.2	7.9	727							
10.9 µg/L	10.9	B	12		22.4	7.4	7.8	694							
10.9 µg/L	10.9	C	12		22.7	7.3	7.7	675							
10.9 µg/L	10.9	D	12		22.5	7.1	7.8	698							
33 µg/L	33	A	12		22.7	7.4	7.7	718							
33 µg/L	33	B	12		22.6	7.7	7.8	723							
33 µg/L	33	C	12		22.6	7.5	7.8	757							
33 µg/L	33	D	12		22.6	7.5	7.9	774							
100 µg/L	100	A	12		22.7	7.4	7.9	731							
100 µg/L	100	B	12		22.4	7.3	8.0	754							
100 µg/L	100	C	12		22.3	7.1	7.8	776							
100 µg/L	100	D	12		22.3	7.1	7.9	770							
(Ctl) 0.0 µg/L	0.0	A	13		22.4										
(Ctl) 0.0 µg/L	0.0	B	13		22.5										
(Ctl) 0.0 µg/L	0.0	C	13		22.3										
(Ctl) 0.0 µg/L	0.0	D	13		22.4										
3.6 µg/L	3.6	A	13		22.3										
3.6 µg/L	3.6	B	13		22.4										
3.6 µg/L	3.6	C	13		22.3										
3.6 µg/L	3.6	D	13		22.3										
10.9 µg/L	10.9	A	13		22.6										

BATT01-00388 FEL

Name of Treatment Group	Nominal Treatment Concentration (µg/L)	Replicate ID	Test Day (0 to 21)	Measured Treatment Concentration (µg/L)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (ug/L)	Conductivity (µS/cm)	Iodide (ug/L)	Chlorine (ug/L)	Comments
10.9 µg/L	10.9	B	13		22.6										
10.9 µg/L	10.9	C	13		22.7										
10.9 µg/L	10.9	D	13		22.5										
33 µg/L	33	A	13		22.5										
33 µg/L	33	B	13		22.5										
33 µg/L	33	C	13		22.4										
33 µg/L	33	D	13		22.5										
100 µg/L	100	A	13		22.3										
100 µg/L	100	B	13		22.3										
100 µg/L	100	C	13		22.3										
100 µg/L	100	D	13		22.4										
(Ctl) 0.0 µg/L	0.0	A	14	0.104	22.3	7.2	7.7	631							**Measured Treatment Concentration** - MQL = 0.208µg/L; For results ≤MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctl) 0.0 µg/L	0.0	B	14	0.104	22.4	7.0	7.6	672							**Measured Treatment Concentration** - MQL = 0.208µg/L; For results ≤MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctl) 0.0 µg/L	0.0	C	14	0.104	22.4	7.9	7.6	641	128	60					**Measured Treatment Concentration** - MQL = 0.208µg/L; For results ≤MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctl) 0.0 µg/L	0.0	D	14	0.104	22.3	6.9	7.8	659							**Measured Treatment Concentration** - MQL = 0.208µg/L; For results ≤MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
3.6 µg/L	3.6	A	14	5.08	22.5	6.3	7.9	662							
3.6 µg/L	3.6	B	14	8.05	22.5	6.5	7.8	664							
3.6 µg/L	3.6	C	14	5.86	22.6	6.7	7.8	703							
3.6 µg/L	3.6	D	14	6.50	22.3	7.4	7.8	715							
10.9 µg/L	10.9	A	14	14.9	22.4	5.0	7.9	680							
10.9 µg/L	10.9	B	14	10.9	22.4	5.4	7.8	676							
10.9 µg/L	10.9	C	14	12.9	22.3	6.8	7.7	616							
10.9 µg/L	10.9	D	14	13.6	22.5	6.2	7.7	674							
33 µg/L	33	A	14	25.8	22.7	4.6	7.6	699							
33 µg/L	33	B	14	28.3	22.6	6.9	7.6	690							
33 µg/L	33	C	14	31.6	22.6	7.2	7.8	715							
33 µg/L	33	D	14	31.2	22.5	5.8	7.7	701							
100 µg/L	100	A	14	51.3	22.4	7.2	7.8	683							**Measured Treatment Concentration** - Based an mean of original [65.5µg/L] and duplicate [37.1µg/L] sample
100 µg/L	100	B	14	58.3	22.3	5.9	7.8	645							**Measured Treatment Concentration** - Based an mean of original [84.4µg/L] and duplicate [32.1µg/L] sample
100 µg/L	100	C	14	62.3	22.3	7.6	7.9	682	132	56					**Measured Treatment Concentration** - Based an mean of original [87.5µg/L] and duplicate [37.0µg/L] sample
100 µg/L	100	D	14	57.0	22.3	6.6	7.9	678							**Measured Treatment Concentration** - Based an mean of original [80.1µg/L] and duplicate [33.9µg/L] sample
(Ctl) 0.0 µg/L	0.0	A	15		22.5										
(Ctl) 0.0 µg/L	0.0	B	15		22.3										
(Ctl) 0.0 µg/L	0.0	C	15		22.4										
(Ctl) 0.0 µg/L	0.0	D	15		22.4										
3.6 µg/L	3.6	A	15		22.6										
3.6 µg/L	3.6	B	15		22.3										
3.6 µg/L	3.6	C	15		22.5										
3.6 µg/L	3.6	D	15		22.4										
10.9 µg/L	10.9	A	15		22.4										
10.9 µg/L	10.9	B	15		22.7										
10.9 µg/L	10.9	C	15		22.6										
10.9 µg/L	10.9	D	15		22.7										
33 µg/L	33	A	15		22.3										
33 µg/L	33	B	15		22.3										
33 µg/L	33	C	15		22.5										
33 µg/L	33	D	15		22.6										
100 µg/L	100	A	15		22.7										
100 µg/L	100	B	15		22.3										
100 µg/L	100	C	15		22.4										
100 µg/L	100	D	15		22.4										
(Ctl) 0.0 µg/L	0.0	A	16		22.4										
(Ctl) 0.0 µg/L	0.0	B	16		22.6										
(Ctl) 0.0 µg/L	0.0	C	16		22.5										
(Ctl) 0.0 µg/L	0.0	D	16		22.6										
3.6 µg/L	3.6	A	16		22.7										
3.6 µg/L	3.6	B	16		22.6										
3.6 µg/L	3.6	C	16		22.3										
3.6 µg/L	3.6	D	16		22.5										
10.9 µg/L	10.9	A	16		22.6										
10.9 µg/L	10.9	B	16		22.7										

DESF

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Analytical Water Quality

Name of Treatment Group	Nominal Treatment Concentration (µg/L)	Replicate ID	Test Day (0 to 21)	Measured Treatment Concentration (µg/L)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (ug/L)	Conductivity (µS/cm)	Iodide (ug/L)	Chlorine (ug/L)	Comments
10.9 µg/L	10.9	C	16		22.4										
10.9 µg/L	10.9	D	16		22.6										
33 µg/L	33	A	16		22.7										
33 µg/L	33	B	16		22.5										
33 µg/L	33	C	16		22.5										
33 µg/L	33	D	16		22.5										
100 µg/L	100	A	16		22.6										
100 µg/L	100	B	16		22.7										
100 µg/L	100	C	16		22.4										
100 µg/L	100	D	16		22.7										
(CF) 0.0 µg/L	0.0	A	17		22.6	6.8	7.7	659							
(CF) 0.0 µg/L	0.0	B	17		22.6	6.9	7.7	672							
(CF) 0.0 µg/L	0.0	C	17		22.7	6.2	7.6	684							
(CF) 0.0 µg/L	0.0	D	17		22.7	6.4	7.6	670							
3.6 µg/L	3.6	A	17		22.6	5.5	7.5	694							
3.6 µg/L	3.6	B	17		22.6	5.6	7.5	901							
3.6 µg/L	3.6	C	17		22.7	4.9	7.5	923							
3.6 µg/L	3.6	D	17		22.7	4.2	7.6	852							
10.9 µg/L	10.9	A	17		22.6	5.4	7.5	734							
10.9 µg/L	10.9	B	17		22.5	5.3	7.6	703							
10.9 µg/L	10.9	C	17		22.6	5.4	7.6	719							
10.9 µg/L	10.9	D	17		22.6	6.3	7.6	698							
33 µg/L	33	A	17		22.3	5.0	7.5	776							
33 µg/L	33	B	17		22.4	5.4	7.5	681							
33 µg/L	33	C	17		22.7	5.4	7.5	675							
33 µg/L	33	D	17		22.6	6.2	7.5	759							
100 µg/L	100	A	17		22.3	5.8	7.5	732							
100 µg/L	100	B	17		22.4	5.9	7.5	724							
100 µg/L	100	C	17		22.4	5.4	7.5	690							
100 µg/L	100	D	17		22.3	5.4	7.5	669							
(CF) 0.0 µg/L	0.0	A	18		22.5										
(CF) 0.0 µg/L	0.0	B	18		22.6										
(CF) 0.0 µg/L	0.0	C	18		22.4										
(CF) 0.0 µg/L	0.0	D	18		22.4										
3.6 µg/L	3.6	A	18		22.5										
3.6 µg/L	3.6	B	18		22.4										
3.6 µg/L	3.6	C	18		22.6										
3.6 µg/L	3.6	D	18		22.5										
10.9 µg/L	10.9	A	18		22.5										
10.9 µg/L	10.9	B	18		22.5										
10.9 µg/L	10.9	C	18		22.4										
10.9 µg/L	10.9	D	18		22.4										
33 µg/L	33	A	18		22.3										
33 µg/L	33	B	18		22.4										
33 µg/L	33	C	18		22.5										
33 µg/L	33	D	18		22.6										
100 µg/L	100	A	18		22.4										
100 µg/L	100	B	18		22.3										
100 µg/L	100	C	18		22.3										
100 µg/L	100	D	18		22.3										
(CF) 0.0 µg/L	0.0	A	19		22.3	7.2	7.7	693							
(CF) 0.0 µg/L	0.0	B	19		22.3	7.3	7.8	645							
(CF) 0.0 µg/L	0.0	C	19		22.3	7.4	7.7	701							
(CF) 0.0 µg/L	0.0	D	19		22.7	7.1	7.7	737							
3.6 µg/L	3.6	A	19		22.7	7.3	7.6	694							
3.6 µg/L	3.6	B	19		22.7	6.8	7.6	688							
3.6 µg/L	3.6	C	19		22.3	7.0	7.7	661							
3.6 µg/L	3.6	D	19		22.3	5.8	7.6	670							
10.9 µg/L	10.9	A	19		22.7	4.8	7.5	692							
10.9 µg/L	10.9	B	19		22.7	5.8	7.4	634							
10.9 µg/L	10.9	C	19		22.4	5.7	7.4	620							
10.9 µg/L	10.9	D	19		22.3	6.2	7.5	695							
33 µg/L	33	A	19		22.7	5.8	7.5	708							
33 µg/L	33	B	19		22.7	5.9	7.5	724							
33 µg/L	33	C	19		22.7	5.4	7.5	739							
33 µg/L	33	D	19		22.7	6.2	7.5	695							
100 µg/L	100	A	19		22.3	5.6	7.5	681							
100 µg/L	100	B	19		22.7	6.0	7.5	772							
100 µg/L	100	C	19		22.7	5.8	7.4	736							

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Name of Treatment Group	Nominal Treatment Concentration (µg/L)	Replicate ID	Test Day (0 to 21)	Measured Treatment Concentration (µg/L)	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (ug/L)	Conductivity (µs/cm)	Iodide (µg/L)	Chlorine (ug/L)	Comments
100 µg/L	100	D	19		22.3	5.7	7.5	816							
(Ctl) 0.0 µg/L	0.0	A	20		22.5	7.3	7.6	754	120	52	<0.06	602		<0.05	Facility dechlorinated
(Ctl) 0.0 µg/L	0.0	B	20		22.5	7.5	7.5	769							tap water
(Ctl) 0.0 µg/L	0.0	C	20		22.4	7.6	7.5	783							
(Ctl) 0.0 µg/L	0.0	D	20		22.5	7.6	7.6	802							
3.6 µg/L	3.6	A	20		22.6	7.2	7.7	750							
3.6 µg/L	3.6	B	20		22.5	7.0	7.6	683							
3.6 µg/L	3.6	C	20		22.5	7.1	7.5	692							
3.6 µg/L	3.6	D	20		22.6	7.2	7.6	783							
10.9 µg/L	10.9	A	20		22.4	6.8	7.7	812							
10.9 µg/L	10.9	B	20		22.5	7.2	7.6	800							
10.9 µg/L	10.9	C	20		22.5	7.0	7.5	816							
10.9 µg/L	10.9	D	20		22.5	7.1	7.5	883							
33 µg/L	33	A	20		22.6	7.0	7.6	796							
33 µg/L	33	B	20		22.5	6.6	7.6	823							
33 µg/L	33	C	20		22.5	6.8	7.6	852							
33 µg/L	33	D	20		22.5	7.1	7.5	824							
100 µg/L	100	A	20		22.6	6.9	7.6	699							
100 µg/L	100	B	20		22.5	6.8	7.7	759							
100 µg/L	100	C	20		22.5	6.5	7.6	825							
100 µg/L	100	D	20		22.5	6.9	7.6	816							
(Ctl) 0.0 µg/L	0.0	A	21	0.104	22.4								9.2		I-measured in facility dilution water; **Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment. Based on mean of original (0.104µg/L) and duplicate (0.104µg/L) sample
(Ctl) 0.0 µg/L	0.0	B	21	0.104	22.4										**Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment. Based on mean of original (0.104µg/L) and duplicate (0.104µg/L) sample
(Ctl) 0.0 µg/L	0.0	C	21	0.104	22.5										**Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment. Based on mean of original (0.104µg/L) and duplicate (0.104µg/L) sample
(Ctl) 0.0 µg/L	0.0	D	21	0.104	22.4				132	48					**Measured Treatment Concentration** - MQL = 0.208µg/L; For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment. Based on mean of original (0.104µg/L) and duplicate (0.104µg/L) sample
3.6 µg/L	3.6	A	21	2.42	22.3										**Measured Treatment Concentration** - Based on mean of original (1.26µg/L) and duplicate (3.58µg/L) sample
3.6 µg/L	3.6	B	21	2.31	22.6										**Measured Treatment Concentration** - Based on mean of original (1.19µg/L) and duplicate (3.42µg/L) sample
3.6 µg/L	3.6	C	21	2.27	22.6										**Measured Treatment Concentration** - Based on mean of original (1.12µg/L) and duplicate (3.42µg/L) sample
3.6 µg/L	3.6	D	21	2.43	22.5										**Measured Treatment Concentration** - Based on mean of original (1.33µg/L) and duplicate (3.52µg/L) sample
10.9 µg/L	10.9	A	21	5.19	22.5										**Measured Treatment Concentration** - Based on mean of original (3.95µg/L) and duplicate (6.42µg/L) sample
10.9 µg/L	10.9	B	21	5.37	22.3										**Measured Treatment Concentration** - Based on mean of original (3.82µg/L) and duplicate (6.91µg/L) sample
10.9 µg/L	10.9	C	21	5.24	22.6										**Measured Treatment Concentration** - Based on mean of original (4.11µg/L) and duplicate (6.36µg/L) sample
10.9 µg/L	10.9	D	21	4.95	22.3										**Measured Treatment Concentration** - Based on mean of original (3.87µg/L) and duplicate (6.03µg/L) sample
33 µg/L	33	A	21	14.6	22.6										**Measured Treatment Concentration** - Based on mean of original (12.5µg/L) and duplicate (16.7µg/L) sample
33 µg/L	33	B	21	16.4	22.5										**Measured Treatment Concentration** - Based on mean of original (16.6µg/L) and duplicate (16.2µg/L) sample
33 µg/L	33	C	21	14.7	22.3										**Measured Treatment Concentration** - Based on mean of original (11.4µg/L) and duplicate (18.0µg/L) sample
33 µg/L	33	D	21	15.8	22.4										**Measured Treatment Concentration** - Based on mean of original (15.7µg/L) and duplicate (15.8µg/L) sample
100 µg/L	100	A	21	41.5	22.4										**Measured Treatment Concentration** - Based on mean of original (44.3µg/L) and duplicate (38.7µg/L) sample
100 µg/L	100	B	21	41.1	22.5										**Measured Treatment Concentration** - Based on mean of original (42.1µg/L) and duplicate (40.0µg/L) sample
100 µg/L	100	C	21	41.2	22.6										**Measured Treatment Concentration** - Based on mean of original (43.2µg/L) and duplicate (39.1µg/L) sample
100 µg/L	100	D	21	39.2	22.4				128	52					**Measured Treatment Concentration** - Based on mean of original (40.2µg/L) and duplicate (38.2µg/L) sample

FEL

DEST BATT01-00388 Mortality (Day 0-7)

Name of Treatment Group	Replicate ID	Initial Number (Day 0)	Test Day (1 to 7)	Mortality (# dead)	Comments
(Ctl) 0.0 µg/L	A	20	1	**0**	
(Ctl) 0.0 µg/L	B	20	1	**0**	
(Ctl) 0.0 µg/L	C	20	1	**0**	
(Ctl) 0.0 µg/L	D	20	1	**0**	
3.6 µg/L	A	20	1	**0**	
3.6 µg/L	B	20	1	**0**	
3.6 µg/L	C	20	1	**0**	
3.6 µg/L	D	20	1	**0**	
10.9 µg/L	A	20	1	**0**	
10.9 µg/L	B	20	1	**0**	
10.9 µg/L	C	20	1	**0**	
10.9 µg/L	D	20	1	**0**	
33 µg/L	A	20	1	**0**	
33 µg/L	B	20	1	**0**	
33 µg/L	C	20	1	**0**	
33 µg/L	D	20	1	**0**	
100 µg/L	A	20	1	**0**	
100 µg/L	B	20	1	**0**	
100 µg/L	C	20	1	**0**	
100 µg/L	D	20	1	**0**	
(Ctl) 0.0 µg/L	A	20	2	**0**	
(Ctl) 0.0 µg/L	B	20	2	**0**	
(Ctl) 0.0 µg/L	C	20	2	**0**	
(Ctl) 0.0 µg/L	D	20	2	**0**	
3.6 µg/L	A	20	2	**0**	
3.6 µg/L	B	20	2	**0**	
3.6 µg/L	C	20	2	**0**	
3.6 µg/L	D	20	2	**0**	
10.9 µg/L	A	20	2	**0**	
10.9 µg/L	B	20	2	**0**	
10.9 µg/L	C	20	2	**0**	
10.9 µg/L	D	20	2	**0**	
33 µg/L	A	20	2	**0**	
33 µg/L	B	20	2	**0**	
33 µg/L	C	20	2	**0**	
33 µg/L	D	20	2	**0**	
100 µg/L	A	20	2	**0**	
100 µg/L	B	20	2	**0**	
100 µg/L	C	20	2	**0**	
100 µg/L	D	20	2	**0**	
(Ctl) 0.0 µg/L	A	20	3	**0**	
(Ctl) 0.0 µg/L	B	20	3	**0**	
(Ctl) 0.0 µg/L	C	20	3	**0**	
(Ctl) 0.0 µg/L	D	20	3	**0**	

DEST BATT01-00388 Mortality (Day 0-7)

Name of Treatment Group	Replicate ID	Initial Number (Day 0)	Test Day (1 to 7)	Mortality (# dead)	Comments
3.6 µg/L	A	20	3	0	
3.6 µg/L	B	20	3	0	
3.6 µg/L	C	20	3	0	
3.6 µg/L	D	20	3	0	
10.9 µg/L	A	20	3	0	
10.9 µg/L	B	20	3	0	
10.9 µg/L	C	20	3	0	
10.9 µg/L	D	20	3	0	
33 µg/L	A	20	3	0	
33 µg/L	B	20	3	0	
33 µg/L	C	20	3	0	
33 µg/L	D	20	3	0	
100 µg/L	A	20	3	0	
100 µg/L	B	20	3	0	
100 µg/L	C	20	3	0	
100 µg/L	D	20	3	0	
(Ctl) 0.0 µg/L	A	20	4	0	
(Ctl) 0.0 µg/L	B	20	4	0	
(Ctl) 0.0 µg/L	C	20	4	0	
(Ctl) 0.0 µg/L	D	20	4	0	
3.6 µg/L	A	20	4	0	
3.6 µg/L	B	20	4	0	
3.6 µg/L	C	20	4	0	
3.6 µg/L	D	20	4	0	
10.9 µg/L	A	20	4	0	
10.9 µg/L	B	20	4	0	
10.9 µg/L	C	20	4	0	
10.9 µg/L	D	20	4	0	
33 µg/L	A	20	4	0	
33 µg/L	B	20	4	0	
33 µg/L	C	20	4	0	
33 µg/L	D	20	4	0	
100 µg/L	A	20	4	0	
100 µg/L	B	20	4	0	
100 µg/L	C	20	4	0	
100 µg/L	D	20	4	0	
(Ctl) 0.0 µg/L	A	20	5	0	
(Ctl) 0.0 µg/L	B	20	5	0	
(Ctl) 0.0 µg/L	C	20	5	0	
(Ctl) 0.0 µg/L	D	20	5	0	
3.6 µg/L	A	20	5	0	
3.6 µg/L	B	20	5	0	
3.6 µg/L	C	20	5	0	
3.6 µg/L	D	20	5	0	

DEST BATT01-00388 Mortality (Day 0-7)

Name of Treatment Group	Replicate ID	Initial Number (Day 0)	Test Day (1 to 7)	Mortality (# dead)	Comments
10.9 µg/L	A	20	5	0	
10.9 µg/L	B	20	5	0	
10.9 µg/L	C	20	5	0	
10.9 µg/L	D	20	5	0	
33 µg/L	A	20	5	0	
33 µg/L	B	20	5	0	
33 µg/L	C	20	5	0	
33 µg/L	D	20	5	0	
100 µg/L	A	20	5	0	
100 µg/L	B	20	5	0	
100 µg/L	C	20	5	0	
100 µg/L	D	20	5	0	
(Ctl) 0.0 µg/L	A	20	6	0	
(Ctl) 0.0 µg/L	B	20	6	0	
(Ctl) 0.0 µg/L	C	20	6	0	
(Ctl) 0.0 µg/L	D	20	6	0	
3.6 µg/L	A	20	6	0	
3.6 µg/L	B	20	6	0	
3.6 µg/L	C	20	6	0	
3.6 µg/L	D	20	6	0	
10.9 µg/L	A	20	6	0	
10.9 µg/L	B	20	6	0	
10.9 µg/L	C	20	6	0	
10.9 µg/L	D	20	6	0	
33 µg/L	A	20	6	0	
33 µg/L	B	20	6	0	
33 µg/L	C	20	6	0	
33 µg/L	D	20	6	0	
100 µg/L	A	20	6	0	
100 µg/L	B	20	6	0	
100 µg/L	C	20	6	0	
100 µg/L	D	20	6	0	
(Ctl) 0.0 µg/L	A	20	7	0	
(Ctl) 0.0 µg/L	B	20	7	0	
(Ctl) 0.0 µg/L	C	20	7	0	
(Ctl) 0.0 µg/L	D	20	7	0	
3.6 µg/L	A	20	7	0	
3.6 µg/L	B	20	7	0	
3.6 µg/L	C	20	7	0	
3.6 µg/L	D	20	7	0	
10.9 µg/L	A	20	7	0	
10.9 µg/L	B	20	7	0	
10.9 µg/L	C	20	7	0	
10.9 µg/L	D	20	7	0	

DEST BATT01-00388 Mortality (Day 0-7)

Name of Treatment Group	Replicate ID	Initial Number (Day 0)	Test Day (1 to 7)	Mortality (# dead)	Comments
33 µg/L	A	20	7	**0**	
33 µg/L	B	20	7	**0**	
33 µg/L	C	20	7	**0**	
33 µg/L	D	20	7	**0**	
100 µg/L	A	20	7	**0**	
100 µg/L	B	20	7	**0**	
100 µg/L	C	20	7	**0**	
100 µg/L	D	20	7	**0**	

DEST BATT01-00388 Mortality (Day 7-21)

Name of Treatment Group	Replicate ID	Initial Number (Day 7)	Test Day (8 to 21)	Mortality (# dead)	Comments
(Ctl) 0.0 µg/L	A	15	8	0	
(Ctl) 0.0 µg/L	B	15	8	0	
(Ctl) 0.0 µg/L	C	15	8	0	
(Ctl) 0.0 µg/L	D	15	8	0	
3.6 µg/L	A	15	8	0	
3.6 µg/L	B	15	8	0	
3.6 µg/L	C	15	8	0	
3.6 µg/L	D	15	8	0	
10.9 µg/L	A	15	8	0	
10.9 µg/L	B	15	8	0	
10.9 µg/L	C	15	8	0	
10.9 µg/L	D	15	8	0	
33 µg/L	A	15	8	0	
33 µg/L	B	15	8	0	
33 µg/L	C	15	8	0	
33 µg/L	D	15	8	0	
100 µg/L	A	15	8	0	
100 µg/L	B	15	8	0	
100 µg/L	C	15	8	0	
100 µg/L	D	15	8	0	
(Ctl) 0.0 µg/L	A	15	9	0	
(Ctl) 0.0 µg/L	B	15	9	0	
(Ctl) 0.0 µg/L	C	15	9	0	
(Ctl) 0.0 µg/L	D	15	9	0	
3.6 µg/L	A	15	9	0	
3.6 µg/L	B	15	9	0	
3.6 µg/L	C	15	9	0	
3.6 µg/L	D	15	9	0	
10.9 µg/L	A	15	9	0	
10.9 µg/L	B	15	9	0	
10.9 µg/L	C	15	9	0	
10.9 µg/L	D	15	9	0	
33 µg/L	A	15	9	0	
33 µg/L	B	15	9	0	
33 µg/L	C	15	9	0	
33 µg/L	D	15	9	0	
100 µg/L	A	15	9	0	
100 µg/L	B	15	9	0	
100 µg/L	C	15	9	0	
100 µg/L	D	15	9	0	
(Ctl) 0.0 µg/L	A	15	10	0	
(Ctl) 0.0 µg/L	B	15	10	0	
(Ctl) 0.0 µg/L	C	15	10	0	
(Ctl) 0.0 µg/L	D	15	10	0	

DEST BATT01-00388 Mortality (Day 7-21)

Name of Treatment Group	Replicate ID	Initial Number (Day 7)	Test Day (8 to 21)	Mortality (# dead)	Comments
3.6 µg/L	A	15	10	0	
3.6 µg/L	B	15	10	0	
3.6 µg/L	C	15	10	0	
3.6 µg/L	D	15	10	0	
10.9 µg/L	A	15	10	0	
10.9 µg/L	B	15	10	0	
10.9 µg/L	C	15	10	0	
10.9 µg/L	D	15	10	0	
33 µg/L	A	15	10	0	
33 µg/L	B	15	10	0	
33 µg/L	C	15	10	0	
33 µg/L	D	15	10	0	
100 µg/L	A	15	10	0	
100 µg/L	B	15	10	0	
100 µg/L	C	15	10	0	
100 µg/L	D	15	10	0	
(Ctl) 0.0 µg/L	A	15	11	0	
(Ctl) 0.0 µg/L	B	15	11	0	
(Ctl) 0.0 µg/L	C	15	11	0	
(Ctl) 0.0 µg/L	D	15	11	0	
3.6 µg/L	A	15	11	0	
3.6 µg/L	B	15	11	0	
3.6 µg/L	C	15	11	0	
3.6 µg/L	D	15	11	0	
10.9 µg/L	A	15	11	0	
10.9 µg/L	B	15	11	0	
10.9 µg/L	C	15	11	0	
10.9 µg/L	D	15	11	0	
33 µg/L	A	15	11	0	
33 µg/L	B	15	11	0	
33 µg/L	C	15	11	0	
33 µg/L	D	15	11	0	
100 µg/L	A	15	11	0	
100 µg/L	B	15	11	0	
100 µg/L	C	15	11	0	
100 µg/L	D	15	11	0	
(Ctl) 0.0 µg/L	A	15	12	0	
(Ctl) 0.0 µg/L	B	15	12	0	
(Ctl) 0.0 µg/L	C	15	12	0	
(Ctl) 0.0 µg/L	D	15	12	0	
3.6 µg/L	A	15	12	0	
3.6 µg/L	B	15	12	0	
3.6 µg/L	C	15	12	0	
3.6 µg/L	D	15	12	0	

DEST BATT01-00388 Mortality (Day 7-21)

Name of Treatment Group	Replicate ID	Initial Number (Day 7)	Test Day (8 to 21)	Mortality (# dead)	Comments
10.9 µg/L	A	15	12	0	
10.9 µg/L	B	15	12	0	
10.9 µg/L	C	15	12	0	
10.9 µg/L	D	15	12	0	
33 µg/L	A	15	12	0	
33 µg/L	B	15	12	0	
33 µg/L	C	15	12	0	
33 µg/L	D	15	12	0	
100 µg/L	A	15	12	0	
100 µg/L	B	15	12	0	
100 µg/L	C	15	12	0	
100 µg/L	D	15	12	0	
(Ctl) 0.0 µg/L	A	15	13	0	
(Ctl) 0.0 µg/L	B	15	13	0	
(Ctl) 0.0 µg/L	C	15	13	0	
(Ctl) 0.0 µg/L	D	15	13	0	
3.6 µg/L	A	15	13	0	
3.6 µg/L	B	15	13	0	
3.6 µg/L	C	15	13	0	
3.6 µg/L	D	15	13	0	
10.9 µg/L	A	15	13	0	
10.9 µg/L	B	15	13	0	
10.9 µg/L	C	15	13	0	
10.9 µg/L	D	15	13	0	
33 µg/L	A	15	13	0	
33 µg/L	B	15	13	0	
33 µg/L	C	15	13	0	
33 µg/L	D	15	13	0	
100 µg/L	A	15	13	0	
100 µg/L	B	15	13	0	
100 µg/L	C	15	13	0	
100 µg/L	D	15	13	0	
(Ctl) 0.0 µg/L	A	15	14	0	
(Ctl) 0.0 µg/L	B	15	14	0	
(Ctl) 0.0 µg/L	C	15	14	0	
(Ctl) 0.0 µg/L	D	15	14	0	
3.6 µg/L	A	15	14	0	
3.6 µg/L	B	15	14	0	
3.6 µg/L	C	15	14	0	
3.6 µg/L	D	15	14	0	
10.9 µg/L	A	15	14	0	
10.9 µg/L	B	15	14	0	
10.9 µg/L	C	15	14	0	
10.9 µg/L	D	15	14	0	

DEST BATT01-00388 Mortality (Day 7-21)

Name of Treatment Group	Replicate ID	Initial Number (Day 7)	Test Day (8 to 21)	Mortality (# dead)	Comments
33 µg/L	A	15	14	0	
33 µg/L	B	15	14	0	
33 µg/L	C	15	14	0	
33 µg/L	D	15	14	0	
100 µg/L	A	15	14	0	
100 µg/L	B	15	14	0	
100 µg/L	C	15	14	0	
100 µg/L	D	15	14	0	
(Ctl) 0.0 µg/L	A	15	15	0	
(Ctl) 0.0 µg/L	B	15	15	0	
(Ctl) 0.0 µg/L	C	15	15	0	
(Ctl) 0.0 µg/L	D	15	15	0	
3.6 µg/L	A	15	15	0	
3.6 µg/L	B	15	15	0	
3.6 µg/L	C	15	15	0	
3.6 µg/L	D	15	15	0	
10.9 µg/L	A	15	15	0	
10.9 µg/L	B	15	15	0	
10.9 µg/L	C	15	15	0	
10.9 µg/L	D	15	15	0	
33 µg/L	A	15	15	0	
33 µg/L	B	15	15	0	
33 µg/L	C	15	15	0	
33 µg/L	D	15	15	0	
100 µg/L	A	15	15	0	
100 µg/L	B	15	15	0	
100 µg/L	C	15	15	0	
100 µg/L	D	15	15	0	
(Ctl) 0.0 µg/L	A	15	16	0	
(Ctl) 0.0 µg/L	B	15	16	0	
(Ctl) 0.0 µg/L	C	15	16	0	
(Ctl) 0.0 µg/L	D	15	16	0	
3.6 µg/L	A	15	16	0	
3.6 µg/L	B	15	16	0	
3.6 µg/L	C	15	16	0	
3.6 µg/L	D	15	16	0	
10.9 µg/L	A	15	16	0	
10.9 µg/L	B	15	16	0	
10.9 µg/L	C	15	16	0	
10.9 µg/L	D	15	16	0	
33 µg/L	A	15	16	0	
33 µg/L	B	15	16	0	
33 µg/L	C	15	16	0	
33 µg/L	D	15	16	0	

DEST BATT01-00388 Mortality (Day 7-21)

Name of Treatment Group	Replicate ID	Initial Number (Day 7)	Test Day (8 to 21)	Mortality (# dead)	Comments
100 µg/L	A	15	16	0	
100 µg/L	B	15	16	0	
100 µg/L	C	15	16	0	
100 µg/L	D	15	16	0	
(Ctl) 0.0 µg/L	A	15	17	0	
(Ctl) 0.0 µg/L	B	15	17	0	
(Ctl) 0.0 µg/L	C	15	17	0	
(Ctl) 0.0 µg/L	D	15	17	0	
3.6 µg/L	A	15	17	0	
3.6 µg/L	B	15	17	0	
3.6 µg/L	C	15	17	0	
3.6 µg/L	D	15	17	0	
10.9 µg/L	A	15	17	0	
10.9 µg/L	B	15	17	0	
10.9 µg/L	C	15	17	0	
10.9 µg/L	D	15	17	0	
33 µg/L	A	15	17	0	
33 µg/L	B	15	17	0	
33 µg/L	C	15	17	0	
33 µg/L	D	15	17	0	
100 µg/L	A	15	17	0	
100 µg/L	B	15	17	0	
100 µg/L	C	15	17	0	
100 µg/L	D	15	17	0	
(Ctl) 0.0 µg/L	A	15	18	0	
(Ctl) 0.0 µg/L	B	15	18	0	
(Ctl) 0.0 µg/L	C	15	18	0	
(Ctl) 0.0 µg/L	D	15	18	0	
3.6 µg/L	A	15	18	0	
3.6 µg/L	B	15	18	0	
3.6 µg/L	C	15	18	0	
3.6 µg/L	D	15	18	0	
10.9 µg/L	A	15	18	0	
10.9 µg/L	B	15	18	0	
10.9 µg/L	C	15	18	0	
10.9 µg/L	D	15	18	0	
33 µg/L	A	15	18	0	
33 µg/L	B	15	18	0	
33 µg/L	C	15	18	0	
33 µg/L	D	15	18	0	
100 µg/L	A	15	18	0	
100 µg/L	B	15	18	0	
100 µg/L	C	15	18	0	
100 µg/L	D	15	18	0	

DEST BATT01-00388 Mortality (Day 7-21)

Name of Treatment Group	Replicate ID	Initial Number (Day 7)	Test Day (8 to 21)	Mortality (# dead)	Comments
(Ctl) 0.0 µg/L	A	15	19	0	
(Ctl) 0.0 µg/L	B	15	19	0	
(Ctl) 0.0 µg/L	C	15	19	0	
(Ctl) 0.0 µg/L	D	15	19	0	
3.6 µg/L	A	15	19	0	
3.6 µg/L	B	15	19	0	
3.6 µg/L	C	15	19	0	
3.6 µg/L	D	15	19	0	
10.9 µg/L	A	15	19	0	
10.9 µg/L	B	15	19	0	
10.9 µg/L	C	15	19	0	
10.9 µg/L	D	15	19	0	
33 µg/L	A	15	19	0	
33 µg/L	B	15	19	0	
33 µg/L	C	15	19	0	
33 µg/L	D	15	19	0	
100 µg/L	A	15	19	0	
100 µg/L	B	15	19	0	
100 µg/L	C	15	19	0	
100 µg/L	D	15	19	0	
(Ctl) 0.0 µg/L	A	15	20	0	
(Ctl) 0.0 µg/L	B	15	20	0	
(Ctl) 0.0 µg/L	C	15	20	0	
(Ctl) 0.0 µg/L	D	15	20	0	
3.6 µg/L	A	15	20	0	
3.6 µg/L	B	15	20	0	
3.6 µg/L	C	15	20	0	
3.6 µg/L	D	15	20	0	
10.9 µg/L	A	15	20	0	
10.9 µg/L	B	15	20	0	
10.9 µg/L	C	15	20	0	
10.9 µg/L	D	15	20	0	
33 µg/L	A	15	20	0	
33 µg/L	B	15	20	0	
33 µg/L	C	15	20	0	
33 µg/L	D	15	20	0	
100 µg/L	A	15	20	0	
100 µg/L	B	15	20	0	
100 µg/L	C	15	20	0	
100 µg/L	D	15	20	0	
(Ctl) 0.0 µg/L	A	15	21	0	
(Ctl) 0.0 µg/L	B	15	21	0	
(Ctl) 0.0 µg/L	C	15	21	0	
(Ctl) 0.0 µg/L	D	15	21	0	

DEST BATT01-00388 Mortality (Day 7-21)

Name of Treatment Group	Replicate ID	Initial Number (Day 7)	Test Day (8 to 21)	Mortality (# dead)	Comments
3.6 µg/L	A	15	21	**0**	
3.6 µg/L	B	15	21	**0**	
3.6 µg/L	C	15	21	**0**	
3.6 µg/L	D	15	21	**0**	
10.9 µg/L	A	15	21	**0**	
10.9 µg/L	B	15	21	**0**	
10.9 µg/L	C	15	21	**0**	
10.9 µg/L	D	15	21	**0**	
33 µg/L	A	15	21	**0**	
33 µg/L	B	15	21	**0**	
33 µg/L	C	15	21	**0**	
33 µg/L	D	15	21	**0**	
100 µg/L	A	15	21	**0**	
100 µg/L	B	15	21	**0**	
100 µg/L	C	15	21	**0**	
100 µg/L	D	15	21	**0**	

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Clinical Signs	Number Affected
(Ctl) 0.0 µg/L	A	0	None	N/A
(Ctl) 0.0 µg/L	B	0	None	N/A
(Ctl) 0.0 µg/L	C	0	None	N/A
(Ctl) 0.0 µg/L	D	0	None	N/A
3.6 µg/L	A	0	None	N/A
3.6 µg/L	B	0	None	N/A
3.6 µg/L	C	0	None	N/A
3.6 µg/L	D	0	None	N/A
10.9 µg/L	A	0	None	N/A
10.9 µg/L	B	0	None	N/A
10.9 µg/L	C	0	None	N/A
10.9 µg/L	D	0	None	N/A
33 µg/L	A	0	None	N/A
33 µg/L	B	0	None	N/A
33 µg/L	C	0	None	N/A
33 µg/L	D	0	None	N/A
100 µg/L	A	0	None	N/A
100 µg/L	B	0	None	N/A
100 µg/L	C	0	None	N/A
100 µg/L	D	0	None	N/A
(Ctl) 0.0 µg/L	A	1	None	N/A
(Ctl) 0.0 µg/L	B	1	None	N/A
(Ctl) 0.0 µg/L	C	1	None	N/A
(Ctl) 0.0 µg/L	D	1	None	N/A
3.6 µg/L	A	1	None	N/A
3.6 µg/L	B	1	None	N/A
3.6 µg/L	C	1	None	N/A
3.6 µg/L	D	1	None	N/A
10.9 µg/L	A	1	None	N/A
10.9 µg/L	B	1	None	N/A
10.9 µg/L	C	1	None	N/A
10.9 µg/L	D	1	None	N/A
33 µg/L	A	1	None	N/A
33 µg/L	B	1	None	N/A
33 µg/L	C	1	None	N/A
33 µg/L	D	1	None	N/A
100 µg/L	A	1	None	N/A
100 µg/L	B	1	None	N/A
100 µg/L	C	1	None	N/A
100 µg/L	D	1	None	N/A
(Ctl) 0.0 µg/L	A	2	None	N/A
(Ctl) 0.0 µg/L	B	2	None	N/A
(Ctl) 0.0 µg/L	C	2	None	N/A
(Ctl) 0.0 µg/L	D	2	None	N/A
3.6 µg/L	A	2	None	N/A

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Clinical Signs	Number Affected
3.6 µg/L	B	2	None	N/A
3.6 µg/L	C	2	None	N/A
3.6 µg/L	D	2	None	N/A
10.9 µg/L	A	2	None	N/A
10.9 µg/L	B	2	None	N/A
10.9 µg/L	C	2	None	N/A
10.9 µg/L	D	2	None	N/A
33 µg/L	A	2	None	N/A
33 µg/L	B	2	None	N/A
33 µg/L	C	2	None	N/A
33 µg/L	D	2	None	N/A
100 µg/L	A	2	None	N/A
100 µg/L	B	2	None	N/A
100 µg/L	C	2	None	N/A
100 µg/L	D	2	None	N/A
(Ctl) 0.0 µg/L	A	3	None	N/A
(Ctl) 0.0 µg/L	B	3	None	N/A
(Ctl) 0.0 µg/L	C	3	None	N/A
(Ctl) 0.0 µg/L	D	3	None	N/A
3.6 µg/L	A	3	None	N/A
3.6 µg/L	B	3	None	N/A
3.6 µg/L	C	3	None	N/A
3.6 µg/L	D	3	None	N/A
10.9 µg/L	A	3	None	N/A
10.9 µg/L	B	3	None	N/A
10.9 µg/L	C	3	None	N/A
10.9 µg/L	D	3	None	N/A
33 µg/L	A	3	None	N/A
33 µg/L	B	3	None	N/A
33 µg/L	C	3	None	N/A
33 µg/L	D	3	None	N/A
100 µg/L	A	3	None	N/A
100 µg/L	B	3	None	N/A
100 µg/L	C	3	None	N/A
100 µg/L	D	3	None	N/A
(Ctl) 0.0 µg/L	A	4	None	N/A
(Ctl) 0.0 µg/L	B	4	None	N/A
(Ctl) 0.0 µg/L	C	4	None	N/A
(Ctl) 0.0 µg/L	D	4	None	N/A
3.6 µg/L	A	4	None	N/A
3.6 µg/L	B	4	None	N/A
3.6 µg/L	C	4	None	N/A
3.6 µg/L	D	4	None	N/A
10.9 µg/L	A	4	None	N/A
10.9 µg/L	B	4	None	N/A

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Clinical Signs	Number Affected
10.9 µg/L	C	4	None	N/A
10.9 µg/L	D	4	None	N/A
33 µg/L	A	4	None	N/A
33 µg/L	B	4	None	N/A
33 µg/L	C	4	None	N/A
33 µg/L	D	4	None	N/A
100 µg/L	A	4	None	N/A
100 µg/L	B	4	None	N/A
100 µg/L	C	4	None	N/A
100 µg/L	D	4	None	N/A
(Ctl) 0.0 µg/L	A	5	None	N/A
(Ctl) 0.0 µg/L	B	5	None	N/A
(Ctl) 0.0 µg/L	C	5	None	N/A
(Ctl) 0.0 µg/L	D	5	None	N/A
3.6 µg/L	A	5	None	N/A
3.6 µg/L	B	5	None	N/A
3.6 µg/L	C	5	None	N/A
3.6 µg/L	D	5	None	N/A
10.9 µg/L	A	5	None	N/A
10.9 µg/L	B	5	None	N/A
10.9 µg/L	C	5	None	N/A
10.9 µg/L	D	5	None	N/A
33 µg/L	A	5	None	N/A
33 µg/L	B	5	None	N/A
33 µg/L	C	5	None	N/A
33 µg/L	D	5	None	N/A
100 µg/L	A	5	None	N/A
100 µg/L	B	5	None	N/A
100 µg/L	C	5	None	N/A
100 µg/L	D	5	None	N/A
(Ctl) 0.0 µg/L	A	6	None	N/A
(Ctl) 0.0 µg/L	B	6	None	N/A
(Ctl) 0.0 µg/L	C	6	None	N/A
(Ctl) 0.0 µg/L	D	6	None	N/A
3.6 µg/L	A	6	None	N/A
3.6 µg/L	B	6	None	N/A
3.6 µg/L	C	6	None	N/A
3.6 µg/L	D	6	None	N/A
10.9 µg/L	A	6	None	N/A
10.9 µg/L	B	6	None	N/A
10.9 µg/L	C	6	None	N/A
10.9 µg/L	D	6	None	N/A
33 µg/L	A	6	None	N/A
33 µg/L	B	6	None	N/A
33 µg/L	C	6	None	N/A

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Clinical Signs	Number Affected
33 µg/L	D	6	None	N/A
100 µg/L	A	6	None	N/A
100 µg/L	B	6	None	N/A
100 µg/L	C	6	None	N/A
100 µg/L	D	6	None	N/A
(Ctl) 0.0 µg/L	A	7	None	N/A
(Ctl) 0.0 µg/L	B	7	None	N/A
(Ctl) 0.0 µg/L	C	7	None	N/A
(Ctl) 0.0 µg/L	D	7	None	N/A
3.6 µg/L	A	7	None	N/A
3.6 µg/L	B	7	None	N/A
3.6 µg/L	C	7	None	N/A
3.6 µg/L	D	7	None	N/A
10.9 µg/L	A	7	None	N/A
10.9 µg/L	B	7	None	N/A
10.9 µg/L	C	7	None	N/A
10.9 µg/L	D	7	None	N/A
33 µg/L	A	7	None	N/A
33 µg/L	B	7	None	N/A
33 µg/L	C	7	None	N/A
33 µg/L	D	7	None	N/A
100 µg/L	A	7	None	N/A
100 µg/L	B	7	None	N/A
100 µg/L	C	7	None	N/A
100 µg/L	D	7	None	N/A
(Ctl) 0.0 µg/L	A	8	None	N/A
(Ctl) 0.0 µg/L	B	8	None	N/A
(Ctl) 0.0 µg/L	C	8	None	N/A
(Ctl) 0.0 µg/L	D	8	None	N/A
3.6 µg/L	A	8	None	N/A
3.6 µg/L	B	8	None	N/A
3.6 µg/L	C	8	None	N/A
3.6 µg/L	D	8	None	N/A
10.9 µg/L	A	8	None	N/A
10.9 µg/L	B	8	None	N/A
10.9 µg/L	C	8	None	N/A
10.9 µg/L	D	8	None	N/A
33 µg/L	A	8	None	N/A
33 µg/L	B	8	None	N/A
33 µg/L	C	8	None	N/A
33 µg/L	D	8	None	N/A
100 µg/L	A	8	None	N/A
100 µg/L	B	8	None	N/A
100 µg/L	C	8	None	N/A
100 µg/L	D	8	None	N/A

DEST BATT01-00388 Clinical Signs (Daily)

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Clinical Signs	Number Affected
(Ctl) 0.0 µg/L	A	9	None	N/A
(Ctl) 0.0 µg/L	B	9	None	N/A
(Ctl) 0.0 µg/L	C	9	None	N/A
(Ctl) 0.0 µg/L	D	9	None	N/A
3.6 µg/L	A	9	None	N/A
3.6 µg/L	B	9	None	N/A
3.6 µg/L	C	9	None	N/A
3.6 µg/L	D	9	None	N/A
10.9 µg/L	A	9	None	N/A
10.9 µg/L	B	9	None	N/A
10.9 µg/L	C	9	None	N/A
10.9 µg/L	D	9	None	N/A
33 µg/L	A	9	None	N/A
33 µg/L	B	9	None	N/A
33 µg/L	C	9	None	N/A
33 µg/L	D	9	None	N/A
100 µg/L	A	9	None	N/A
100 µg/L	B	9	None	N/A
100 µg/L	C	9	None	N/A
100 µg/L	D	9	None	N/A
(Ctl) 0.0 µg/L	A	10	None	N/A
(Ctl) 0.0 µg/L	B	10	None	N/A
(Ctl) 0.0 µg/L	C	10	None	N/A
(Ctl) 0.0 µg/L	D	10	None	N/A
3.6 µg/L	A	10	None	N/A
3.6 µg/L	B	10	None	N/A
3.6 µg/L	C	10	None	N/A
3.6 µg/L	D	10	None	N/A
10.9 µg/L	A	10	None	N/A
10.9 µg/L	B	10	None	N/A
10.9 µg/L	C	10	None	N/A
10.9 µg/L	D	10	None	N/A
33 µg/L	A	10	None	N/A
33 µg/L	B	10	None	N/A
33 µg/L	C	10	None	N/A
33 µg/L	D	10	None	N/A
100 µg/L	A	10	None	N/A
100 µg/L	B	10	None	N/A
100 µg/L	C	10	None	N/A
100 µg/L	D	10	None	N/A
(Ctl) 0.0 µg/L	A	11	None	N/A
(Ctl) 0.0 µg/L	B	11	None	N/A
(Ctl) 0.0 µg/L	C	11	None	N/A
(Ctl) 0.0 µg/L	D	11	None	N/A
3.6 µg/L	A	11	None	N/A

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Clinical Signs	Number Affected
3.6 µg/L	B	11	None	N/A
3.6 µg/L	C	11	None	N/A
3.6 µg/L	D	11	None	N/A
10.9 µg/L	A	11	None	N/A
10.9 µg/L	B	11	None	N/A
10.9 µg/L	C	11	None	N/A
10.9 µg/L	D	11	None	N/A
33 µg/L	A	11	None	N/A
33 µg/L	B	11	None	N/A
33 µg/L	C	11	None	N/A
33 µg/L	D	11	None	N/A
100 µg/L	A	11	None	N/A
100 µg/L	B	11	None	N/A
100 µg/L	C	11	None	N/A
100 µg/L	D	11	None	N/A
(Ctl) 0.0 µg/L	A	12	None	N/A
(Ctl) 0.0 µg/L	B	12	None	N/A
(Ctl) 0.0 µg/L	C	12	None	N/A
(Ctl) 0.0 µg/L	D	12	None	N/A
3.6 µg/L	A	12	None	N/A
3.6 µg/L	B	12	None	N/A
3.6 µg/L	C	12	None	N/A
3.6 µg/L	D	12	None	N/A
10.9 µg/L	A	12	None	N/A
10.9 µg/L	B	12	None	N/A
10.9 µg/L	C	12	None	N/A
10.9 µg/L	D	12	None	N/A
33 µg/L	A	12	None	N/A
33 µg/L	B	12	None	N/A
33 µg/L	C	12	None	N/A
33 µg/L	D	12	None	N/A
100 µg/L	A	12	None	N/A
100 µg/L	B	12	None	N/A
100 µg/L	C	12	None	N/A
100 µg/L	D	12	None	N/A
(Ctl) 0.0 µg/L	A	13	None	N/A
(Ctl) 0.0 µg/L	B	13	None	N/A
(Ctl) 0.0 µg/L	C	13	None	N/A
(Ctl) 0.0 µg/L	D	13	None	N/A
3.6 µg/L	A	13	None	N/A
3.6 µg/L	B	13	None	N/A
3.6 µg/L	C	13	None	N/A
3.6 µg/L	D	13	None	N/A
10.9 µg/L	A	13	None	N/A
10.9 µg/L	B	13	None	N/A

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Clinical Signs	Number Affected
10.9 µg/L	C	13	None	N/A
10.9 µg/L	D	13	None	N/A
33 µg/L	A	13	None	N/A
33 µg/L	B	13	None	N/A
33 µg/L	C	13	None	N/A
33 µg/L	D	13	None	N/A
100 µg/L	A	13	None	N/A
100 µg/L	B	13	None	N/A
100 µg/L	C	13	None	N/A
100 µg/L	D	13	None	N/A
(Ctl) 0.0 µg/L	A	14	None	N/A
(Ctl) 0.0 µg/L	B	14	None	N/A
(Ctl) 0.0 µg/L	C	14	None	N/A
(Ctl) 0.0 µg/L	D	14	None	N/A
3.6 µg/L	A	14	None	N/A
3.6 µg/L	B	14	None	N/A
3.6 µg/L	C	14	None	N/A
3.6 µg/L	D	14	None	N/A
10.9 µg/L	A	14	None	N/A
10.9 µg/L	B	14	None	N/A
10.9 µg/L	C	14	None	N/A
10.9 µg/L	D	14	None	N/A
33 µg/L	A	14	None	N/A
33 µg/L	B	14	None	N/A
33 µg/L	C	14	None	N/A
33 µg/L	D	14	None	N/A
100 µg/L	A	14	None	N/A
100 µg/L	B	14	None	N/A
100 µg/L	C	14	None	N/A
100 µg/L	D	14	None	N/A
(Ctl) 0.0 µg/L	A	15	None	N/A
(Ctl) 0.0 µg/L	B	15	None	N/A
(Ctl) 0.0 µg/L	C	15	None	N/A
(Ctl) 0.0 µg/L	D	15	None	N/A
3.6 µg/L	A	15	None	N/A
3.6 µg/L	B	15	None	N/A
3.6 µg/L	C	15	None	N/A
3.6 µg/L	D	15	None	N/A
10.9 µg/L	A	15	None	N/A
10.9 µg/L	B	15	None	N/A
10.9 µg/L	C	15	None	N/A
10.9 µg/L	D	15	None	N/A
33 µg/L	A	15	None	N/A
33 µg/L	B	15	None	N/A
33 µg/L	C	15	None	N/A

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Clinical Signs	Number Affected
33 µg/L	D	15	None	N/A
100 µg/L	A	15	None	N/A
100 µg/L	B	15	None	N/A
100 µg/L	C	15	None	N/A
100 µg/L	D	15	None	N/A
(Ctl) 0.0 µg/L	A	16	None	N/A
(Ctl) 0.0 µg/L	B	16	None	N/A
(Ctl) 0.0 µg/L	C	16	None	N/A
(Ctl) 0.0 µg/L	D	16	None	N/A
3.6 µg/L	A	16	None	N/A
3.6 µg/L	B	16	None	N/A
3.6 µg/L	C	16	None	N/A
3.6 µg/L	D	16	None	N/A
10.9 µg/L	A	16	None	N/A
10.9 µg/L	B	16	None	N/A
10.9 µg/L	C	16	None	N/A
10.9 µg/L	D	16	None	N/A
33 µg/L	A	16	None	N/A
33 µg/L	B	16	None	N/A
33 µg/L	C	16	None	N/A
33 µg/L	D	16	None	N/A
100 µg/L	A	16	None	N/A
100 µg/L	B	16	None	N/A
100 µg/L	C	16	None	N/A
100 µg/L	D	16	None	N/A
(Ctl) 0.0 µg/L	A	17	None	N/A
(Ctl) 0.0 µg/L	B	17	None	N/A
(Ctl) 0.0 µg/L	C	17	None	N/A
(Ctl) 0.0 µg/L	D	17	None	N/A
3.6 µg/L	A	17	None	N/A
3.6 µg/L	B	17	None	N/A
3.6 µg/L	C	17	None	N/A
3.6 µg/L	D	17	None	N/A
10.9 µg/L	A	17	None	N/A
10.9 µg/L	B	17	None	N/A
10.9 µg/L	C	17	None	N/A
10.9 µg/L	D	17	None	N/A
33 µg/L	A	17	None	N/A
33 µg/L	B	17	None	N/A
33 µg/L	C	17	None	N/A
33 µg/L	D	17	None	N/A
100 µg/L	A	17	None	N/A
100 µg/L	B	17	None	N/A
100 µg/L	C	17	None	N/A
100 µg/L	D	17	None	N/A

DEST BATT01-00388 Clinical Signs (Daily)

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Clinical Signs	Number Affected
(Ctl) 0.0 µg/L	A	18	None	N/A
(Ctl) 0.0 µg/L	B	18	None	N/A
(Ctl) 0.0 µg/L	C	18	None	N/A
(Ctl) 0.0 µg/L	D	18	None	N/A
3.6 µg/L	A	18	None	N/A
3.6 µg/L	B	18	None	N/A
3.6 µg/L	C	18	None	N/A
3.6 µg/L	D	18	None	N/A
10.9 µg/L	A	18	None	N/A
10.9 µg/L	B	18	None	N/A
10.9 µg/L	C	18	None	N/A
10.9 µg/L	D	18	None	N/A
33 µg/L	A	18	None	N/A
33 µg/L	B	18	None	N/A
33 µg/L	C	18	None	N/A
33 µg/L	D	18	None	N/A
100 µg/L	A	18	None	N/A
100 µg/L	B	18	None	N/A
100 µg/L	C	18	None	N/A
100 µg/L	D	18	None	N/A
(Ctl) 0.0 µg/L	A	19	None	N/A
(Ctl) 0.0 µg/L	B	19	None	N/A
(Ctl) 0.0 µg/L	C	19	None	N/A
(Ctl) 0.0 µg/L	D	19	None	N/A
3.6 µg/L	A	19	None	N/A
3.6 µg/L	B	19	None	N/A
3.6 µg/L	C	19	None	N/A
3.6 µg/L	D	19	None	N/A
10.9 µg/L	A	19	None	N/A
10.9 µg/L	B	19	None	N/A
10.9 µg/L	C	19	None	N/A
10.9 µg/L	D	19	None	N/A
33 µg/L	A	19	None	N/A
33 µg/L	B	19	None	N/A
33 µg/L	C	19	None	N/A
33 µg/L	D	19	None	N/A
100 µg/L	A	19	None	N/A
100 µg/L	B	19	None	N/A
100 µg/L	C	19	None	N/A
100 µg/L	D	19	None	N/A
(Ctl) 0.0 µg/L	A	20	None	N/A
(Ctl) 0.0 µg/L	B	20	None	N/A
(Ctl) 0.0 µg/L	C	20	None	N/A
(Ctl) 0.0 µg/L	D	20	None	N/A
3.6 µg/L	A	20	None	N/A

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Clinical Signs	Number Affected
3.6 µg/L	B	20	None	N/A
3.6 µg/L	C	20	None	N/A
3.6 µg/L	D	20	None	N/A
10.9 µg/L	A	20	None	N/A
10.9 µg/L	B	20	None	N/A
10.9 µg/L	C	20	None	N/A
10.9 µg/L	D	20	None	N/A
33 µg/L	A	20	None	N/A
33 µg/L	B	20	None	N/A
33 µg/L	C	20	None	N/A
33 µg/L	D	20	None	N/A
100 µg/L	A	20	None	N/A
100 µg/L	B	20	None	N/A
100 µg/L	C	20	None	N/A
100 µg/L	D	20	None	N/A
(Ctl) 0.0 µg/L	A	21	None	N/A
(Ctl) 0.0 µg/L	B	21	None	N/A
(Ctl) 0.0 µg/L	C	21	None	N/A
(Ctl) 0.0 µg/L	D	21	None	N/A
3.6 µg/L	A	21	None	N/A
3.6 µg/L	B	21	None	N/A
3.6 µg/L	C	21	None	N/A
3.6 µg/L	D	21	None	N/A
10.9 µg/L	A	21	None	N/A
10.9 µg/L	B	21	None	N/A
10.9 µg/L	C	21	None	N/A
10.9 µg/L	D	21	None	N/A
33 µg/L	A	21	None	N/A
33 µg/L	B	21	None	N/A
33 µg/L	C	21	None	N/A
33 µg/L	D	21	None	N/A
100 µg/L	A	21	None	N/A
100 µg/L	B	21	None	N/A
100 µg/L	C	21	None	N/A
100 µg/L	D	21	None	N/A

Name of Treatment Group	Replicate ID	Day 7 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Comments
(Ctl) 0.0 µg/L	A	1	0.165	13.5	1.6	0.1	54		
(Ctl) 0.0 µg/L	A	2	0.193	15.6	2.3	0.1	54		
(Ctl) 0.0 µg/L	A	3	0.202	14.7	1.6	0.1	54		
(Ctl) 0.0 µg/L	A	4	0.207	16.3	1.4	0.1	54		
(Ctl) 0.0 µg/L	A	5	0.268	17.1	2.1	0.1	54		
(Ctl) 0.0 µg/L	B	1	0.156	15.0	1.3	0.1	54		
(Ctl) 0.0 µg/L	B	2	0.215	15.0	1.9	0.1	54		
(Ctl) 0.0 µg/L	B	3	0.249	16.6	1.9	0.1	54		
(Ctl) 0.0 µg/L	B	4	0.201	15.0	1.3	0.1	54		
(Ctl) 0.0 µg/L	B	5	0.250	16.9	1.7	0.1	54		
(Ctl) 0.0 µg/L	C	1	0.160	14.3	1.3	0.1	54		
(Ctl) 0.0 µg/L	C	2	0.163	14.6	1.4	0.1	54		
(Ctl) 0.0 µg/L	C	3	0.145	14.3	1.4	0.1	54		
(Ctl) 0.0 µg/L	C	4	0.195	15.1	1.7	0.1	54		
(Ctl) 0.0 µg/L	C	5	0.254	15.3	1.8	0.1	54		
(Ctl) 0.0 µg/L	D	1	0.167	15.2	1.9	0.1	54		
(Ctl) 0.0 µg/L	D	2	0.199	15.9	1.8	0.1	54		
(Ctl) 0.0 µg/L	D	3	0.202	15.6	1.5	0.1	54		
(Ctl) 0.0 µg/L	D	4	0.234	16.6	1.7	0.1	54		
(Ctl) 0.0 µg/L	D	5	0.296	16.9	1.6	0.1	54		
3.6 µg/L	A	1	0.232	13.3	1.5	0.1	54		
3.6 µg/L	A	2	0.216	12.9	1.6	0.1	54		
3.6 µg/L	A	3	0.258	14.0	1.3	0.1	54		
3.6 µg/L	A	4	0.244	14.3	1.8	0.1	54		
3.6 µg/L	A	5	0.270	14.2	2.3	0.2	54		
3.6 µg/L	B	1	0.202	12.9	1.6	0.1	54		
3.6 µg/L	B	2	0.226	14.5	2.0	0.1	54		
3.6 µg/L	B	3	0.233	14.3	1.7	0.1	54		
3.6 µg/L	B	4	0.272	14.8	2.4	0.2	54		
3.6 µg/L	B	5	0.266	14.7	2.0	0.1	54		
3.6 µg/L	C	1	0.146	11.7	1.2	0.1	54		

FEL

Name of Treatment Group	Replicate ID	Day 7 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Comments
3.6 µg/L	C	2	0.199	13.6	1.9	0.1	54		
3.6 µg/L	C	3	0.166	12.4	1.3	0.1	54		
3.6 µg/L	C	4	0.286	14.0	1.8	0.1	54		
3.6 µg/L	C	5	0.294	15.4	2.0	0.1	54		
3.6 µg/L	D	1	0.269	14.6	1.7	0.1	54		
3.6 µg/L	D	2	0.340	16.2	2.0	0.1	54		
3.6 µg/L	D	3	0.372	16.4	2.5	0.2	54		
3.6 µg/L	D	4	0.355	16.9	2.2	0.1	54		
3.6 µg/L	D	5	0.430	16.8	2.4	0.1	54		
10.9 µg/L	A	1	0.260	14.7	1.8	0.1	54		
10.9 µg/L	A	2	0.188	13.7	1.7	0.1	54		
10.9 µg/L	A	3	0.274	14.2	1.7	0.1	54		
10.9 µg/L	A	4	0.333	16.4	2.2	0.1	54		
10.9 µg/L	A	5	0.243	13.2	1.7	0.1	54		
10.9 µg/L	B	1	0.161	12.6	1.8	0.1	54		
10.9 µg/L	B	2	0.196	12.6	1.5	0.1	54		
10.9 µg/L	B	3	0.233	13.5	1.9	0.1	54		
10.9 µg/L	B	4	0.233	14.3	2.0	0.1	54		
10.9 µg/L	B	5	0.408	17.3	2.3	0.1	54		
10.9 µg/L	C	1	0.240	13.8	1.4	0.1	54		
10.9 µg/L	C	2	0.237	14.2	1.5	0.1	54		
10.9 µg/L	C	3	0.236	13.7	1.6	0.1	54		
10.9 µg/L	C	4	0.367	16.6	2.2	0.1	54		
10.9 µg/L	C	5	0.547	18.3	2.3	0.1	54		
10.9 µg/L	D	1	0.141	11.0	1.2	0.1	54		
10.9 µg/L	D	2	0.201	12.8	1.6	0.1	54		
10.9 µg/L	D	3	0.141	11.6	1.2	0.1	54		
10.9 µg/L	D	4	0.168	11.8	1.4	0.1	54		
10.9 µg/L	D	5	0.254	14.4	1.5	0.1	54		
33 µg/L	A	1	0.202	12.7	1.4	0.1	54		
33 µg/L	A	2	0.273	15.1	1.8	0.1	54		

FEL

Name of Treatment Group	Replicate ID	Day 7 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Comments
33 µg/L	A	3	0.313	15.0	2.0	0.1	54		
33 µg/L	A	4	0.426	17.5	2.2	0.1	54		
33 µg/L	A	5	0.410	16.6	1.7	0.1	54		
33 µg/L	B	1	0.223	13.6	1.8	0.1	54		
33 µg/L	B	2	0.233	15.5	1.9	0.1	54		
33 µg/L	B	3	0.290	14.6	1.7	0.1	54		
33 µg/L	B	4	0.458	18.3	1.9	0.1	54		
33 µg/L	B	5	0.439	17.4	2.4	0.1	54		
33 µg/L	C	1	0.297	15.2	1.9	0.1	54		
33 µg/L	C	2	0.287	14.2	1.5	0.1	54		
33 µg/L	C	3	0.283	14.7	1.7	0.1	54		
33 µg/L	C	4	0.314	15.5	2.0	0.1	54		
33 µg/L	C	5	0.317	15.6	1.8	0.1	54		
33 µg/L	D	1	0.157	11.7	1.6	0.1	54		
33 µg/L	D	2	0.221	14.2	1.4	0.1	54		
33 µg/L	D	3	0.218	13.6	1.7	0.1	54		
33 µg/L	D	4	0.238	14.7	1.9	0.1	54		
33 µg/L	D	5	0.222	13.7	1.9	0.1	54		
100 µg/L	A	1	0.278	14.4	1.6	0.1	54		
100 µg/L	A	2	0.284	16.3	1.6	0.1	54		
100 µg/L	A	3	0.275	15.4	1.8	0.1	54		
100 µg/L	A	4	0.390	17.8	2.1	0.1	54		
100 µg/L	A	5	0.390	17.8	2.3	0.1	54		
100 µg/L	B	1	0.387	17.2	1.7	0.1	54		
100 µg/L	B	2	0.271	14.9	1.9	0.1	54		
100 µg/L	B	3	0.359	16.7	2.1	0.1	54		
100 µg/L	B	4	0.326	16.4	1.9	0.1	54		
100 µg/L	B	5	0.429	18.2	2.6	0.1	54		
100 µg/L	C	1	0.229	15.0	1.9	0.1	54		
100 µg/L	C	2	0.273	15.0	1.4	0.1	54		
100 µg/L	C	3	0.350	15.4	1.8	0.1	54		

FEL

Name of Treatment Group	Replicate ID	Day 7 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Comments
100 µg/L	C	4	0.321	16.8	2.2	0.1	54		
100 µg/L	C	5	0.442	15.9	2.2	0.1	54		
100 µg/L	D	1	0.224	15.0	2.0	0.1	54		
100 µg/L	D	2	0.219	16.3	2.1	0.1	54		
100 µg/L	D	3	0.242	16.8	2.3	0.1	54		
100 µg/L	D	4	0.326	16.4	2.4	0.1	54		
100 µg/L	D	5	0.246	18.2	2.4	0.1	54		

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Histopathology? (Y or N)	Comments
(Ctl) 0.0 µg/L	A	1	1.028	26.9	5.8	0.2	58		Y	
(Ctl) 0.0 µg/L	A	2	0.944	24.5	7.8	0.3	58		N	
(Ctl) 0.0 µg/L	A	3	1.324	27.7	7.2	0.3	58		Y	
(Ctl) 0.0 µg/L	A	4	0.641	28.0	8.6	0.3	58		N	
(Ctl) 0.0 µg/L	A	5	0.738	21.7	5.2	0.2	58		N	
(Ctl) 0.0 µg/L	A	6	1.214	27.7	6.9	0.3	58		Y	
(Ctl) 0.0 µg/L	A	7	1.146	26.9	7.5	0.3	58		N	
(Ctl) 0.0 µg/L	A	8	0.579	21.7	4.3	0.2	58		N	
(Ctl) 0.0 µg/L	A	9	0.796	24.0	5.2	0.2	58		N	
(Ctl) 0.0 µg/L	A	10	0.834	24.2	4.7	0.2	58		N	
(Ctl) 0.0 µg/L	A	11	1.119	26.7	7.2	0.3	58		N	
(Ctl) 0.0 µg/L	A	12	1.012	26.9	5.3	0.2	58		Y	
(Ctl) 0.0 µg/L	A	13	0.999	25.5	4.9	0.2	58		N	
(Ctl) 0.0 µg/L	A	14	0.941	25.6	6.6	0.3	58		Y	
(Ctl) 0.0 µg/L	A	15	0.629	22.1	5.5	0.2	58		N	
(Ctl) 0.0 µg/L	B	1	1.148	28.1	9.1	0.3	59		N	
(Ctl) 0.0 µg/L	B	2	1.282	28.8	8.7	0.3	59		N	
(Ctl) 0.0 µg/L	B	3	1.399	29.1	8.0	0.3	59		N	
(Ctl) 0.0 µg/L	B	4	1.197	28.2	7.2	0.3	58		Y	
(Ctl) 0.0 µg/L	B	5	0.578	21.7	5.2	0.2	58		N	
(Ctl) 0.0 µg/L	B	6	1.348	30.4	6.7	0.2	59		N	
(Ctl) 0.0 µg/L	B	7	1.075	27.6	6.2	0.2	58		Y	
(Ctl) 0.0 µg/L	B	8	1.450	28.1	6.1	0.2	58		Y	
(Ctl) 0.0 µg/L	B	9	1.140	28.8	7.3	0.3	59		N	
(Ctl) 0.0 µg/L	B	10	1.005	26.0	6.4	0.2	59		N	
(Ctl) 0.0 µg/L	B	11	1.507	29.7	9.2	0.3	59		N	
(Ctl) 0.0 µg/L	B	12	0.897	26.4	5.4	0.2	58		Y	
(Ctl) 0.0 µg/L	B	13	0.573	22.3	4.5	0.2	58		N	
(Ctl) 0.0 µg/L	B	14	0.956	26.4	6.5	0.2	59		N	
(Ctl) 0.0 µg/L	B	15	1.095	27.6	6.5	0.2	58		Y	
(Ctl) 0.0 µg/L	C	1	1.338	29.1	7.0	0.2	59		N	
(Ctl) 0.0 µg/L	C	2	1.569	31.2	9.0	0.3	59		N	
(Ctl) 0.0 µg/L	C	3	0.841	25.2	6.5	0.3	58		N	
(Ctl) 0.0 µg/L	C	4	1.214	28.9	6.6	0.2	59		N	
(Ctl) 0.0 µg/L	C	5	1.459	30.4	9.2	0.3	59		N	
(Ctl) 0.0 µg/L	C	6	1.444	30.3	7.0	0.2	59		N	
(Ctl) 0.0 µg/L	C	7	1.191	28.7	7.1	0.2	59		N	

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Histopathology? (Y or N)	Comments
(Ctl) 0.0 µg/L	C	8	1.204	28.8	6.6	0.2	58		Y	
(Ctl) 0.0 µg/L	C	9	1.242	28.5	7.2	0.3	58		N	
(Ctl) 0.0 µg/L	C	10	1.014	26.3	5.9	0.2	58		Y	
(Ctl) 0.0 µg/L	C	11	1.223	28.1	7.3	0.3	58		Y	
(Ctl) 0.0 µg/L	C	12	1.071	27.5	6.6	0.2	58		N	
(Ctl) 0.0 µg/L	C	13	1.431	30.2	7.9	0.3	58		Y	
(Ctl) 0.0 µg/L	C	14	1.082	27.2	6.4	0.2	58		N	
(Ctl) 0.0 µg/L	C	15	1.317	28.0	5.6	0.2	58		Y	
(Ctl) 0.0 µg/L	D	1	1.549	30.7	10.5	0.3	59		N	
(Ctl) 0.0 µg/L	D	2	0.787	24.5	5.0	0.2	58		N	
(Ctl) 0.0 µg/L	D	3	1.377	28.8	12.5	0.4	59		N	
(Ctl) 0.0 µg/L	D	4	1.052	27.1	5.9	0.2	58		Y	
(Ctl) 0.0 µg/L	D	5	1.276	28.6	7.2	0.3	58		Y	
(Ctl) 0.0 µg/L	D	6	1.210	29.7	6.8	0.2	58		Y	
(Ctl) 0.0 µg/L	D	7	1.389	27.2	7.9	0.3	58		N	
(Ctl) 0.0 µg/L	D	8	1.285	28.1	6.2	0.2	58		Y	
(Ctl) 0.0 µg/L	D	9	1.219	27.8	6.2	0.2	58		N	
(Ctl) 0.0 µg/L	D	10	1.121	31.4	8.3	0.3	58		Y	
(Ctl) 0.0 µg/L	D	11	1.473	28.2	8.8	0.3	58		N	
(Ctl) 0.0 µg/L	D	12	1.183	28.6	6.5	0.2	58		N	
(Ctl) 0.0 µg/L	D	13	1.185	28.7	5.8	0.2	58		N	
(Ctl) 0.0 µg/L	D	14	1.204	26.8	5.1	0.2	58		N	
(Ctl) 0.0 µg/L	D	15	1.014	25.4	5.2	0.2	58		N	
3.6 µg/L	A	1	1.745	32.2	12.0	0.4	59		N	
3.6 µg/L	A	2	1.430	30.2	9.4	0.3	58		Y	
3.6 µg/L	A	3	1.227	27.7	7.1	0.3	58		Y	
3.6 µg/L	A	4	1.637	30.3	10.0	0.3	59		N	
3.6 µg/L	A	5	1.770	33.1	11.9	0.4	59		N	
3.6 µg/L	A	6	1.754	32.4	11.0	0.3	58		N	
3.6 µg/L	A	7	2.023	33.5	11.1	0.3	59		N	
3.6 µg/L	A	8	2.136	33.7	14.9	0.4	59		N	
3.6 µg/L	A	9	1.356	28.4	9.7	0.3	58		Y	
3.6 µg/L	A	10	1.815	31.8	11.8	0.4	58		Y	
3.6 µg/L	A	11	0.905	25.9	7.3	0.3	58		N	
3.6 µg/L	A	12	2.185	31.2	16.6	0.5	59		N	
3.6 µg/L	A	13	1.252	27.2	8.0	0.3	58		N	
3.6 µg/L	A	14	1.640	29.8	13.8	0.5	59		N	

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Histopathology? (Y or N)	Comments
3.6 µg/L	A	15	1.429	30.5	8.4	0.3	58		Y	
3.6 µg/L	B	1	1.760	30.7	11.6	0.4	58		Y	
3.6 µg/L	B	2	1.428	30.2	8.9	0.3	58		N	
3.6 µg/L	B	3	1.580	30.8	10.7	0.3	59		N	
3.6 µg/L	B	4	1.647	30.3	15.5	0.5	59		N	
3.6 µg/L	B	5	1.877	30.2	16.5	0.5	59		N	
3.6 µg/L	B	6	1.663	30.6	16.5	0.5	59		N	
3.6 µg/L	B	7	1.240	26.9	10.8	0.4	58		N	
3.6 µg/L	B	8	1.003	26.6	7.0	0.3	58		N	
3.6 µg/L	B	9	1.378	29.4	10.2	0.3	58		Y	
3.6 µg/L	B	10	1.265	29.0	8.1	0.3	58		N	
3.6 µg/L	B	11	1.532	30.6	13.7	0.4	58		Y	
3.6 µg/L	B	12	1.601	28.5	16.2	0.6	59		N	
3.6 µg/L	B	13	1.478	30.5	10.7	0.4	58		Y	
3.6 µg/L	B	14	1.469	29.1	13.9	0.5	58		N	
3.6 µg/L	B	15	1.377	30.2	8.5	0.3	58		Y	
3.6 µg/L	C	1	0.845	25.4	5.5	0.2	58		N	
3.6 µg/L	C	2	1.131	27.0	7.4	0.3	58		Y	
3.6 µg/L	C	3	1.125	27.0	6.6	0.2	58		Y	
3.6 µg/L	C	4	0.585	21.8	4.6	0.2	58		N	
3.6 µg/L	C	5	0.852	25.7	5.3	0.2	58		Y	
3.6 µg/L	C	6	1.517	30.4	10.2	0.3	59		N	
3.6 µg/L	C	7	0.890	25.3	6.4	0.3	58		N	
3.6 µg/L	C	8	0.556	22.5	4.1	0.2	58		N	
3.6 µg/L	C	9	0.777	23.9	6.7	0.3	58		N	
3.6 µg/L	C	10	0.768	24.4	6.3	0.3	58		N	
3.6 µg/L	C	11	0.999	26.8	7.1	0.3	58		Y	
3.6 µg/L	C	12	1.615	29.9	10.0	0.3	59		N	
3.6 µg/L	C	13	1.235	29.2	5.2	0.2	58		Y	
3.6 µg/L	C	14	0.493	21.4	5.0	0.2	57		N	
3.6 µg/L	C	15	0.662	23.5	3.9	0.2	57		N	
3.6 µg/L	D	1	2.021	27.7	13.8	0.5	59		N	
3.6 µg/L	D	2	1.765	27.0	17.4	0.6	59		N	
3.6 µg/L	D	3	1.979	28.5	9.3	0.3	58		Y	
3.6 µg/L	D	4	2.072	29.5	14.9	0.5	59		N	
3.6 µg/L	D	5	1.760	27.3	12.6	0.5	59		N	
3.6 µg/L	D	6	1.266	25.6	7.7	0.3	58		Y	

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Histopathology? (Y or N)	Comments
3.6 µg/L	D	7	2.189	29.8	10.9	0.4	59		N	
3.6 µg/L	D	8	1.916	28.2	14.7	0.5	59		N	
3.6 µg/L	D	9	1.385	25.9	11.1	0.4	58		Y	
3.6 µg/L	D	10	1.342	25.5	8.1	0.3	58		Y	
3.6 µg/L	D	11	2.031	27.1	22.4	0.8	59		N	
3.6 µg/L	D	12	2.402	30.7	14.1	0.5	59		N	
3.6 µg/L	D	13	1.822	28.7	11.8	0.4	59		N	
3.6 µg/L	D	14	1.623	28.1	11.0	0.4	58		Y	
3.6 µg/L	D	15	1.260	26.0	7.7	0.3	58		N	
10.9 µg/L	A	1	1.753	27.9	9.7	0.3	58		Y	
10.9 µg/L	A	2	1.576	28.8	9.7	0.3	58		N	
10.9 µg/L	A	3	1.466	27.9	9.0	0.3	58		N	
10.9 µg/L	A	4	1.278	27.3	8.1	0.3	58		N	
10.9 µg/L	A	5	1.694	28.9	8.7	0.3	58		Y	
10.9 µg/L	A	6	1.327	26.7	7.5	0.3	58		N	
10.9 µg/L	A	7	1.113	24.8	7.7	0.3	58		N	
10.9 µg/L	A	8	1.199	25.5	8.0	0.3	58		N	
10.9 µg/L	A	9	1.566	27.2	8.6	0.3	58		N	
10.9 µg/L	A	10	1.405	25.4	10.7	0.4	58		N	
10.9 µg/L	A	11	1.888	28.2	10.2	0.4	59		N	
10.9 µg/L	A	12	1.506	27.6	11.0	0.4	58		Y	
10.9 µg/L	A	13	1.041	23.9	7.8	0.3	58		N	
10.9 µg/L	A	14	1.696	28.1	11.0	0.4	58		Y	
10.9 µg/L	A	15	1.589	27.8	10.7	0.4	58		Y	
10.9 µg/L	B	1	1.535	26.9	8.6	0.3	58		Y	
10.9 µg/L	B	2	1.744	28.5	10.5	0.4	59		N	
10.9 µg/L	B	3	1.412	26.1	9.1	0.3	58		Y	
10.9 µg/L	B	4	1.068	24.1	8.5	0.4	58		N	
10.9 µg/L	B	5	1.387	26.8	10.8	0.4	58		Y	
10.9 µg/L	B	6	1.504	27.2	10.4	0.4	58		N	
10.9 µg/L	B	7	0.586	21.1	3.9	0.2	57		N	
10.9 µg/L	B	8	1.163	24.5	9.4	0.4	58		N	
10.9 µg/L	B	9	0.786	21.6	6.1	0.3	57		N	
10.9 µg/L	B	10	1.601	28.5	11.4	0.4	58		Y	
10.9 µg/L	B	11	1.138	25.0	6.3	0.3	58		N	
10.9 µg/L	B	12	1.014	24.0	7.6	0.3	58		Y	
10.9 µg/L	B	13	1.016	24.1	7.0	0.3	58		N	

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Histopathology? (Y or N)	Comments
10.9 µg/L	B	14	1.875	28.0	18.9	0.7	59		N	
10.9 µg/L	B	15	1.675	26.9	16.5	0.6	59		N	
10.9 µg/L	C	1	1.523	27.4	10.6	0.4	58		Y	
10.9 µg/L	C	2	1.805	27.3	11.5	0.4	58		Y	
10.9 µg/L	C	3	1.583	28.1	9.1	0.3	58		Y	
10.9 µg/L	C	4	1.719	28.2	13.6	0.5	59		N	
10.9 µg/L	C	5	1.333	25.7	7.5	0.3	58		N	
10.9 µg/L	C	6	1.575	27.3	10.8	0.4	59		N	
10.9 µg/L	C	7	1.171	24.9	7.7	0.3	58		N	
10.9 µg/L	C	8	1.518	26.9	11.4	0.4	58		Y	
10.9 µg/L	C	9	1.649	28.2	8.9	0.3	58		Y	
10.9 µg/L	C	10	1.129	24.2	7.2	0.3	58		N	
10.9 µg/L	C	11	1.259	25.6	7.8	0.3	58		N	
10.9 µg/L	C	12	1.234	26.5	7.5	0.3	58		N	
10.9 µg/L	C	13	0.938	23.1	9.0	0.4	58		N	
10.9 µg/L	C	14	1.261	24.8	10.6	0.4	58		N	
10.9 µg/L	C	15	0.882	22.4	7.1	0.3	58		N	
10.9 µg/L	D	1	0.828	23.1	4.9	0.2	57		N	
10.9 µg/L	D	2	1.451	25.7	10.0	0.4	58		Y	
10.9 µg/L	D	3	1.629	27.5	9.9	0.4	58		Y	
10.9 µg/L	D	4	0.982	24.3	5.1	0.2	57		N	
10.9 µg/L	D	5	0.782	22.1	5.0	0.2	58		N	
10.9 µg/L	D	6	1.225	25.8	6.3	0.2	58		Y	
10.9 µg/L	D	7	1.323	25.7	9.5	0.4	58		Y	
10.9 µg/L	D	8	1.181	25.4	6.0	0.2	58		N	
10.9 µg/L	D	9	1.211	25.9	6.9	0.3	58		N	
10.9 µg/L	D	10	0.841	22.2	4.8	0.2	57		N	
10.9 µg/L	D	11	0.758	22.2	5.8	0.3	57		N	
10.9 µg/L	D	12	1.713	27.0	12.8	0.5	59		N	
10.9 µg/L	D	13	1.294	26.7	7.8	0.3	58		N	
10.9 µg/L	D	14	1.384	26.6	6.5	0.2	58		Y	
10.9 µg/L	D	15	0.906	22.6	4.8	0.2	58		N	
33 µg/L	A	1	1.267	26.3	10.0	0.4	59		N	
33 µg/L	A	2	1.507	27.3	10.5	0.4	59		N	
33 µg/L	A	3	1.713	28.6	17.8	0.6	59		N	
33 µg/L	A	4	2.149	29.9	22.3	0.7	59		N	
33 µg/L	A	5	1.241	24.8	9.0	0.4	58		Y	

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Histopathology? (Y or N)	Comments
33 µg/L	A	6	1.710	28.3	13.3	0.5	59		Y	
33 µg/L	A	7	1.448	25.8	15.1	0.6	59		N	
33 µg/L	A	8	1.457	25.9	10.0	0.4	59		N	
33 µg/L	A	9	1.263	25.5	11.6	0.5	59		N	
33 µg/L	A	10	1.049	23.2	3.9	0.2	58		Y	
33 µg/L	A	11	1.716	23.9	7.3	0.3	58		Y	
33 µg/L	A	12	1.358	28.5	12.2	0.4	59		N	
33 µg/L	A	13	1.565	26.0	11.9	0.5	59		N	
33 µg/L	A	14	1.510	27.0	15.0	0.6	59		N	
33 µg/L	A	15	1.522	27.1	9.7	0.4	58		Y	
33 µg/L	B	1	1.563	27.5	9.0	0.3	59		N	
33 µg/L	B	2	1.418	27.2	7.6	0.3	58		Y	
33 µg/L	B	3	1.414	27.1	6.9	0.3	58		Y	
33 µg/L	B	4	1.534	27.8	9.6	0.3	59		N	
33 µg/L	B	5	1.254	25.5	8.4	0.3	59		N	
33 µg/L	B	6	1.550	28.0	9.1	0.3	58		Y	
33 µg/L	B	7	1.926	29.8	11.4	0.4	59		N	
33 µg/L	B	8	1.231	26.0	6.4	0.2	58		Y	
33 µg/L	B	9	1.043	25.1	5.9	0.2	58		Y	
33 µg/L	B	10	2.118	30.4	9.6	0.3	59		N	
33 µg/L	B	11	2.051	30.8	16.3	0.5	59		N	
33 µg/L	B	12	2.005	30.8	9.6	0.3	59		N	
33 µg/L	B	13	2.041	29.1	13.8	0.5	59		N	
33 µg/L	B	14	1.789	28.9	9.8	0.3	59		N	
33 µg/L	B	15	2.066	29.8	11.9	0.4	59		N	
33 µg/L	C	1	1.624	27.2	14.7	0.5	59		Y	
33 µg/L	C	2	1.895	28.8	14.6	0.5	59		N	
33 µg/L	C	3	2.207	30.4	15.7	0.5	59		N	
33 µg/L	C	4	1.909	29.2	14.7	0.5	59		N	
33 µg/L	C	5	1.980	30.0	11.8	0.4	59		N	
33 µg/L	C	6	1.528	27.6	8.9	0.3	58		Y	
33 µg/L	C	7	1.758	28.2	14.3	0.5	59		N	
33 µg/L	C	8	2.079	25.4	8.2	0.3	59		N	
33 µg/L	C	9	1.539	29.7	14.0	0.5	59		N	
33 µg/L	C	10	1.585	28.1	7.9	0.3	58		Y	
33 µg/L	C	11	1.983	27.3	7.3	0.3	58		Y	
33 µg/L	C	12	1.861	29.1	11.8	0.4	59		N	

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Histopathology? (Y or N)	Comments
33 µg/L	C	13	1.830	29.7	10.6	0.4	59		N	
33 µg/L	C	14	1.564	26.8	13.0	0.5	58		Y	
33 µg/L	C	15	1.465	27.0	11.9	0.4	59		N	
33 µg/L	D	1	1.941	30.6	10.7	0.4	58		Y	
33 µg/L	D	2	1.507	27.8	7.9	0.3	58		Y	
33 µg/L	D	3	1.479	27.3	7.4	0.3	58		Y	
33 µg/L	D	4	1.407	26.8	6.5	0.2	58		Y	
33 µg/L	D	5	1.891	29.6	13.0	0.4	59		N	
33 µg/L	D	6	1.508	26.5	9.2	0.3	59		N	
33 µg/L	D	7	0.820	22.3	5.0	0.2	58		N	
33 µg/L	D	8	1.844	28.7	8.5	0.3	58		Y	
33 µg/L	D	9	1.853	28.7	13.4	0.5	59		N	
33 µg/L	D	10	1.384	26.8	7.5	0.3	58		N	
33 µg/L	D	11	2.915	30.9	9.8	0.3	59		N	
33 µg/L	D	12	1.608	28.2	8.7	0.3	58		N	
33 µg/L	D	13	1.660	27.9	8.2	0.3	59		N	
33 µg/L	D	14	1.420	26.4	5.2	0.2	58		N	
33 µg/L	D	15	1.036	25.0	5.1	0.2	58		N	
100 µg/L	A	1	1.992	29.4	10.0	0.3	59		N	
100 µg/L	A	2	1.574	27.4	11.6	0.4	59		N	
100 µg/L	A	3	1.866	29.1	11.3	0.4	59		N	
100 µg/L	A	4	1.718	28.6	10.5	0.4	59		N	
100 µg/L	A	5	1.789	29.5	11.6	0.4	59		N	
100 µg/L	A	6	1.468	26.7	11.6	0.4	58		Y	
100 µg/L	A	7	1.131	24.3	8.9	0.4	58		Y	
100 µg/L	A	8	1.574	27.4	13.1	0.5	59		N	
100 µg/L	A	9	1.751	28.1	10.5	0.4	59		N	
100 µg/L	A	10	1.249	26.3	7.5	0.3	58		Y	
100 µg/L	A	11	1.855	28.7	8.7	0.3	59		N	
100 µg/L	A	12	1.501	27.0	8.8	0.3	58		Y	
100 µg/L	A	13	1.633	27.7	13.5	0.5	59		N	
100 µg/L	A	14	1.050	24.4	7.0	0.3	58		Y	
100 µg/L	A	15	1.540	27.7	12.4	0.4	59		N	
100 µg/L	B	1	1.564	28.0	10.8	0.4	58		Y	
100 µg/L	B	2	1.383	26.0	8.5	0.3	58		Y	
100 µg/L	B	3	1.384	26.4	10.7	0.4	59		N	
100 µg/L	B	4	1.459	26.4	8.2	0.3	59		N	

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Histopathology? (Y or N)	Comments
100 µg/L	B	5	1.684	27.9	8.6	0.3	58		Y	
100 µg/L	B	6	1.242	25.5	8.1	0.3	58		Y	
100 µg/L	B	7	1.689	27.3	9.3	0.3	59		N	
100 µg/L	B	8	1.714	28.0	12.0	0.4	59		N	
100 µg/L	B	9	1.473	26.9	11.6	0.4	59		N	
100 µg/L	B	10	1.853	29.2	10.4	0.4	59		N	
100 µg/L	B	11	2.048	29.7	14.1	0.5	59		N	
100 µg/L	B	12	1.820	30.0	8.8	0.3	58		N	
100 µg/L	B	13	1.707	28.5	9.8	0.3	58		Y	
100 µg/L	B	14	1.727	28.9	11.1	0.4	59		N	
100 µg/L	B	15	1.338	25.9	9.5	0.4	59		N	
100 µg/L	C	1	1.707	29.1	10.3	0.4	58		Y	
100 µg/L	C	2	1.644	27.9	10.9	0.4	59		N	
100 µg/L	C	3	1.721	28.2	10.9	0.4	58		Y	
100 µg/L	C	4	1.788	29.4	8.5	0.3	58		Y	
100 µg/L	C	5	1.719	28.6	10.4	0.4	59		N	
100 µg/L	C	6	2.262	32.1	11.0	0.3	59		N	
100 µg/L	C	7	1.404	26.8	8.9	0.3	58		Y	
100 µg/L	C	8	0.955	22.9	6.2	0.3	58		N	
100 µg/L	C	9	1.358	26.5	8.5	0.3	59		N	
100 µg/L	C	10	1.582	27.2	10.5	0.4	59		N	
100 µg/L	C	11	1.550	27.8	8.1	0.3	59		N	
100 µg/L	C	12	2.024	29.3	13.3	0.5	59		N	
100 µg/L	C	13	1.482	27.0	9.6	0.4	58		N	
100 µg/L	C	14	1.891	24.2	7.9	0.3	58		Y	
100 µg/L	C	15	1.662	28.5	7.5	0.3	58		N	
100 µg/L	D	1	1.720	29.1	8.5	0.3	58		N	
100 µg/L	D	2	1.668	28.6	7.1	0.2	58		Y	
100 µg/L	D	3	2.018	31.4	10.2	0.3	59		N	
100 µg/L	D	4	1.689	28.6	7.9	0.3	58		Y	
100 µg/L	D	5	1.618	28.6	8.4	0.3	58		N	
100 µg/L	D	6	1.257	26.1	6.9	0.3	58		Y	
100 µg/L	D	7	1.536	28.0	8.8	0.3	58		N	
100 µg/L	D	8	1.915	30.5	10.3	0.3	59		N	
100 µg/L	D	9	1.338	27.4	8.8	0.3	58		Y	
100 µg/L	D	10	1.733	29.1	10.6	0.4	59		N	
100 µg/L	D	11	1.747	29.0	10.1	0.3	58		N	

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Body Weight (g)	Snout-Vent Length (mm)	Hind Limb Length (mm)	HLL:SVL	NF Stage	Clinical Signs	Histopathology? (Y or N)	Comments
100 µg/L	D	12	1.357	26.5	6.6	0.2	58		Y	
100 µg/L	D	13	1.495	27.8	7.0	0.3	58		N	
100 µg/L	D	14	2.475	32.6	12.9	0.4	59		N	
100 µg/L	D	15	1.615	28.5	8.0	0.3	59		N	

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Follicular Cell Hypertrophy	Follicular Cell Hyperplasia	Comments
(Ctl) 0.0 µg/L	A	210			
(Ctl) 0.0 µg/L	A	212			
(Ctl) 0.0 µg/L	A	215			
(Ctl) 0.0 µg/L	A	221	1		
(Ctl) 0.0 µg/L	A	223	1		
(Ctl) 0.0 µg/L	B	228			
(Ctl) 0.0 µg/L	B	231	1		
(Ctl) 0.0 µg/L	B	232	1		
(Ctl) 0.0 µg/L	B	236			
(Ctl) 0.0 µg/L	B	239	1		
(Ctl) 0.0 µg/L	C	247			
(Ctl) 0.0 µg/L	C	249			
(Ctl) 0.0 µg/L	C	250	1		
(Ctl) 0.0 µg/L	C	252			
(Ctl) 0.0 µg/L	C	254			
(Ctl) 0.0 µg/L	D	258	1		
(Ctl) 0.0 µg/L	D	259			
(Ctl) 0.0 µg/L	D	260	1		
(Ctl) 0.0 µg/L	D	262	1	1	
(Ctl) 0.0 µg/L	D	264	1		
3.6 µg/L	A	271	1		
3.6 µg/L	A	272			
3.6 µg/L	A	278			
3.6 µg/L	A	279	1		
3.6 µg/L	A	284	1		
3.6 µg/L	B	285			
3.6 µg/L	B	293			
3.6 µg/L	B	295			
3.6 µg/L	B	297			
3.6 µg/L	B	299			
3.6 µg/L	C	301	1		

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Follicular Cell Hypertrophy	Follicular Cell Hyperplasia	Comments
3.6 µg/L	C	302			
3.6 µg/L	C	304			
3.6 µg/L	C	310			
3.6 µg/L	C	312			
3.6 µg/L	D	317	2	1	
3.6 µg/L	D	320			
3.6 µg/L	D	323	1		
3.6 µg/L	D	324	1		
3.6 µg/L	D	328			
10.9 µg/L	A	330			
10.9 µg/L	A	334	1		
10.9 µg/L	A	341	1		
10.9 µg/L	A	343	1		
10.9 µg/L	A	344	1		
10.9 µg/L	B	345	1		
10.9 µg/L	B	347	1		
10.9 µg/L	B	349	1		
10.9 µg/L	B	354	1		
10.9 µg/L	B	356			
10.9 µg/L	C	360	1		
10.9 µg/L	C	361	1		
10.9 µg/L	C	362			
10.9 µg/L	C	367			
10.9 µg/L	C	368			
10.9 µg/L	D	376	1		
10.9 µg/L	D	377	1		
10.9 µg/L	D	380			
10.9 µg/L	D	381			
10.9 µg/L	D	388	1		
33 µg/L	A	394			
33 µg/L	A	395	1		

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Follicular Cell Hypertrophy	Follicular Cell Hyperplasia	Comments
33 µg/L	A	399			
33 µg/L	A	400			
33 µg/L	A	404	1		
33 µg/L	B	406	1		
33 µg/L	B	407	1		
33 µg/L	B	410			
33 µg/L	B	412			
33 µg/L	B	413			
33 µg/L	C	420	1		
33 µg/L	C	425	1		
33 µg/L	C	429	1		
33 µg/L	C	430	2		
33 µg/L	C	433	1	1	
33 µg/L	D	435	1	1	
33 µg/L	D	436			
33 µg/L	D	437	1		
33 µg/L	D	438	1		
33 µg/L	D	442	1	1	
100 µg/L	A	455			
100 µg/L	A	456	1		
100 µg/L	A	459			
100 µg/L	A	461	1		
100 µg/L	A	463	1		
100 µg/L	B	465	2		
100 µg/L	B	466	1	1	
100 µg/L	B	469	1		
100 µg/L	B	470	1		
100 µg/L	B	477			
100 µg/L	C	480			
100 µg/L	C	482			
100 µg/L	C	483		1	

FEL

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Follicular Cell Hypertrophy	Follicular Cell Hyperplasia	Comments
100 µg/L	C	486	1		
100 µg/L	C	493			
100 µg/L	D	496	1		
100 µg/L	D	498	1		
100 µg/L	D	500			
100 µg/L	D	503	1		
100 µg/L	D	506	1		

FEL

DUPLICATES

Analytical results, IQR calculations, and means.

Week 0					Week 1					Week 2					Week 3				
ID	Desc.	Orig	Dup	Avg	ID	Desc.	Orig	Dup	Avg	ID	Desc.	Orig	Dup	Avg	ID	Desc.	Orig	Dup	Avg
34	0.0 µg/L A	0.104	0.104	0.104											511	0.0 µg/L A	0.104	0.104	0.104
35	0.0 µg/L B	0.104	0.104	0.104											512	0.0 µg/L B	0.104	0.104	0.104
36	0.0 µg/L C	0.104	0.104	0.104											513	0.0 µg/L C	0.104	0.104	0.104
37	0.0 µg/L D	0.104	0.315	0.210											514	0.0 µg/L D	0.104	0.104	0.104
38	3.6 µg/L A	1.61	5.64	3.63											515	3.6 µg/L A	1.26	3.58	2.42
39	3.6 µg/L B	1.64	5.52	3.58											516	3.6 µg/L B	1.19	3.42	2.31
40	3.6 µg/L C	1.59	5.60	3.60	168	3.6 µg/L C	11.2	0.887	6.04						517	3.6 µg/L C	1.12	3.42	2.27
41	3.6 µg/L D	1.68	5.85	3.77											518	3.6 µg/L D	1.33	3.52	2.43
42	10.9 µg/L A	3.07	15.0	9.04											519	10.9 µg/L A	3.95	6.42	5.19
43	10.9 µg/L B	3.41	13.9	8.66											520	10.9 µg/L B	3.82	6.91	5.37
44	10.9 µg/L C	3.13	15.9	9.52											521	10.9 µg/L C	4.11	6.36	5.24
45	10.9 µg/L D	3.35	16.7	10.0											522	10.9 µg/L D	3.87	6.03	4.95
46	33.0 µg/L A	8.17	37.5	22.8											523	33.0 µg/L A	12.5	16.7	14.6
47	33.0 µg/L B	7.97	36.7	22.3											524	33.0 µg/L B	16.6	16.2	16.4
48	33.0 µg/L C	7.84	42.7	25.3											525	33.0 µg/L C	11.4	18.0	14.7
49	33.0 µg/L D	8.04	37.5	22.8											526	33.0 µg/L D	15.7	15.8	15.8
50	100 µg/L A	22.9	97.0	60.0						203	100 µg/L A	65.5	37.1	51.3	527	100 µg/L A	44.3	38.7	41.5
51	100 µg/L B	24.1	94.3	59.2						204	100 µg/L B	84.4	32.1	58.3	528	100 µg/L B	42.1	40.0	41.1
52	100 µg/L C	25.3	131	78.2						205	100 µg/L C	87.5	37.0	62.3	529	100 µg/L C	43.2	39.1	41.2
53	100 µg/L D	26.2	107	66.6						206	100 µg/L D	80.1	33.9	57.0	530	100 µg/L D	40.2	38.2	39.2

This color indicates the value was determined to be outside IQR, therefore not used in calculating average.

This color indicates the value used in the Concentration Analysis sheet.

IQR Calculations

	0	3.6	10.9	33	100
1st Quartile	0.104	1.61	3.93	14.9	37.1
3rd Quartile	0.104	5.85	13.7	31.3	84.9
IQR	0.000	4.24	9.75	16.4	47.9
Upper	0.104	12.2	28.3	55.9	157
Lower	0.104	-4.75	-10.7	-9.70	-34.7

FEL

Substance 2-EHHB
Study BATT01-00388

CONCENTRATION ANALYSIS

Treatment and Replicate	Nominal Conc. (µg/L)	Measured Concentration (µg/L)				Mean (µg/L)	Overall treatment			% Nominal
		Week 0	Week 1	Week 2	Week 3		Mean (µg/L)	Mean CVs [Between] (%)	Replicate Means CV [Within]	
0.0 µg/L A	<MQL	0.104	0.104	0.104	0.104	0.104	0.104			
0.0 µg/L B	<MQL	0.104	0.104	0.104	0.104	0.104				
0.0 µg/L C	<MQL	0.104	0.104	0.104	0.104	0.104				
0.0 µg/L D	<MQL	0.104	0.104	0.104	0.104	0.104				
0.0 µg/L Mean		0.104	0.104	0.104	0.104	0.104				
Replicate CV %		0.0	0.0	0.0	0.0					
3.6 µg/L A	3.60	3.63	4.47	5.08	2.42	3.90	4.58	11.2	12.70%	127
3.6 µg/L B	3.60	3.58	7.28	8.05	2.31	5.30				
3.6 µg/L C	3.60	3.60	6.04	5.86	2.27	4.44				
3.6 µg/L D	3.60	3.77	5.94	6.50	2.43	4.66				
3.6 µg/L Mean		3.64	5.93	6.37	2.36	4.58				
Replicate CV %		2.3	19.4	19.8	3.4					
10.9 µg/L A	10.90	9.04	13.3	14.9	5.19	10.6	10.3	7.2	3.33%	94.2
10.9 µg/L B	10.90	8.66	14.7	10.9	5.37	9.91				
10.9 µg/L C	10.90	9.52	12.6	12.9	5.24	10.1				
10.9 µg/L D	10.90	10.0	13.5	13.6	4.95	10.5				
10.9 µg/L Mean		9.31	13.53	13.08	5.18	10.3				
Replicate CV %		6.4	6.5	12.8	3.3					
33 µg/L A	33.0	22.8	35.4	26.8	14.6	24.9	25.0	6.6	1.84%	75.9
33 µg/L B	33.0	22.3	30.5	28.9	16.4	24.5				
33 µg/L C	33.0	25.3	31.0	31.6	14.7	25.6				
33 µg/L D	33.0	22.8	30.5	31.2	15.8	25.1				
33 µg/L Mean		23.3	31.9	29.6	15.4	25.0				
Replicate CV %		5.7	7.5	7.5	5.6					
100 µg/L A	100	60.0	83.3	51.3	41.5	59.0	62.0	7.1	5.81%	62.0
100 µg/L B	100	59.2	86.5	58.3	41.1	61.3				
100 µg/L C	100	78.2	87.1	62.3	41.2	67.2				
100 µg/L D	100	66.6	78.6	57.0	39.2	60.4				
100 µg/L Mean		66.0	83.9	57.2	40.7	62.0				
Replicate CV %		13.3	4.6	7.9	2.5					

Indicates value from Duplicates sheet.

NOTE: <MQL reported as 1/2 of MQL (.208 µg/L)

FEL

Appendix F

BATTELLE STATISTICAL ANALYSIS REPORT

FEL

Endocrine Disruptor Screening Program

USEPA Contract No: EP-W-11-063

Task Order No: 14

Final Statistical Analysis Report for

21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, Xenopus laevis

FEL Study No. BATT01-00388

(AMA #388)

February 26, 2018

BATTELLE

505 King Avenue

Columbus, Ohio 43201

COMPLIANCE STATEMENT

FEL Study Number: BATT01-00388

Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, Xenopus laevis

Battelle Statistician / Report Originator: Ying-Liang Chou
Battelle Statistician / Technical Reviewer: Po-Hsu Chen

The statistical analysis portion of the above-referenced study was conducted in compliance with the Good Laboratory Practice regulations of the Environmental Protection Agency as stipulated by 40 CFR Part 160 (FIFRA), the study protocol, and all applicable amendments. Deviations, if present, can be found in study data file.

Ying-Liang Chou 2/26/2018
Battelle Study Statistician Date

SIGNATURES

Name (Role)	Signature	Date
Ying-Liang Chou (Battelle Statistician / Report Originator)	Ying-Liang Chou	2/26/2018
Po-Hsu Chen (Battelle Statistician / Technical Reviewer)		2/26/2018
Vince Brown (Program Manager)	Vince Brown	2/26/2018

USEPA Contract No.: EP-W-11-063, TO 14
FEL Study No. BATT01-00388 (AMA #388)

Quality Assurance Statement
Battelle

USEPA Contract No.: EP-W-11-063, TO 14
Study No. BATT01-00388

This study was inspected by the Quality Assurance Unit. Reports were submitted to the Study Director and Management as follows:

Audit	Date of Audit	Date Reported to Study Director and Management
Audit Study Data	May 24-25, 2017	May 25, 2017
Audit Draft Statistical Report	May 24-25, 2017 July 5-6, 2017	May 25, 2017 July 7, 2017
Audit Study File Addendum	July 5-6, 2017	July 7, 2017
Audit Final Statistical Report	February 23, 2018	February 26, 2018

Quality Assurance Unit, Battelle — Date: 2-26-18

INTRODUCTION

This report summarizes the statistical analysis of the data collected under the Fort Environmental Laboratories (FEL) Study Number BATT01-00388, "21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, Xenopus laevis" (AMA #388).

An amphibian metamorphosis assay was performed in which Nieuwkoop and Faber (NF) (1) stage 51 *Xenopus laevis* larvae were exposed to different concentrations of the test substance (2-ethylhexyl 4-hydroxybenzoate; 2-EHHB) for 21-days. Tadpoles were exposed to four different concentrations of the test chemical (n = 4 replicates per concentration) and dilution water control (n = 4 replicates). Larval density at test initiation was 20 tadpoles per test tank (i.e., replicate) for all treatment groups. Larvae selected were stage matched to the greatest extent possible based on stage distribution and from those specimens were randomly selected. The treatment tanks were randomly assigned to a position in the exposure system to account for possible variations in temperature and light intensity. The primary endpoints were hind limb length (HLL), body length (snout-to-vent [SVL]), developmental stage, wet body weight, thyroid histology, and daily mortality. The experimental design is presented in Table 1.

Table 1. Experimental Design

2-EHHB Treatment Group (µg/L)	Number of Replicates	Number of Tadpoles per Replicate	Total Number of Tadpoles per Treatment Group
0.0 (control)	4	20	80
3.6	4	20	80
10.9	4	20	80[a]
33.0	4	20	80
100.0	4	20	80
TOTAL			400

[a] Thyroid gland tissue was not recovered from one tadpole of this group

STATISTICAL METHODS

Statistical analyses were performed and consistent with OPPTS 890.1100 test guidelines (2), the TO 14 QAPP (3), and generally follow procedures described in the document "Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application" (4).

Preliminary analyses were performed on continuous quantitative endpoints: hind limb length (HLL), snout-to-vent length (SVL), normalized hind limb length, and wet body weight, separately for Day 7 and Day 21. Hind limb length was normalized by taking the ratio of hind limb length to snout-to-vent length of an individual (HLL:SVL). Concentration-response monotonicity was assessed visually from the replicate and treatment means. Normality was evaluated by Shapiro-Wilk's test and homogeneity of variance was evaluated by Levene's test.

For endpoints that followed a monotonic concentration-response, the Jonckheere-Terpstra test was applied in step-down manner to establish significant treatment effects. For endpoints that were not consistent with a monotonic concentration-response, the data were evaluated for normality (Shapiro-Wilk's test) and homogeneity (Levene's test). If a data set was found to have a non-normal distribution or a heterogeneous distribution of variance, a normalizing, variance stabilizing transformation was used. If data sets were normally distributed with homogeneous variance following transformation, the data set was evaluated using Dunnett's test. If the data set was normally distributed with heterogeneous variance following data transformation, the Mann-Whitney-Wilcoxon test (with Bonferroni-Holm adjustment) was used to evaluate the data. Where no normalizing transformation was found, the Mann-Whitney-Wilcoxon test with a Bonferroni-Holm adjustment to the p-values was used to evaluate the data sets. Statistical analyses were performed on replicate means.

A RSCABS (Rao-Scott Cochran-Armitage by Slices) test (5), which uses a step-down Rao-Scott adjusted Cochran-Armitage trend test on each level of severity in a histopathology response, was used to evaluate histopathology data. The by slices (BS) portion of the test allows for testing at each severity score. By slices works by splitting the severity scores associated with an endpoint into two groups based on the severity score being tested. One group contains all severity scores less than the target severity score and the other group contains all severity scores equal to or greater than the target severity score.

Potential statistical outliers were assessed for all continuous quantitative endpoints. If the studentized residual from an analysis of variance model fit was greater than 3 in absolute value, then the observed value was flagged as potential statistical outlier. Statistical analyses were performed on data with and without potential statistical outliers.

All tadpoles in the study survived. Therefore, no statistical analysis was performed on mortality data. Treatment effect significance for developmental stage was determined on the replicate median values using the Jonckheere-Terpstra step-down test. The two histopathologic findings recorded in this study were follicular cell hypertrophy and follicular cell hyperplasia. The histopathologic findings occurrence rates were summarized.

Except for the RSCABS analysis on histopathology data, statistical analysis software SAS® (version 9.3 or later) was used in all other statistical analyses, with the statistical significance assessed at the two-sided 0.05 level. An R-based RSCABS software, version 0.9.01 (6) was used in the RSCABS analysis on histopathology data, with the statistical significance assessed at the one-sided 0.05 level at each slice.

RESULTS

Table 2 summarizes mortality and NF stage for Days 7 and 21. Jonckheere-Terpstra step-down test results on Day 21 replicate median values are also presented in Table 2. As noted above, all tadpoles survived in the study; therefore, no statistical analysis was performed on mortality data. Also, no statistical test was conducted for Day 7 NF stage since all tadpoles were recorded as NF stage 54 on Day 7. Jonckheere-Terpstra step-down test results for NF stage on Day 21 showed that all four treatment groups were compatible with the control group.

Tables 3a through 3d present replicate means for each treatment group and descriptive statistics (number of samples, mean, standard error of the mean, and percent coefficient of variation) for each treatment group for hind limb length (HLL), snout-to-vent length (SVL), normalized hind limb length (HLL:SVL), and wet body weight by Days 7 and 21, respectively. Figures 1 through 8 present treatment mean ± 2 standard errors plots.

Table 4 presents statistical analysis results. A significant increasing trend was seen for the highest treatment group (100 µg/L) for HLL at Day 7 and for the two highest treatment groups (33 µg/L and 100 µg/L) for body weight at both Days 7 and 21. For pairwise comparisons to control, there was no significant difference between treatment groups and control. Note that while the comparisons were based on group means (or geometric means) for normally (or log-normally) distributed data for the analysis of variance (ANOVA) Dunnett's test, the comparisons were based on mean scores for the non-parametric Mann-Whitney-Wilcoxon test.

Tables 5a and 5b summarize occurrence rates for histopathologic findings for follicular cell hypertrophy and follicular cell hyperplasia, respectively. Note that thyroid gland tissue was not recovered from one tadpole in replicate A of the 10.9 µg/L treatment group. RSCABS tests showed that there was no statistically significant treatment effect on the level of severity for histopathologic endpoints.

Figures A-1 to A-8 in Appendix A present plots for replicate and treatment means for the visual assessment of concentration-response monotonicity.

Tables and figures in Appendix B present statistical analyses performed on data with potential statistical outliers removed. Table B-1 lists the nine potential statistical outliers. The statistical analysis results were not changed with outliers removed.

STUDY ARCHIVAL

Supporting data and the final report were archived at Battelle.

REFERENCES

1. Nieuwkoop, P.D. and Faber, J. Normal Tables of Xenopus laevis (Daudin). Garland Publishing, London, 1994.

2. QAPP, Endocrine Disruptor Screening Program (EDSP) Tier 1 and Tier 2 In Vivo Testing of Selected Chemicals for Potential Endocrine Effects in Non-Mammals. EPA Contract No. EP-W-11-063 Task Order 14, version 1.2, August 28, 2015.

3. OPPTS. 890.1100 Amphibian Metamorphosis (Frog), United States Environmental Protection Agency, Washington DC, EPA 740-C-09-002, October 2009.

4. OECD. Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. Environmental Health and Safety Publications. Series on Testing and Assessment, No. 54, Paris, France, 2006.

5. Green J.W., Springer T.A., Saulnier A.N., Swintek J. 2014. Statistical analysis of histopathology endpoints. Environmental Toxicology and Chemistry. 33(5):1108-1116.

6. Swintek J. https://cran.r-project.org/web/packages/RSCABS/RSCABS.pdf, December 12, 2016.

Table 2. Summary of Mortality and NF Stage (with Jonckheere-Terpstra Test[a]).

Treatment (µg/L)	Replicate	Mortality (Day 7)		Mortality (Day 21)		NF Stage (Day 7)			NF Stage (Day 21)			Jonckheere-Terpstra Test on Day 21 NF Stage (p-value)
		N	Dead	N	Dead	N	Median	IQR	N	Median	IQR	
0.0	A	20	0	15	0	5	54	54-54	15	58	58-58	
	B	20	0	15	0	5	54	54-54	15	59	58-59	
	C	20	0	15	0	5	54	54-54	15	58	58-59	
	D	20	0	15	0	5	54	54-54	15	58	58-59	
	Overall	80	0	60	0	20	54	54-54	60	58	58-59	
3.6	A	20	0	15	0	5	54	54-54	15	58	58-59	
	B	20	0	15	0	5	54	54-54	15	58	58-59	
	C	20	0	15	0	5	54	54-54	15	58	57-59	NP
	D	20	0	15	0	5	54	54-54	15	59	58-59	
	Overall	80	0	60	0	20	54	54-54	60	58	58-59	
10.9	A	20	0	15	0	5	54	54-54	15	58	58-59	
	B	20	0	15	0	5	54	54-54	15	58	57-59	
	C	20	0	15	0	5	54	54-54	15	58	58-59	NP
	D	20	0	15	0	5	54	54-54	15	58	57-59	
	Overall	80	0	60	0	20	54	54-54	60	58	58-59	
33	A	20	0	15	0	5	54	54-54	15	59	58-59	
	B	20	0	15	0	5	54	54-54	15	59	58-59	
	C	20	0	15	0	5	54	54-54	15	59	58-59	NP
	D	20	0	15	0	5	54	54-54	15	58	58-59	
	Overall	80	0	60	0	20	54	54-54	60	59	58-59	
100	A	20	0	15	0	5	54	54-54	15	59	58-59	
	B	20	0	15	0	5	54	54-54	15	59	58-59	
	C	20	0	15	0	5	54	54-54	15	58	58-59	0.1962
	D	20	0	15	0	5	54	54-54	15	58	58-59	
	Overall	80	0	60	0	20	54	54-54	60	59	58-59	

a. Jonckheere-Terpstra tests were conducted on NF stage Day 21 replicate median values in a stepdown fashion at the 0.05 level (2-sided). The test was not conducted for NF stage Day 7 since all tadpoles were recorded as NF stage 54 on Day 7.

IQR Interquartile range, 10^{th} to 90^{th} percentiles.

NP Jonckheere-Terpstra step-down test was not performed since the highest treatment group at 100 µg/L was not statistically significant at the 0.05 level.

Table 3a. Descriptive Statistics for Hind Limb Length (mm) by Study Days 7 and 21.

		Hind Limb Length (mm) Day 7					Hind Limb Length (mm) Day 21				
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	SEM	CV (%)	N	Replicate Mean	Mean	SEM	CV (%)
0.0	A	5	1.80	1.66	0.06	7.16	15	6.18	6.83	0.23	6.60
	B	5	1.62				15	6.87			
	C	5	1.52				15	7.06			
	D	5	1.70				15	7.19			
3.6	A	5	1.70	1.86	0.12	12.81	15	10.87	10.39	1.41	27.13
	B	5	1.94				15	11.92			
	C	5	1.64				15	6.29			
	D	5	2.16				15	12.50			
10.9	A	5	1.82	1.73	0.12	13.57	15	9.23	8.83	0.59	13.43
	B	5	1.90				15	9.67			
	C	5	1.80				15	9.35			
	D	5	1.38				15	7.07			
33	A	5	1.82	1.81	0.05	5.52	15	11.97	10.51	0.88	16.80
	B	5	1.94				15	9.69			
	C	5	1.78				15	11.96			
	D	5	1.70				15	8.41			
100	A	5	1.88	2.02	0.08	8.24	15	10.47	9.72	0.36	7.48
	B	5	2.04				15	10.10			
	C	5	1.90				15	9.50			
	D	5	2.24				15	8.81			

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.

Table 3b. Descriptive Statistics for Snout -to-Vent Length (mm) by Study Days 7 and 21.

		Snout-to-Vent Length (mm) Day 7					Snout-to-Vent Length (mm) Day 21				
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	SEM	CV (%)	N	Replicate Mean	Mean	SEM	CV (%)
0.0	A	5	15.44	15.48	0.28	3.62	15	25.34	27.32	0.71	5.21
	B	5	15.70				15	27.28			
	C	5	14.72				15	28.56			
	D	5	16.04				15	28.11			
3.6	A	5	13.74	14.40	0.62	8.59	15	30.53	28.36	1.09	7.66
	B	5	14.24				15	29.57			
	C	5	13.42				15	25.61			
	D	5	16.18				15	27.71			
10.9	A	5	14.44	14.04	0.63	8.97	15	27.07	25.88	0.46	3.59
	B	5	14.06				15	25.55			
	C	5	15.32				15	26.04			
	D	5	12.32				15	24.85			
33	A	5	15.38	14.97	0.49	6.61	15	26.54	27.67	0.41	2.97
	B	5	15.88				15	28.25			
	C	5	15.04				15	28.30			
	D	5	13.58				15	27.57			
100	A	5	16.34	16.30	0.24	2.89	15	27.49	27.90	0.30	2.13
	B	5	16.68				15	27.64			
	C	5	15.62				15	27.70			
	D	5	16.54				15	28.79			

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.

Table 3c. Descriptive Statistics for Normalized Hind Limb Length (ratio of HLL:SVL) by Study Days 7 and 21.

		Normalized Hind Limb Length (ratio of HLL:SVL) Day 7					Normalized Hind Limb Length (ratio of HLL:SVL) Day 21				
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	SEM	CV (%)	N	Replicate Mean	Mean	SEM	CV (%)
0.0	A	5	0.10	0.10	0.00	0.00	15	0.25	0.24	0.00	1.58
	B	5	0.10				15	0.24			
	C	5	0.10				15	0.24			
	D	5	0.10				15	0.25			
3.6	A	5	0.12	0.12	0.01	8.70	15	0.35	0.36	0.04	23.67
	B	5	0.12				15	0.40			
	C	5	0.10				15	0.25			
	D	5	0.12				15	0.45			
10.9	A	5	0.10	0.10	0.00	0.00	15	0.33	0.34	0.02	12.58
	B	5	0.10				15	0.38			
	C	5	0.10				15	0.35			
	D	5	0.10				15	0.28			
33	A	5	0.10	0.10	0.00	0.00	15	0.46	0.38	0.04	20.10
	B	5	0.10				15	0.33			
	C	5	0.10				15	0.42			
	D	5	0.10				15	0.30			
100	A	5	0.10	0.10	0.00	0.00	15	0.38	0.35	0.02	9.81
	B	5	0.10				15	0.36			
	C	5	0.10				15	0.35			
	D	5	0.10				15	0.30			

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.

Table 3d. Descriptive Statistics for Wet Body Weight (g) by Study Days 7 and 21.

		Body Weight (g) Day 7					Body Weight (g) Day 21				
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	SEM	CV (%)	N	Replicate Mean	Mean	SEM	CV (%)
0.0	A	5	0.2070	0.2061	0.0080	7.7446	15	0.9296	1.1260	0.0716	12.7242
	B	5	0.2142				15	1.1100			
	C	5	0.1834				15	1.2427			
	D	5	0.2196				15	1.2216			
3.6	A	5	0.2440	0.2638	0.0303	22.9956	15	1.6203	1.4581	0.1845	25.3048
	B	5	0.2398				15	1.4865			
	C	5	0.2182				15	0.9367			
	D	5	0.3532				15	1.7889			
10.9	A	5	0.2596	0.2531	0.0296	23.3964	15	1.4731	1.3281	0.0643	9.6833
	B	5	0.2462				15	1.3003			
	C	5	0.3254				15	1.3719			
	D	5	0.1810				15	1.1672			
33	A	5	0.3248	0.2911	0.0274	18.8170	15	1.4983	1.6426	0.0598	7.2788
	B	5	0.3286				15	1.6669			
	C	5	0.2996				15	1.7871			
	D	5	0.2112				15	1.6182			
100	A	5	0.3234	0.3131	0.0218	13.9443	15	1.5794	1.6284	0.0222	2.7267
	B	5	0.3544				15	1.6057			
	C	5	0.3230				15	1.6499			
	D	5	0.2514				15	1.6787			

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.

Table 4. Statistical Analysis Results

Parameter	Monotonicity Assessment[1]	Normality Test[2]	Homogeneity of Variance Test[4]	Jonckheere-Terpstra Test[5] (p-value)	Significant Pairwise Comparisons to Control[6] (p-value)
HLL (mm) (Day 7)	Monotonic	NP	NP	Group 5 (0.0180)	NP
HLL (mm) (Day 21)	Non-Monotonic	Log-normal[3]	Heterogeneous	NP	NS
SVL (mm) (Day 7)	Non-Monotonic	Normal	Homogeneous	NP	NS
SVL (mm) (Day 21)	Non-Monotonic	Non-normal	Heterogeneous	NP	NS
HLL:SVL (Day 7)	Non-Monotonic	Non-normal	Heterogeneous	NP	NS
HLL:SVL (Day 21)	Non-Monotonic	Non-normal	Heterogeneous	NP	NS
Body Weight (g) (Day 7)	Monotonic	NP	NP	Group 5 (0.0079) Group 4 (0.0399)	NP
Body Weight (g) (Day 21)	Monotonic	NP	NP	Group 5 (0.0053) Group 4 (0.0196)	NP

1. Monotonicity was assessed visually from the replicate and treatment means.
2. Shapiro-Wilk test for normality.
3. Normal when log-transformed.
4. LeveneTest for homogeneity of variance.
5. Jonckheere-Terpstra step-down trend test was performed on monotonic concentration-response data. Only statistically significant treatment trends were listed.
6. Control is group 1 and test concentration groups are groups 2 to 5. Only statistically significant pairwise comparisons were listed.
   - For non-monotonic, normally distributed, and homogeneous variance data, Dunnett's test was used in the pairwise comparisons to control.
   - For other types of data (e.g., non-monotonic, normally distributed, and heterogeneous variance data), Mann-Whitney-Wilcoxon test was used in the pairwise comparisons to control with Bonferroni-Holm multiple comparison adjustment.

NP Statistical test or comparison was not performed.

NS No statistically significant differences were found for Dunnett's tests or Mann-Whitney-Wilcoxon tests after Bonferroni-Holm adjustment.

Table 5a. Summary of Histopathologic Findings for Follicular Cell Hypertrophy.

Treatment (µg/L)	Replicate	Mild (1)		Moderate (2)	
		No. Findings/ No. in Group	Proportion	No. Findings/ No. in Group	Proportion
0.0	A	2/5	0.40	0/5	0.00
	B	3/5	0.60	0/5	0.00
	C	1/5	0.20	0/5	0.00
	D	4/5	0.80	0/5	0.00
	Overall	10/20	0.50	0/20	0.00
3.6	A	3/5	0.60	0/5	0.00
	B	0/5	0.00	0/5	0.00
	C	1/5	0.20	0/5	0.00
	D	2/5	0.40	1/5	0.20
	Overall	6/20	0.30	1/20	0.05
10.9	A	4/4[a]	1.00	0/4[a]	0.00
	B	4/5	0.80	0/5	0.00
	C	2/5	0.40	0/5	0.00
	D	3/5	0.60	0/5	0.00
	Overall	13/19[a]	0.68	0/19[a]	0.00
33	A	2/5	0.40	0/5	0.00
	B	2/5	0.40	0/5	0.00
	C	4/5	0.80	1/5	0.20
	D	4/5	0.80	0/5	0.00
	Overall	12/20	0.60	1/20	0.05
100	A	3/5	0.60	0/5	0.00
	B	3/5	0.60	1/5	0.20
	C	1/5	0.20	0/5	0.00
	D	4/5	0.80	0/5	0.00
	Overall	11/20	0.55	1/20	0.05

(1) The RSCABS test showed that there was no statistically significant treatment effect for the mild (p-value=0.1501).

(2) The RSCABS test showed that there was no statistically significant treatment effect for the moderate (p-value=0.2025).

a. Thyroid gland tissue was not recovered from one tadpole in replicate A of treatment group 10.9 µg/L.

Table 5b. Summary of Histopathologic Findings for Follicular Cell Hyperplasia.

Treatment (µg/L)	Replicate	Mild (1)	
		No. Findings/ No. in Group	Proportion
0.0	A	0/5	0.00
	B	0/5	0.00
	C	0/5	0.00
	D	1/5	0.20
	Overall	1/20	0.05
3.6	A	0/5	0.00
	B	0/5	0.00
	C	0/5	0.00
	D	1/5	0.20
	Overall	1/20	0.05
10.9	A	0/4[a]	0.00
	B	0/5	0.00
	C	0/5	0.00
	D	0/5	0.00
	Overall	0/19[a]	0.00
33	A	0/5	0.00
	B	0/5	0.00
	C	1/5	0.20
	D	2/5	0.40
	Overall	3/20	0.15
100	A	0/5	0.00
	B	1/5	0.20
	C	1/5	0.20
	D	0/5	0.00
	Overall	2/20	0.10

(1) The RSCABS test showed that there was no statistically significant treatment effect for the mild (p-value=0.1553).

a. Thyroid gland tissue was not recovered from one tadpole in replicate A of treatment group 10.9 µg/L.

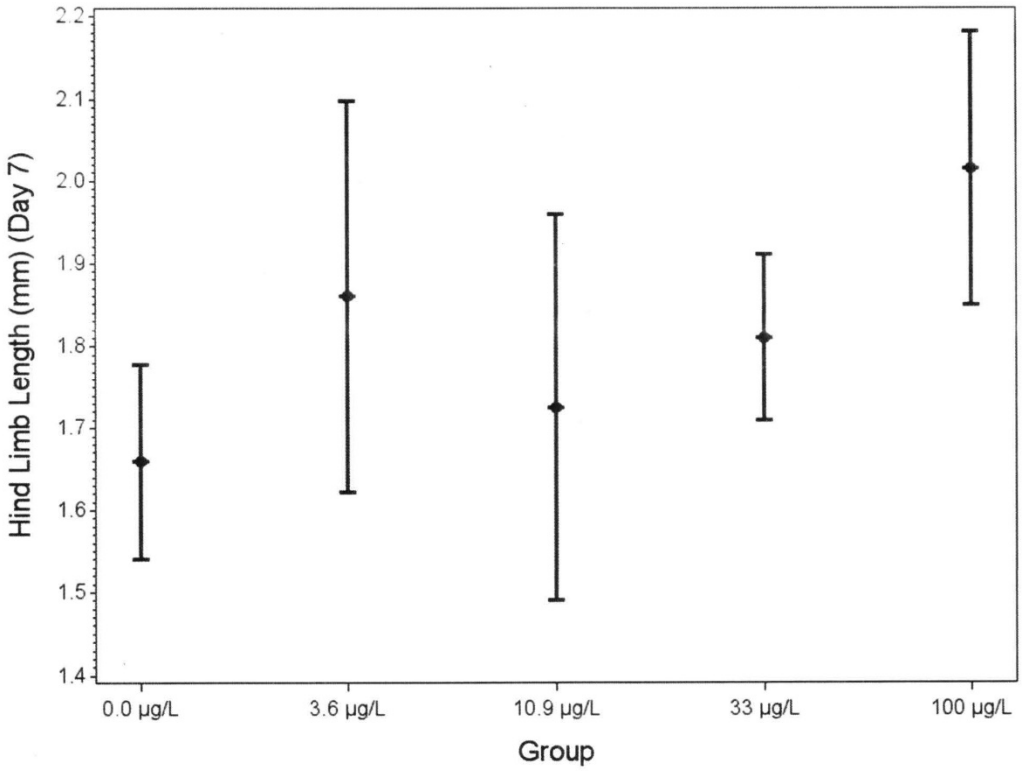

Figure 1. Treatment Mean ± 2 Standard Errors for Hind Limb Length (mm), Day 7.

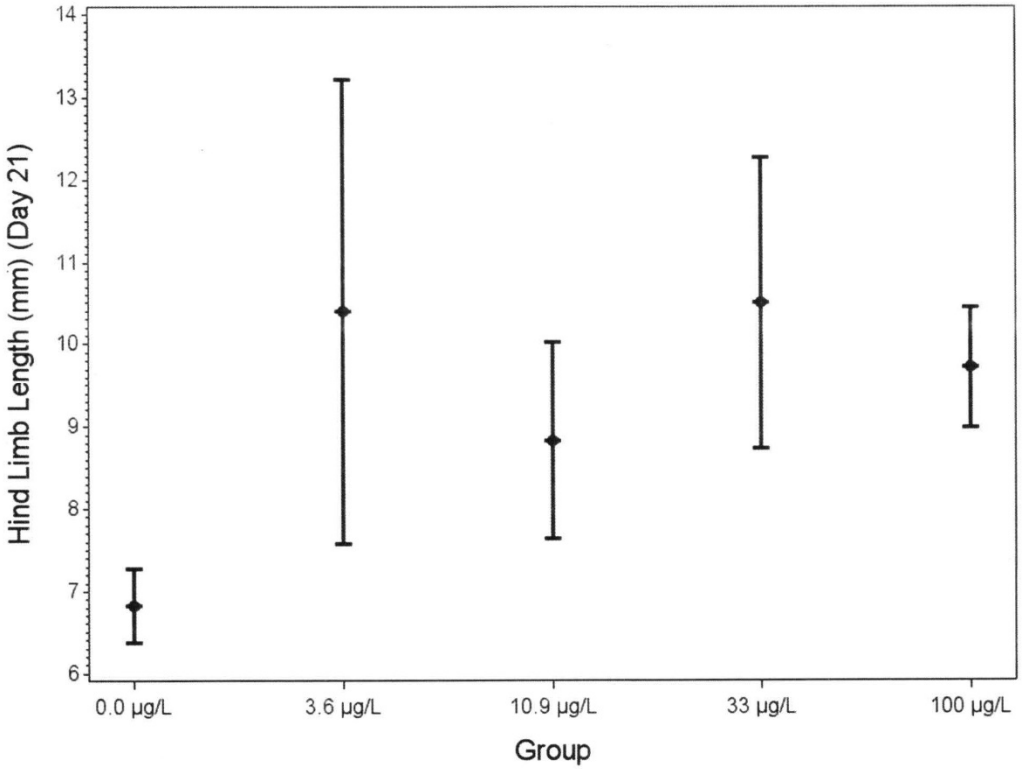

Figure 2. Treatment Mean ± 2 Standard Errors for Hind Limb Length (mm), Day 21.

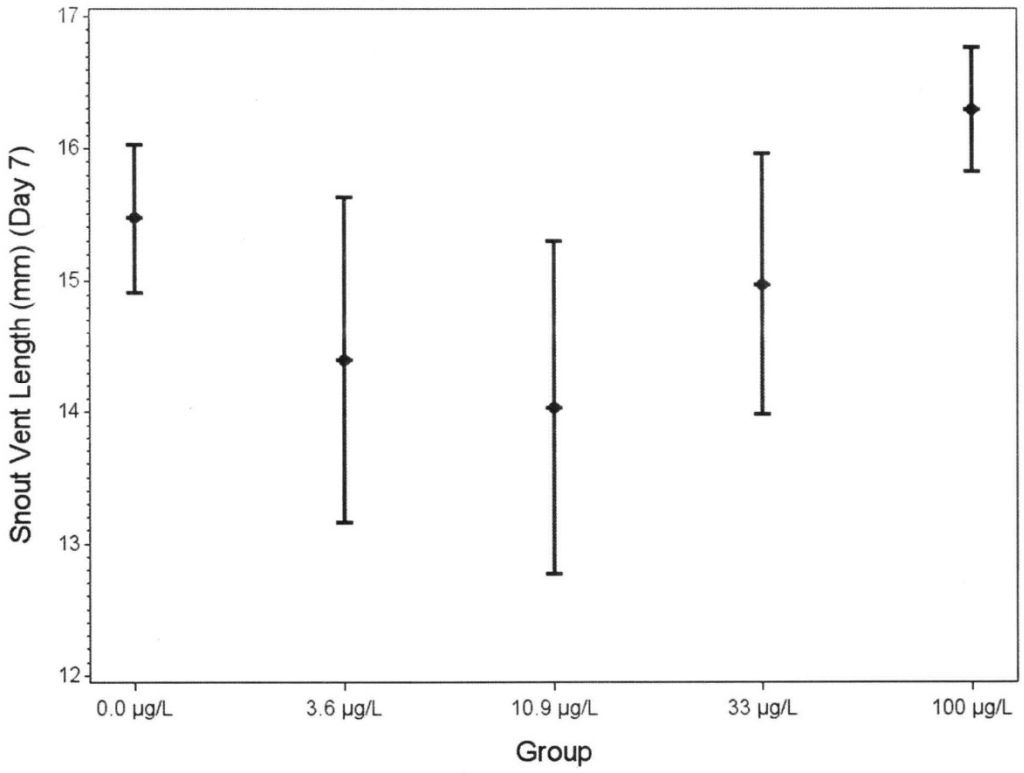

Figure 3. **Treatment Mean ± 2 Standard Errors for Snout-to-Vent Length (mm), Day 7.**

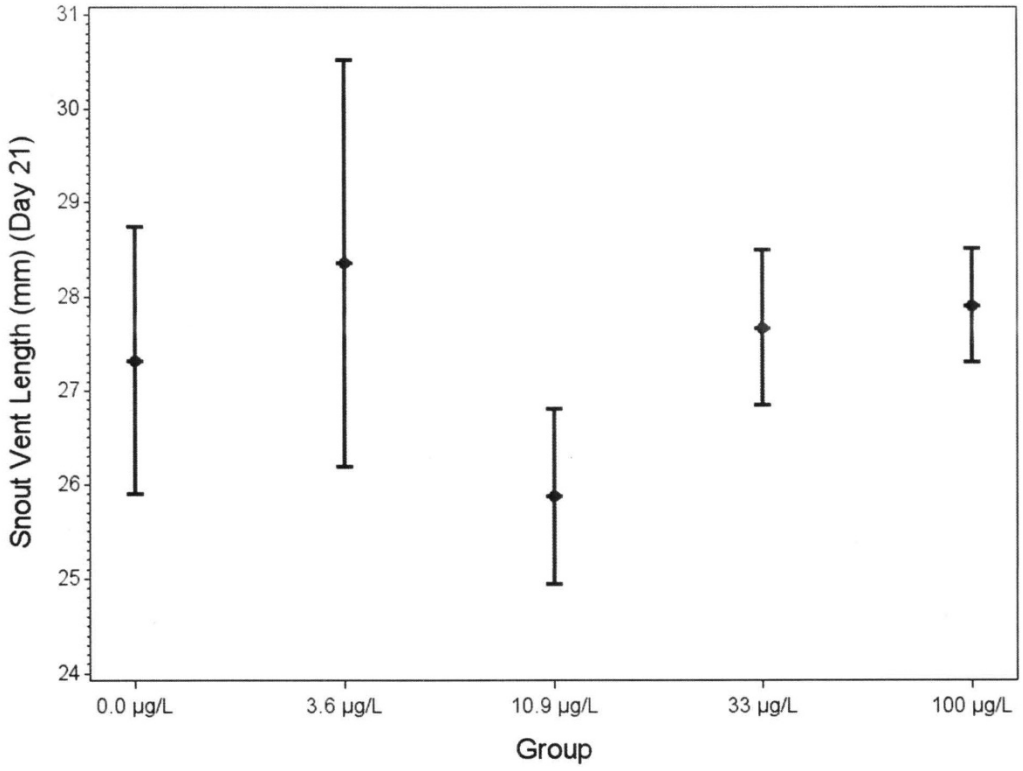

Figure 4. **Treatment Mean ± 2 Standard Errors for Snout-to-Vent Length (mm), Day 21.**

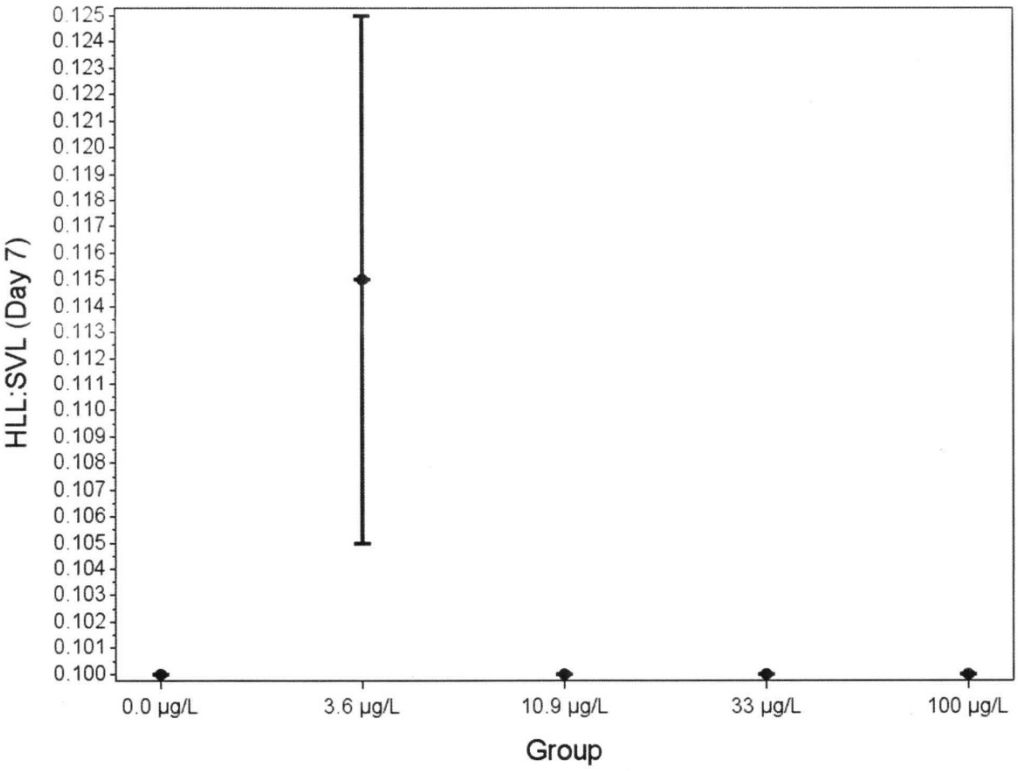

Figure 5. **Treatment Mean ± 2 Standard Errors for Normalized Hind Limb Length (HLL:SVL), Day 7.**

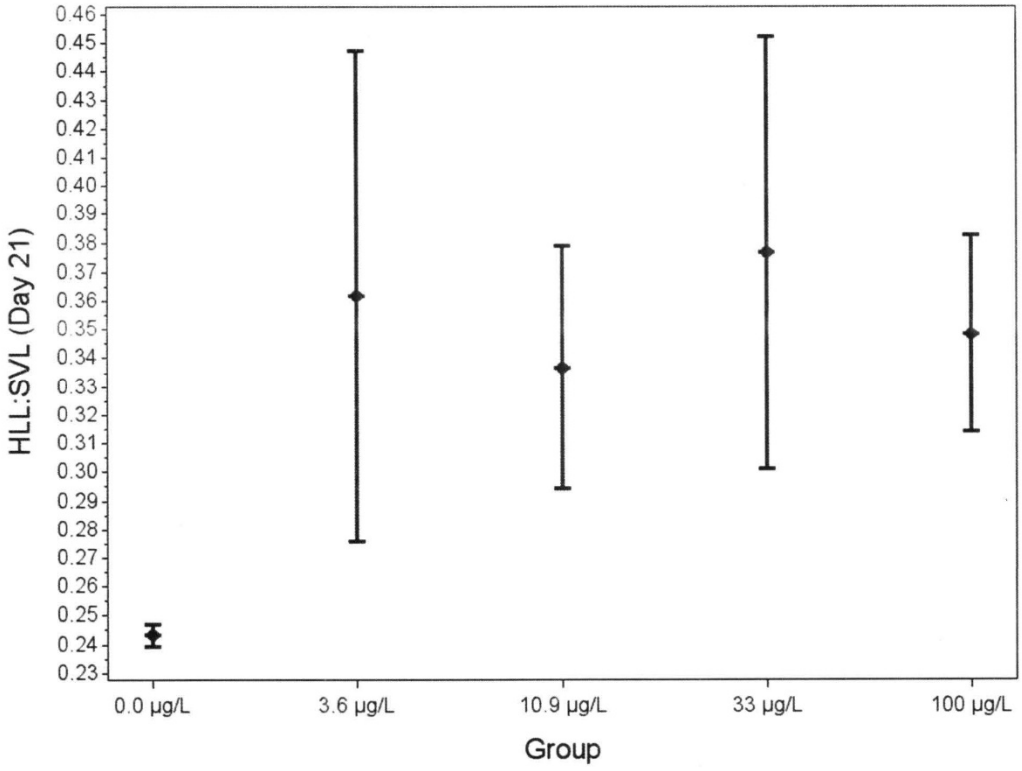

Figure 6. **Treatment Mean ± 2 Standard Errors for Normalized Hind Limb Length (HLL:SVL), Day 21.**

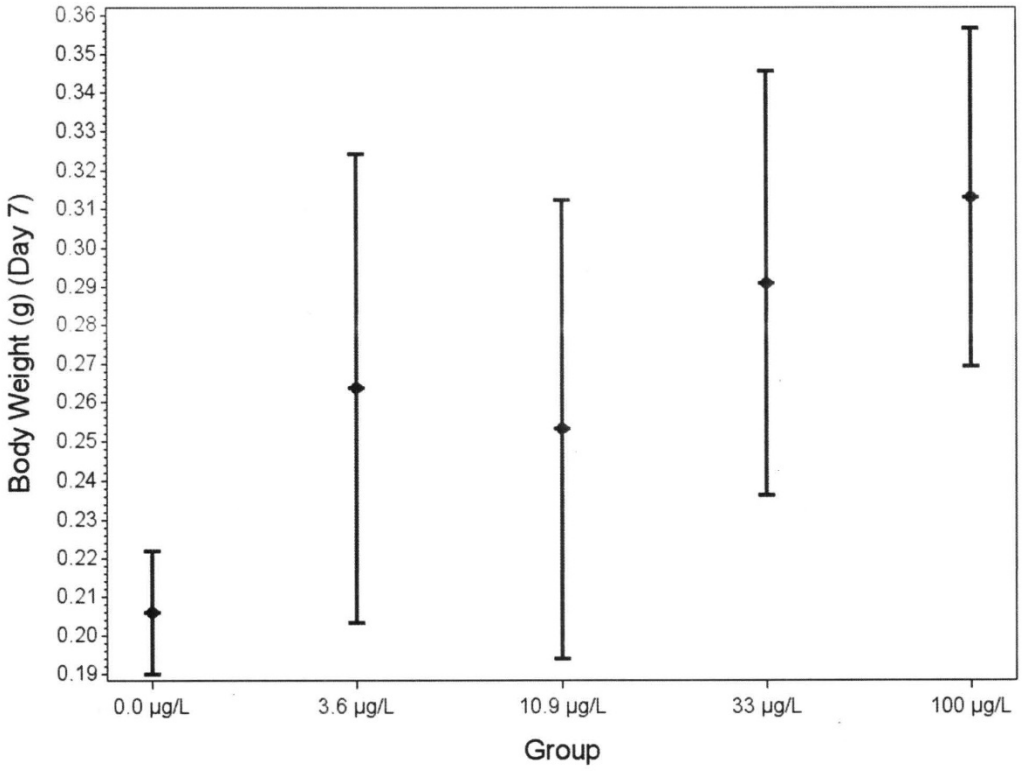

Figure 7. Treatment Mean ± 2 Standard Errors for Body Weight (g), Day 7.

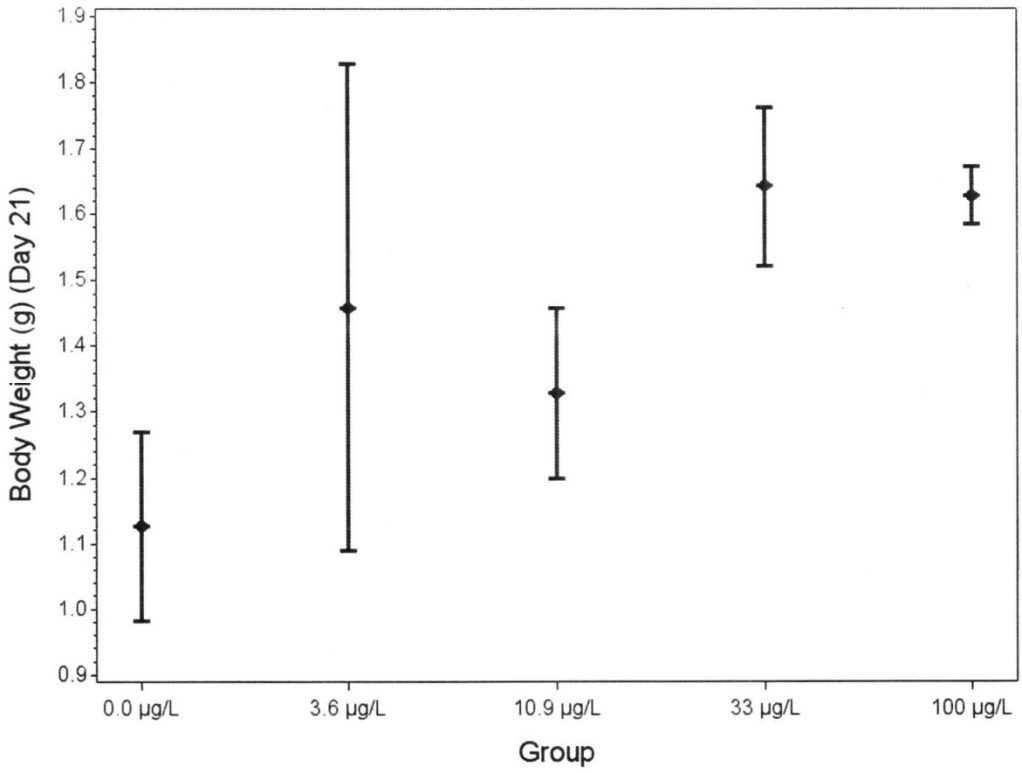

Figure 8. Treatment Mean ± 2 Standard Errors for Body Weight (g), Day 21.

Appendix A

Plots Used for the Visual Assessment of Concentration-Response Monotonicity

USEPA Contract No.: EP-W-11-063, TO 14
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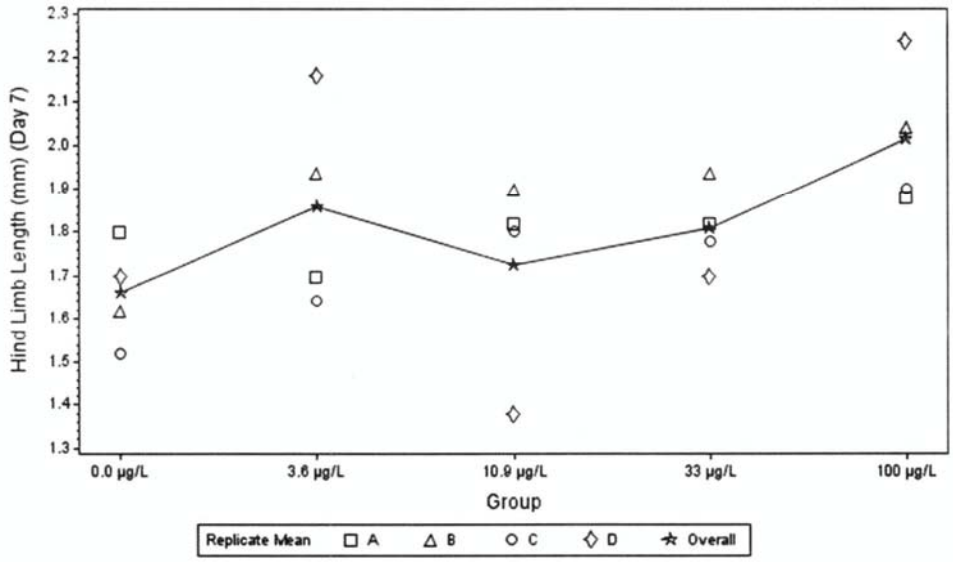

Figure A-1. Treatment and Replicate Means for Hind Limb Length (mm), Day 7.

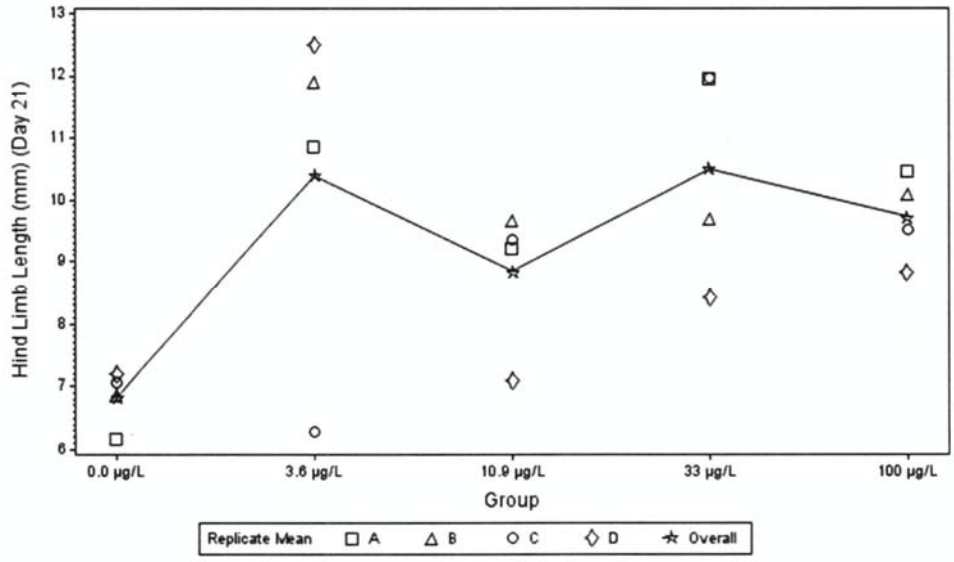

Figure A-2. Treatment and Replicate Means for Hind Limb Length (mm), Day 21.

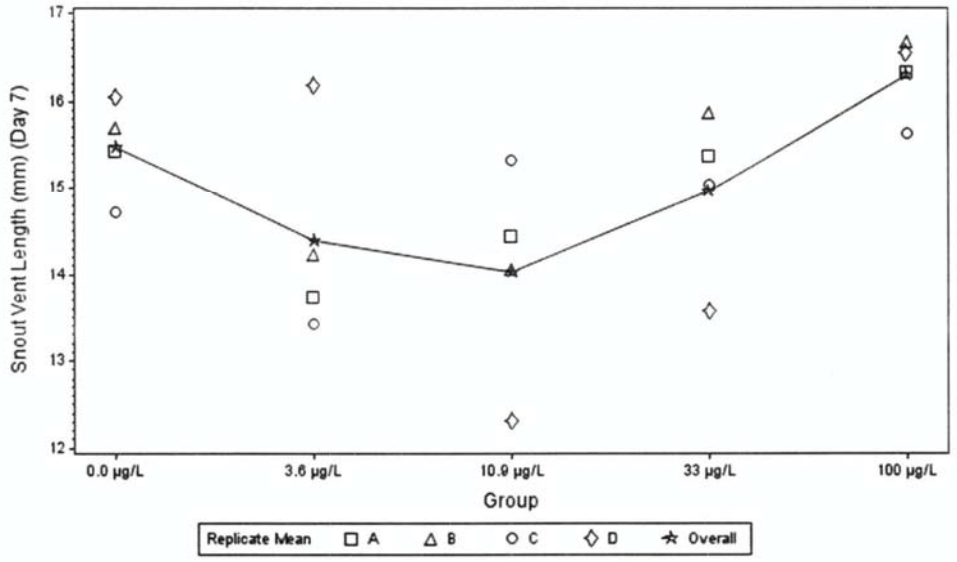

Figure A-3. Treatment and Replicate Means for Snout-to-Vent Length (mm), Day 7.

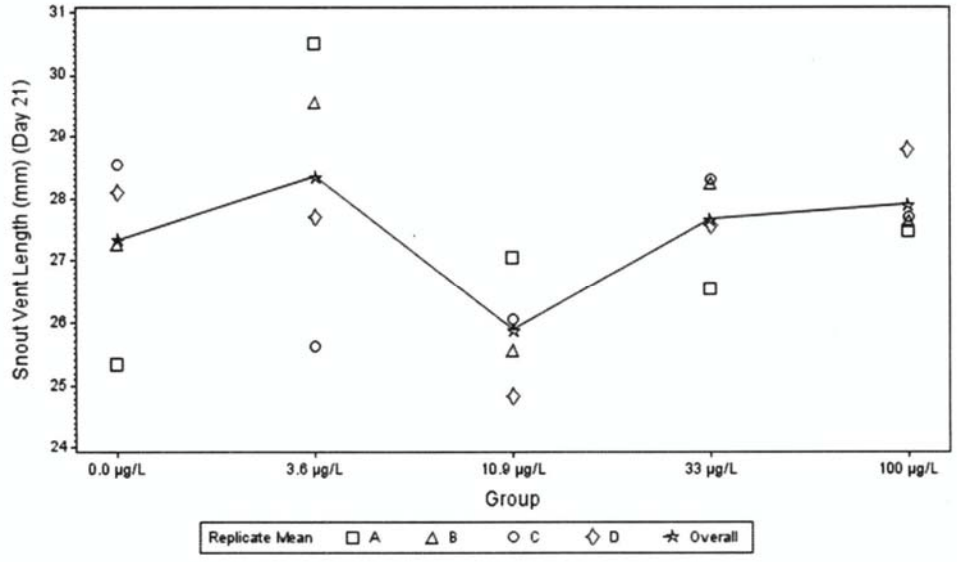

Figure A-4. Treatment and Replicate Means for Snout-to-Vent Length (mm), Day 21.

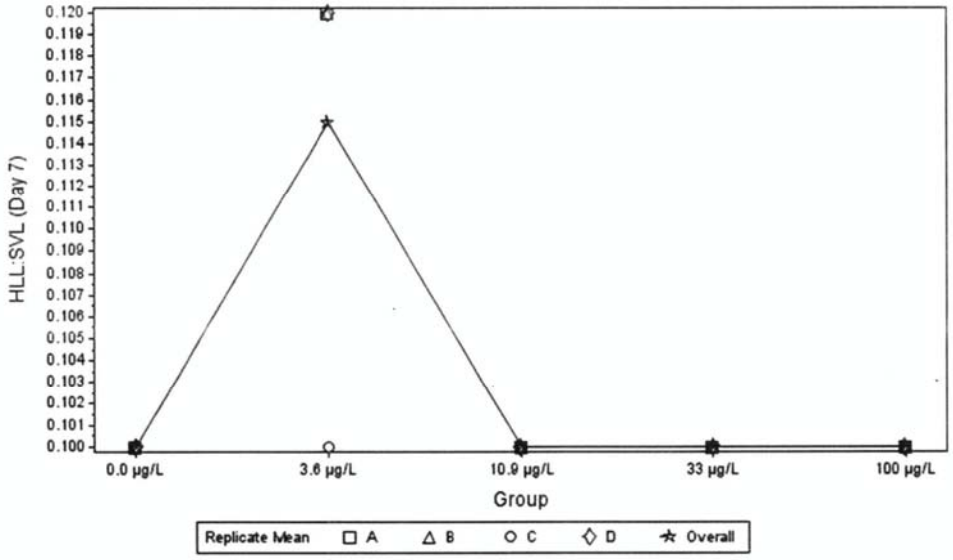

Figure A-5. Treatment and Replicate Means for Normalized Hind Limb Length (HLL:SVL), Day 7.

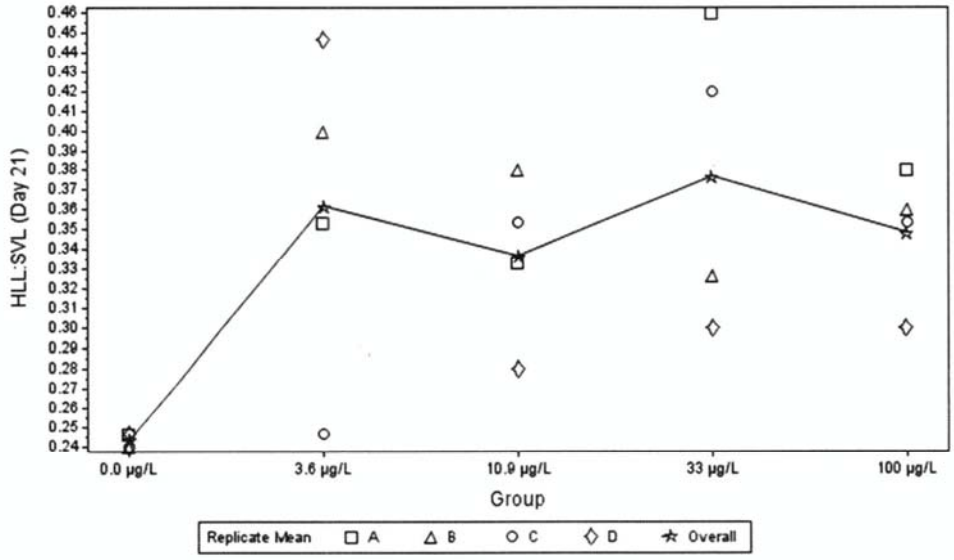

Figure A-6. Treatment and Replicate Means for Normalized Hind Limb Length (HLL:SVL), Day 21.

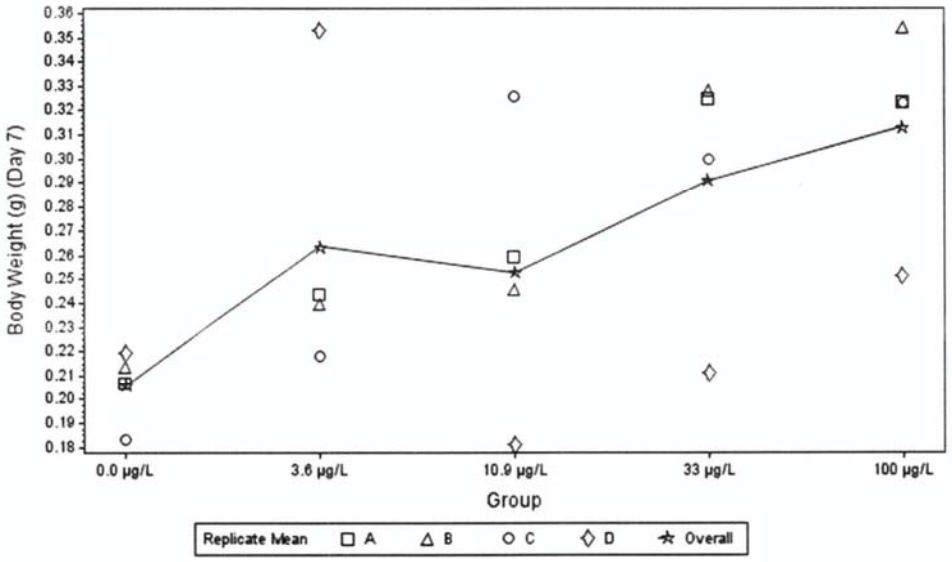

Figure A-7. Treatment and Replicate Means for Body Weight (g), Day 7.

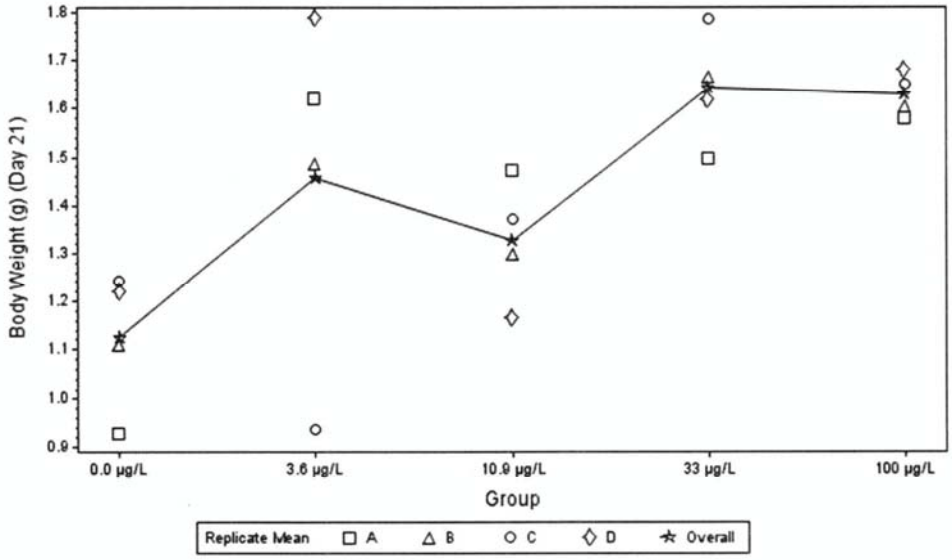

Figure A-8. Treatment and Replicate Means for Body Weight (g), Day 21.

Appendix B

Statistical Analyses Performed on Data with Potential Statistical Outliers Removed

USEPA Contract No.: EP-W-11-063, TO 14
FEL Study No. BATT01-00388 (AMA #388)

Table B-1. Potential Statistical Outliers.

Parameter	Treatment	Replicate	Observed Value	Predicted Value	Residual	Studentized Residual
HLL (mm) (Day 21)	0.0 µg/L	D	12.500	6.825	5.675	3.787
	3.6 µg/L	D	22.400	10.393	12.007	3.161
	10.9 µg/L	B	18.900	8.830	10.070	3.784
	33 µg/L	A	22.300	10.507	11.793	3.424
HLL:SVL (Day 21)	3.6 µg/L	D	0.800	0.362	0.438	3.732
	10.9 µg/L	B	0.700	0.337	0.363	3.865
Body Weight (g) (Day 7)	10.9 µg/L	C	0.547	0.253	0.294	3.154
Body Weight (g) (Day 21)	33 µg/L	D	2.915	1.643	1.272	3.698
	100 µg/L	D	2.475	1.628	0.847	3.164

Table B-2a. Descriptive Statistics for Hind Limb Length (mm) Day 21 and Normalized Hind Limb Length (HLL:SVL ratio) Day 21, Potential Statistical Outliers Removed.

		Hind Limb Length (mm) Day 21					Normalized Hind Limb Length (ratio of HLL:SVL) Day 21				
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	SEM	CV (%)	N	Replicate Mean	Mean	SEM	CV (%)
0.0	A	15	6.18	6.73	0.19	5.67	15	0.25	0.24	0.00	1.58
	B	15	6.87				15	0.24			
	C	15	7.06				15	0.24			
	D	14	6.81				15	0.25			
3.6	A	15	10.87	10.22	1.33	26.05	15	0.35	0.36	0.04	21.90
	B	15	11.92				15	0.40			
	C	15	6.29				15	0.25			
	D	14	11.79				14	0.42			
10.9	A	15	9.23	8.67	0.54	12.36	15	0.33	0.33	0.02	10.74
	B	14	9.01				14	0.36			
	C	15	9.35				15	0.35			
	D	15	7.07				15	0.28			
33	A	14	11.24	10.32	0.80	15.41	15	0.46	0.38	0.04	20.10
	B	15	9.69				15	0.33			
	C	15	11.96				15	0.42			
	D	15	8.41				15	0.30			
100	A	15	10.47	9.72	0.36	7.48	15	0.38	0.35	0.02	9.81
	B	15	10.10				15	0.36			
	C	15	9.50				15	0.35			
	D	15	8.81				15	0.30			

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.

Table B-2b. Descriptive Statistics for Body Weight (g) by Day 7 and 21, Potential Statistical Outliers Removed.

		Body Weight (g) Day 7					Body Weight (g) Day 21				
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	SEM	CV (%)	N	Replicate Mean	Mean	SEM	CV (%)
0.0	A	5	0.2070	0.2061	0.0080	7.7446	15	0.9296	1.1260	0.0716	12.7242
	B	5	0.2142				15	1.1100			
	C	5	0.1834				15	1.2427			
	D	5	0.2196				15	1.2216			
3.6	A	5	0.2440	0.2638	0.0303	22.9956	15	1.6203	1.4581	0.1845	25.3048
	B	5	0.2398				15	1.4865			
	C	5	0.2182				15	0.9367			
	D	5	0.3532				15	1.7889			
10.9	A	5	0.2596	0.2392	0.0200	16.7242	15	1.4731	1.3281	0.0643	9.6833
	B	5	0.2462				15	1.3003			
	C	4	0.2700				15	1.3719			
	D	5	0.1810				15	1.1672			
33	A	5	0.3248	0.2911	0.0274	18.8170	15	1.4983	1.6195	0.0670	8.2728
	B	5	0.3286				15	1.6669			
	C	5	0.2996				15	1.7871			
	D	5	0.2112				14	1.5256			
100	A	5	0.3234	0.3131	0.0218	13.9443	15	1.5794	1.6142	0.0148	1.8305
	B	5	0.3544				15	1.6057			
	C	5	0.3230				15	1.6499			
	D	5	0.2514				14	1.6219			

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.

Table B-3. Statistical Analysis Results, Potential Statistical Outliers Removed.

Parameter	Monotonicity Assessment[1]	Normality Test[2]	Homogeneity of Variance Test[4]	Jonckheere-Terpstra Test[5] (p-value)	Significant Pairwise Comparisons to Control[6] (p-value)
HLL (mm) (Day 21)	Non-Monotonic	Log-normal[3]	Heterogeneous	NP	NS
HLL:SVL (Day 21)	Non-Monotonic	Non-normal	Heterogeneous	NP	NS
Body Weight (g) (Day 7)	Monotonic	NP	NP	Group 5 (0.0079) Group 4 (0.0399)	NP
Body Weight (g) (Day 21)	Monotonic	NP	NP	Group 5 (0.0053) Group 4 (0.0196)	NP

1. Monotonicity was assessed visually from the replicate and treatment means.
2. Shapiro-Wilk test for normality.
3. Normal when log-transformed.
4. LeveneTest for homogeneity of variance.
5. Jonckheere-Terpstra step-down trend test was performed on monotonic concentration-response data. Only statistically significant treatment trends were listed.
6. Control is group 1 and test concentration groups are groups 2 to 5. Only statistically significant pairwise comparisons were listed.
   - For non-monotonic, normally distributed, and homogeneous variance data, Dunnett's test was used in the pairwise comparisons to control.
   - For other types of data (e.g., non-monotonic, normally distributed, and heterogeneous variance data), Mann-Whitney-Wilcoxon test was used in the pairwise comparisons to control with Bonferroni-Holm multiple comparison adjustment.

NP Statistical test or comparison was not performed.

NS No statistically significant differences were found for Dunnett's tests or Mann-Whitney-Wilcoxon tests after Bonferroni-Holm adjustment.

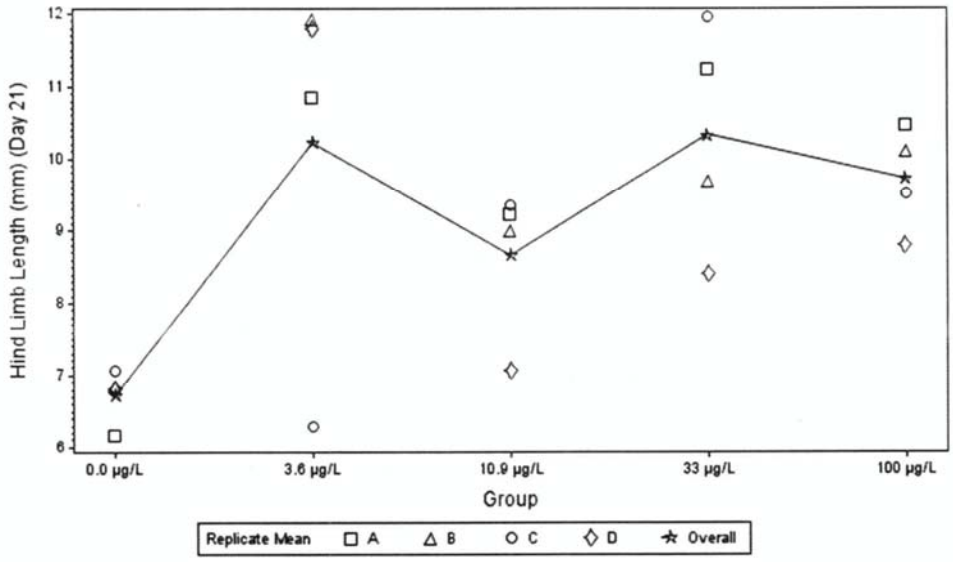

Figure B-1. Treatment and Replicate Means for Hind Limb Length (mm), Day 21, Potential Statistical Outliers Removed.

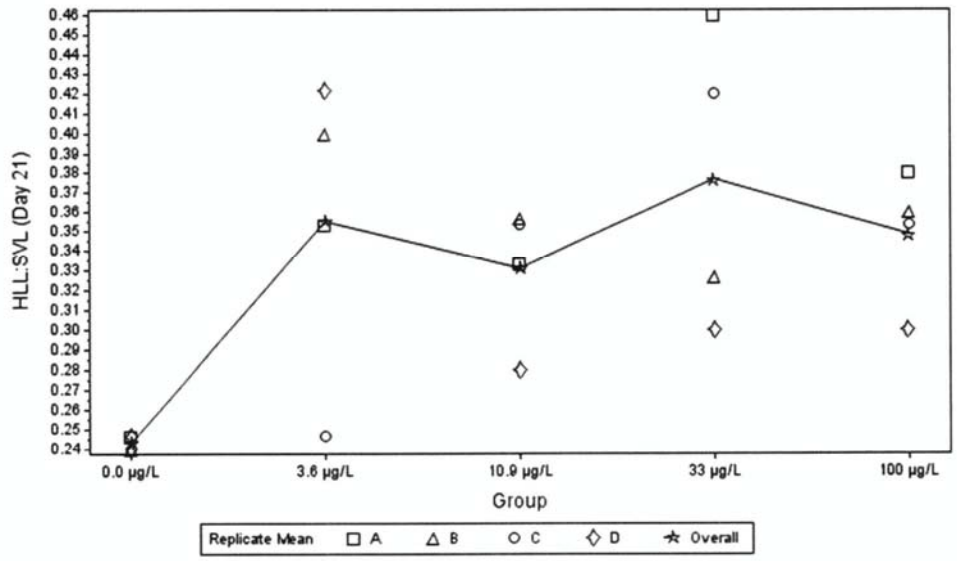

Figure B-2. Treatment and Replicate Means for Hind Limb Length Normalized by Snout Vent Length, Day 21, Potential Statistical Outliers Removed.

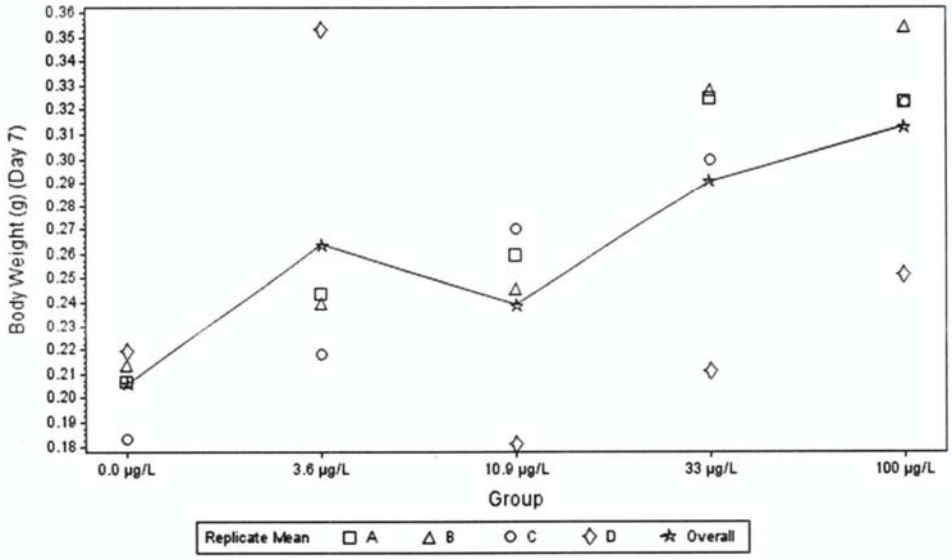

Figure B-3. Treatment and Replicate Means for Body Weight (g), Day 7, Potential Statistical Outliers Removed.

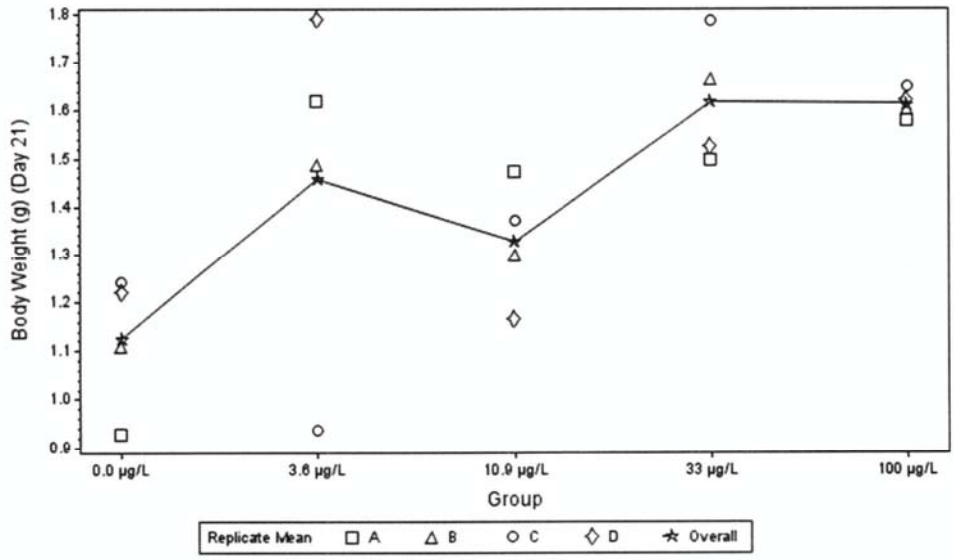

Figure B-4. Treatment and Replicate Means for Body Weight (g), Day 21, Potential Statistical Outliers Removed.

Appendix G

RANGE-FINDING DATA (RANGE-FINDING STUDIES WERE NOT PERFORMED IN A GLP-COMPLIANT MANNER PER EXCEPTION NOTED IN SECTION 1)

FEL

GENERAL WATER CHEMISTRY

FEL

Client/Project-WO No: BATT01-00385 Test Species: 2-EHHB - AMA NF 51 - X. Laevis

Analysis Date	Tech Initials	Sample ID	Temp (C)	pH (su)	DO (mg/L)
10/19/15	DJF	Control Renewal	22.4	7.3	7.4
10/19/15	DJF	0.001 mg/L Renewal	22.3	7.2	7.3
10/19/15	DJF	0.01 mg/L Renewal	22.3	7.2	7.2
10/19/15	DJF	0.1 mg/L Renewal	22.4	7.2	7.3
10/19/15	DJF	1.0 mg/L Renewal	22.3	7.1	7.4
10/19/15	DJF	5.0 mg/L Renewal	22.4	7.1	7.4
10/20/15	DJF	Control Renewal	22.3	7.2	7.4
10/20/15	DJF	0.001 mg/L Renewal	22.3	7.3	7.3
10/20/15	DJF	0.01 mg/L Renewal	22.4	7.2	7.4
10/20/15	DJF	0.1 mg/L Renewal	22.3	7.1	7.4
10/20/15	DJF	Control Spent	22.2	7.2	6.5
10/20/15	DJF	0.001 mg/L Spent	22.3	7.2	6.2
10/20/15	DJF	0.01 mg/L Spent	22.3	7.2	6.3
10/20/15	DJF	0.1 mg/L Spent	22.2	7.1	6.2
10/21/15	DJF	Control Renewal	22.3	7.3	7.3
10/21/15	DJF	0.001 mg/L Renewal	22.4	7.3	7.2
10/21/15	DJF	0.01 mg/L Renewal	22.3	7.3	7.3
10/21/15	DJF	0.1 mg/L Renewal	22.3	7.1	7.2
10/21/15	DJF	Control Spent	22.3	7.2	6.0
10/21/15	DJF	0.001 mg/L Spent	22.3	7.1	6.2
10/21/15	DJF	0.01 mg/L Spent	22.4	7.2	6.1
10/21/15	DJF	0.1 mg/L Spent	22.3	7.0	6.3
10/22/15	DJF	Control Renewal	22.3	7.3	7.3
10/22/15	DJF	0.001 mg/L Renewal	22.2	7.3	7.2
10/22/15	DJF	0.01 mg/L Renewal	22.3	7.2	7.2
10/22/15	DJF	0.1 mg/L Renewal	22.3	7.2	7.3
10/22/15	DJF	Control Spent	22.2	7.2	6.0
10/22/15	DJF	0.001 mg/L Spent	22.2	7.2	6.1
10/22/15	DJF	0.01 mg/L Spent	22.2	7.1	6.0
10/22/15	DJF	0.1 mg/L Spent	22.3	7.1	6.2
10/23/15	DJF	Control Spent	22.2	7.2	6.7
10/23/15	DJF	0.001 mg/L Spent	22.3	7.2	6.6
10/23/15	DJF	0.01 mg/L Spent	22.2	7.1	6.5
10/23/15	DJF	0.1 mg/L Spent	22.3	7.1	6.5

FEL

FEL

CULTURE SURVIVAL/STAGE DATA SHEET

Client/Project-WO No: **BATT01-00385** Test Type: **AMA NF 51 RF**

Sample No. / ID: **2-EHHB** Test No.: **1** Species: ***X. Laevis***

	Replicate-Concentration	Control	0.001 mg/L	0.01 mg/L	0.1 mg/L	1.0 mg/L	5.0 mg/L	
	Initial Culture No.:	20	20	20	20	20	20	
Test Day	**Test Date**	**Tech Init.**	**No. Survived (Rep A / Rep B)**					**Observations**
0	10/19/15	DJF	10 / 10	10 / 10	10 / 10	10 / 10	10 / 10	10 / 10	Normal behaving and no abnormalities in Control - 5.0 mg/L
1	10/20/15	DJF	10 / 10	10 / 10	10 / 10	10 / 10	0 / 0	0 / 0	Normal behaving and no abnormalities in Control - 0.1 mg/L
2	10/21/15	DJF	10 / 10	10 / 10	10 / 10	9 / 10	0 / 0	0 / 0	Normal behaving and no abnormalities in Control - 0.1 mg/L
3	10/22/15	DJF	10 / 10	10 / 10	10 / 10	9 / 10	0 / 0	0 / 0	Normal behaving and no abnormalities in Control - 0.1 mg/L
4	10/23/15	DJF	10 / 10	10 / 10	10 / 10	9 / 10	0 / 0	0 / 0	Normal behaving and no abnormalities in Control - 0.1 mg/L

Appendix H
REPORT AMENDMENTS

FEL

BATT01-00388
Report Amendment 01

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: 21-d Amphibian Metamorphosis Assay (AMA) of 2-Ethylhexyl 4-Hydroxybenzoate with African Clawed Frog, *Xenopus laevis*	
Amendment Number: 01	**Document ID Number:** BATT01-3 (00388)
Submitted By: Douglas J. Fort	**Date:** 5/17/2018

Amendment Relating To: BATT01-00388

[ ] Protocol [ ] Study Plan [ ] QAPP [ ] QAMP [ ] SOP

[X] Other (describe): Final Report

Original Specifications:

1. Appendix C - EAG Laboratories (Columbia, MO) Analytical Report, pages 98-140.

Changed To:

1. Replace entire EAG Laboratories (Columbia, MO) Analytical Report in Appendix C with amended EAG Laboratories (Columbia, MO) Analytical Report.

Reason for Change:

Correct spelling error for test substance, 2-Ethylhexyl 4-Hydroxybenzoate throughout entire EAG Laboratories (Columbia, MO) Analytical Report.

Approval:

Study Director: _____ Date: 5/18/2018

Sponsor Representative: _____ Date: 5/18/2018