SPONSOR

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

USEPA TASK ORDER/BATTELLE CONTRACT NO.

TO 17/ EP-W-11-063

TEST SUBSTANCE

2-Ethylhexyl 4-Hydroxybenzoate

STUDY TITLE

Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

TEST GUIDELINE

U.S. EPA, OPPTS 890.1350 (October 2009), modified and OECD guideline 229 (October 2012), modified

STUDY DIRECTOR

Douglas J. Fort, Ph.D.

STUDY INITIATION DATE

January 8, 2017

STUDY COMPLETION DATE

May 21, 2018

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REPORT NUMBER

BATT01-00404

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1. 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, BATT01-00404, 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 were 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 was not expected to impact on the study results. Lack of GLP-compliant chemical characterization or GLP-compliant range-finding studies was not expected to impact study results, nor change the conclusions drawn from the study.

GLP Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

FEL Study No.: BATT01-00404

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

DJF — 5/21/2018

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

Date

Vincent J. Brown — 5/21/2018

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

Date

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	1/10/2017	1/10/2017	1/10/2017
Technical Systems Audit	2/8/2017	2/8/2017	2/8/2017
Audit of Data Quality	8/11/2017	8/11/2017	8/11/2017
Draft Report Audit	9/28/2017	9/28/2017	9/28/2017
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). One protocol deviation 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

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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5. EXECUTIVE SUMMARY

5.1. INTRODUCTION

FEL was contracted by Battelle Memorial Institute to perform a modified Fish Short-Term Reproduction Assay (FSTRA) under EPA Test Guidelines OPPTS 890.1350 and OECD Guideline 229 using 2-ethylhexyl 4-hydroxybenzoate [2-EHHB] (test substance) as directed by USEPA Task Order (TO) 17 under USEPA/Battelle Memorial Institute contract EP-W-11-063 and sponsor-approved Quality Assurance Project Plan (QAPP) (1). This study was conducted in accordance with the USEPA FIFRA, Good Laboratory Practice (GLP) Standards, Final Rule, 40 CFR Part 160 with exceptions noted in Section 1 (2), EPA Test Guidelines OPPTS 890.1350 (3) and OECD Guideline 229 (4), FEL Quality Assurance Management Plan (QAMP) (5), relevant facility standard operating procedures (SOPs), and the following Study Protocol No. BATT01-10, prepared for FEL Study No. BATT01-00404. The in-life exposure phase of the study was initiated on February 6, 2017 and concluded February 27, 2017. 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.

5.2. METHOD

The primary objective of the study was to evaluate the potential reproductive toxicity of 2-EHHB to Japanese medaka in accordance with the guidelines established in OPPTS 890.1350 Fish Short-Term Reproduction Assay (3) and OECD Guideline 229 (4) with modification to use medaka *in lieu* of fathead minnow. The Fish Short-Term Reproduction Assay measures a suite of histological and biochemical endpoints that are designed to reflect effects associated with disturbance to the hypothalamus-pituitary-gonadal (HPG) endocrine axis (6,7). The assay is traditionally initiated with sexually mature male and female fathead minnows, but medaka were used as a modification in the present study (00404) per OECD Guideline 229. During a 21-d in-life exposure phase, survival, reproductive behavior, secondary sexual characteristics, fecundity, and fertility were monitored. At the conclusion of the in-life exposure phase, measurements were made that were indicative of the status of the reproductive endocrine system, including secondary sex characteristics; gonado-somatic index (GSI); liver vitellogenin (VTG) mRNA; and gonad histopathology. These endpoints were generally inclusive of the traditional FSTRA endpoints (3,4), but contained additional endpoints to provide supplemental data, namely genetic sex and liver and thyroid histopathology, with statistical analysis of the histopathology results.

The pre-exposure phase was 12 days, per OECD guideline 229 (4), which specifies 7 to 14 days. The assay used fish that were approximately 16±2 weeks post-fertilization (wpf), previously maintained in communal culture tanks. Five sexually mature female medaka and five sexually mature male medaka were randomly assigned to each replicate exposure chamber at each treatment concentration. Any specimens whose phenotypic sex could not be identified were excluded from the assay. Additional exposure chambers were set up for pre-exposure to account for a lack of spawning in some chambers and/or mortality during the pre-exposure phase. The pre-exposure phase of the assay was conducted under conditions (temperature, photo period, feeding, etc.) identical to those used during the chemical exposure phase. The animals were fed

brine shrimp twice daily *ad libidum*. One 4 in. breeding mop head constructed from inert acrylic yarn was placed in each tank to collect eggs per facility SOP. FEL has experienced markedly more consistent and productive breeding with medaka using an artificial substrate that simulates rice grass. During the daily egg collection, eggs were assessed for fecundity. The mop heads were removed from the tank daily and the eggs were cultured directly on the mop head in a separate brood chamber. For each assay, successful pre-exposure (suitability for testing) was established when regular spawning occurred in each replicate test chamber at least two times in the immediately preceding 7 days.

After successful spawning was verified during pre-exposure as per the requirements of the assay, the chemical exposure was initiated and continued for 21 days. Five female and five male fish per replicate tank during exposure phases were used. One 4 in. breeding mop head constructed from inert acrylic yarn was placed in each tank as an artificial substrate to collect eggs. During the egg collection phases, eggs were cultured directly on the mop heads in a separate brood chamber. The assays were conducted at four chemical concentrations, as well as a dilution water control, with four experimental units (replicates) per treatment. The nominal treatment concentrations were 3.6, 10.9, 33.0, and 100 µg/L. The replicate tanks were assigned to treatment levels according to randomized complete block design, with the four highest-spawning replicates assigned (one each) to one treatment level or control first, followed by the assignment of the next four highest-spawning replicates by distribution to the four test groups, until all groups were established. The test chemical was delivered to the exposure chambers using a continuous-flow diluter. The exposure phase was conducted for 21 days, during which time the appearance, behavior, and reproductive fecundity of the fish were assessed daily. At termination of the exposure period, the remaining endpoints were recorded.

5.3. RESULTS AND CONCLUSIONS

A summary of study results is provided in Table 1. The following results were considered, using the framework outlined in Ankley and Jensen (18), as a basis for concluding if 2-EHHB has the potential to interact with the endocrine system.

- The mean measured concentrations for the 0.0 (control), 3.6, 10.9, 33.0, and 100 µg/L 2-EHHB treatments (nominal) were (<0.212 µg/L [minimum quantitation level, MQL]), 2.98, 10.6, 34.1, and 105 µg/L 2-EHHB (see Table 6).

- Survival rates in the control and 2-EHHB treatments were both 100%.

- The mean egg production in all of the 2-EHHB treatments was significantly less than the control (Jonckheere-Terpstra test, p=0.0433, 0.0021, <0.0001, and <0.0001 for the 3.6, 10.9, 33.0 and 100 µg/L treatments, respectively). The mean fertilization in the 10.9, 33, and 100 µg/L 2-EHHB treatments was significantly less than the control (Jonckheere-Terpstra test, p=0.0034, <0.0001, and <0.0001, respectively). It is plausible that only the effects on reproductive fecundity observed in the 33.0 and 100 µg/L treatments were toxicologically-relevant based on the magnitude and longevity of effect during the study.

- The mean anal fin papillae count in the 33.0 and 100 µg/L 2-EHHB treatments was significantly less than the control (Jonckheere-Terpstra test, p=0.0394 and 0.0012, respectively). No papillae were found in control or 2-EHHB-treated female fish. A reduction in mean male anal fin papillae counts in response to treatment can be considered indicative of endocrine activity.

- Male liver VTG mRNA expression was significantly greater in the 3.6 and 10.9 µg/L 2-EHHB treatments than the control expression (Dunnett's test, p=0.0017 and 0.0069, respectively). Male liver VTG mRNA expression represented a non-monotonic concentration-response which can occur in studies focusing on endocrine active compounds (13) and might be indicative of possible low-concentration effects associated with 2-EHHB exposure (14-17). Further, these results suggested that 2-EHHB may induce VTG expression at low concentrations indicative of a weak estrogen. Exposure to 2-EHHB did not affect female liver VTG mRNA expression. Variability in the data obtained; however, may have affected data interpretation.

- Exposure to 2-EHHB did not affect gonad weight or GSI in male or female medaka relative to the control.

- Exposure to 2-EHHB did not affect body weight or standard length in male or female medaka relative to the control.

- **THYROID:** Modest increase in the severity of follicular cell hypertrophy (minimal to mild) in male medaka exposed to 100.0 µg/L 2-EHHB (4/11 had mild hypertrophy) compared to controls (2/12 had mild hypertrophy) was observed. However, this difference was most likely attributable to inter-specimen variation. In addition, modest decreases in the severity of follicular cell hyperplasia in male medaka exposed to 10.9 and 33.0 µg/L 2-EHHB (7/12 and 8/12 had mild hyperplasia, respectively) relative to controls (10/12 had mild hyperplasia) which was most likely due to inter-specimen variation since no concentration-response pattern was associated with this finding. Reduced presence of minimal hyaline degeneration (follicular cells) was observed in in the thyroid of female medaka from the 33.0 and 100 µg/L 2-EHHB treatments relative to control (RSCABS, p=0.0374 and 0.0263, respectively). However, this particular finding, as is true of most degenerative-type changes, was not considered to be toxicologically relevant (see Appendix D).

- **MALE LIVER:** Increased presence of mild cystic degeneration in liver from male medaka exposed to 100 µg/L 2-EHHB (RSCABS, p=0.0315), but decreased presence of mild cystic degeneration in male medaka exposed to 33.0 and 10.9 µg/L 2-EHHB (RSCABS, p=0.0188 and 0.0343, respectively) compared to control were observed. A reduced occurrence of minimal eosinophilic cytoplasmic inclusions was noted in liver from male medaka from the 10.9, 33.0, and 100 µg/L 2-EHHB treatments compared to control (RSCABS, p=0.0142, 0.0047, and 0.0273, respectively). Increased minimal (RSCABS, p=0.0273), mild (RSCABS, p=0.0025), and moderate (RSCABS, p=0.0263) granulomatous

inflammation in male medaka from the 100 µg/L 2-EHHB treatment relative to control was noted (RSCABS, p=0.0273). Although these findings were statistically significant, they were not considered to be toxicologically relevant by the pathologist (see Appendix D).

- **FEMALE LIVER:** Decreased occurrence of mild pigmented macrophage aggregates in liver from female medaka exposed to 100 µg/L 2-EHHB (RSCABS, p=0.0307) compared to control was observed. In addition, increased presence of mild and moderate vacuolation in liver from female medaka exposed to 33.0 and 100 100 µg/L 2-EHHB compared to control was observed (RSCABS, p=0.0148 and 0.0028) for mild and p=0.0278 and 0.228 for moderate, respectively). A treatment-related decrease in the occurrence of minimal, mild, and moderate basophilia was observed in liver from female medaka exposed to 33.0 and 100 µg/L 2-EHHB compared to control (RSCABS, p=0.0005 and 0.0438 [minimal], 0.0001 and 0.0008 [mild], and 0.0020 and 0.0195 [moderate], respectively). A slight decrease in basophilia in fish of the 10.9 µg/L treatment group relative to controls was insubstantial and not statistically significant. This occurred as decrease in the severity of this finding in the 100.0 µg/L dose group relative to controls, and a decrease of both the severity and prevalence of basophilia in the 33.0 µg/L dose group. On an individual fish basis, the presence of zero, minimal, or mild basophilia in females was often (but not always) associated with a low (i.e., Grade 1 or 2) ovarian stage score and/or oocyte atresia. An increase in the presence of minimal, mild, and moderate focus of cellular alteration was observed in liver from female medaka exposed to the 100 µg/L 2-EHHB treatment relative to control (RSCABS, p=0.0307, 0.0263, and 0.0263 for minimal, mild, and moderated severity). Although these findings were statistically significant, they were not considered to be toxicologically relevant by the pathologist (see Appendix D).

- **TESTIS:** No histopathological findings were noted in the testis of male medaka exposed to 2-EHHB compared to the control.

- **OVARY:** An increase in mild oocyte atresia in female medaka exposed to 100 µg/L 2-EHHB compared to control (RSCABS, p=0.0075). Decreased occurrence of minimal and mild egg debris in oviduct in female medaka exposed to 33.0 and 100 µg/L 2-EHHB compared to control was observed (RSCABS, p=0.0374 and 0.0263, respectively [for both minimal and mild]. Mean ovarian stage scores were slightly greater than controls in females of the 100.0 mg/L dose group, but were lower than controls in 10.9 and 33.0 µg/L group females. Especially in the 33.0 µg/L dose group, the high prevalence of oocyte atresia appeared to contribute to the low stage scores, as atresia appeared to affect the more mature vitellogenic follicles preferentially. Decreased occurrence of minimal and mild egg debris in oviduct in female medaka exposed to 33.0 and 100 µg/L 2-EHHB compared to control was observed (RSCABS, p=0.0374 and 0.0263, respectively [for both minimal and mild]; however, these decreases in egg debris were not deemed likely to be toxicologically relevant by the pathologist.

- **OTHER FINDINGS – GONADS:** There were six gonadal germ cell neoplasms observed in this study (Table 19), none of which were thought to be treatment related. Benign seminomas were found in the testes of one control male (replicate B) and two 100.0 μg/L treatment group males (replicates A and C). Malignant seminomas, with intra-coelomic metastasis (to the anterior coelomic wall in one case, and ventral liver capsule in another), were identified in the ovaries of one control female (replicate A) and one 100.0 μg/L treatment group female (replicate D). Interestingly, while the malignant seminomas of the ovaries were of the spermatocytic type (which is characteristic of most teleosts), the testicular seminomas were of the less common spermatogonial type. A benign dysgerminoma occurred in the testis of a single 100.0 μg/L treatment group male (replicate A). The labeling of benign versus malignant for these tumors was based solely on the presence or absence of metastasis, as the cytologic features were not generally a useful indicator of malignancy. The relatively high prevalence (for medaka) of these germ cell tumors and their presence in controls suggests that this is likely a strain-related background issue. In the current study, gonadal germ cell tumors were observed in four male and two female medaka. Although the occurrence of these neoplasms was not considered treatment-related, two germ cell neoplasms were identified in the control and four in the 100 μg/L 2-EHHB treatment group.

  Overall, the recording of gonadal tumors in six of 72 examined medaka (control - 2.8% and 8% overall) was somewhat unusual, based on the experience of the pathologist and the results of at least one published report (18), in which germ cell neoplasms were detected in only 26 of approximately 10,000 medaka (0.3%). The investigators (18) reported that the tumors were most likely associated with age rather than chemical exposure suggesting that the development of the neoplasms was most likely attributable to culturing techniques, genetic factors, or a virus, based on the tendency for multiple occurrences to cluster in the fish of the same cohort. Overall, this finding is perplexing as culturing practices for medaka have been consistently maintained with a low background rate of occurrence similar to that reported (18). The age of the fish used in the present study (15.5 wpf) and the breeders used to produce the fish used in the study are controlled to eliminate age as a confounding factor. The genotype of the fish affected with the neoplasms matched the phenotype identified. Most importantly for the present study, the occurrence of the six germ cell neoplasms was distributed among control and high dose groups from both sexes, and thus their presence did not affect the interpretation of the study.

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

In conclusion, survival rates in the control and 2-EHHB treatments were both 100%. Exposure to 2-EHHB did not affect body weight or standard length in male or female medaka relative to the control. Exposure to 2-EHHB increased cystic degeneration, eosinophilic cytoplasmic inclusions, and granulomatous inflammation of the liver in male medaka, and

decreased occurrence of pigmented macrophage aggregates, vacuolation, basophilia, and increased focus of cellular alteration in the liver of female medaka exposed to 2-EHHB. Although these findings were statistically significant, they were not considered to be toxicologically relevant by the pathologist with the potential exception of basophilia. In this case, a slight decrease in basophilia in fish of the 10.9 µg/L treatment group relative to controls was insubstantial and not statistically significant. This occurred as decrease in the severity of this finding in the 100.0 µg/L dose group relative to controls, and a decrease of both the severity and prevalence of basophilia in the 33.0 µg/L dose group. On an individual fish basis, the presence of zero, minimal, or mild basophilia in females was often (but not always) associated with a low (i.e., Grade 1 or 2) ovarian stage score and/or oocyte atresia.

Results from the present study indicated that 2-EHHB reduced reproductive fecundity, including both egg production and fertility. No significant differences were found in gonad weight or GSI in male or female medaka relative to the control. Exposure to 2-EHHB reduced anal fin papillae counts in male medaka compared to control. No anal fin papillae were found in female medaka. The reduction in mean male anal fin papillae counts suggested a decrease in male sexual characteristics. Male liver VTG mRNA expression was increased relative to the control in the 3.6 and 10.9 µg/L 2-EHHB treatments. Male liver VTG mRNA expression represented a non-monotonic concentration-response which can occur in studies focusing on endocrine active compounds (13) and might be indicative of possible low-concentration effects associated with 2-EHHB exposure (14-17). However, care in interpreting potential low-concentration effects should be tempered based on variability in the data set. Further, these results suggested that 2-EHHB may induce VTG expression at low concentrations indicative of a weak estrogen. Exposure to 2-EHHB did not affect female liver VTG mRNA expression.

A modest increase in the severity of follicular cell hypertrophy (minimal to mild) in male medaka exposed to 100.0 µg/L 2-EHHB compared to controls was observed. In addition, modest decreases in the severity of follicular cell hyperplasia in male medaka exposed to 10.9 and 33.0 µg/L 2-EHHB relative to controls which was most likely due to inter-animal variation since no concentration-response pattern was associated with this finding. Reduced presence of minimal hyaline degeneration (follicular cells) was observed in in the thyroid of female medaka from the 33.0 and 100 µg/L 2-EHHB treatments relative to control. However, this particular finding, as is true of most degenerative-type changes, was not considered to be toxicologically relevant. No histopathological findings were noted in the testis of male medaka exposed to 2-EHHB compared to the control. Increased mild oocyte atresia in female medaka exposed to 2-EHHB compared to control and decreased occurrence of egg debris in oviduct in female medaka exposed to 2-EHHB compared to control was observed. However, these decreases in egg debris were not deemed likely to be toxicologically relevant by the pathologist. Mean ovarian stage scores were slightly greater than controls in females of the 100.0 mg/L dose group, but were lower than controls in 10.9 and 33.0 mg/L group females.

Six gonadal germ cell neoplasms were observed in this study, none of which were thought to be treatment related. Benign seminomas were found in the testes of one control male (replicate B) and two 100.0 µg/L treatment group males (replicates A and C). Malignant seminomas, with intra-coelomic metastasis (to the anterior coelomic wall in one case, and ventral liver capsule in another), were identified in the ovaries of one control female (replicate A) and

one 100.0 µg/L treatment group female (replicate D). Interestingly, while the malignant seminomas of the ovaries were of the spermatocytic type (which is characteristic of most teleosts), the testicular seminomas were of the less common spermatogonial type. A benign dysgerminoma occurred in the testis of a single 100.0 µg/L treatment group male (replicate A). Although the occurrence of these neoplasms was not considered treatment-related, two germ cell neoplasms were identified in the control and four in the 100 µg/L 2-EHHB treatment group. Overall, the recording of gonadal tumors in six of 72 examined medaka (control - 2.8% and 8% overall) was somewhat unusual, based on the experience of the pathologist and the results of at least one published report (18), in which germ cell neoplasms were detected in only 26 of approximately 10,000 medaka (0.3%). The investigators (18) reported that the tumors were most likely associated with age rather than chemical exposure suggesting that the development of the neoplasms was most likely attributable to culturing techniques, genetic factors, or a virus, based on the tendency for multiple occurrences to cluster in the fish of the same cohort. This finding is perplexing as culturing practices for medaka have been consistently maintained with a low background rate of occurrence similar to that reported (18). The age of the fish used in the present study (15.5 wpf) and the breeders used to produce the fish used in the study are controlled to eliminate age as a confounding factor. The genotype of the fish affected with the neoplasms matched the phenotype identified. Most importantly for the present study, the occurrence of the six germ cell neoplasms was distributed among control and high dose groups from both sexes, and thus their presence did not affect the interpretation of the study. Based on endpoints measured in the present modified FSTRA study and the decision criteria in the FSTRA test guideline (OCSPP 890.1350) (3) and OECD Guideline 229 (4), 2-EHHB appeared to interfere with the function of the endocrine system in medaka.

6. INTRODUCTION

FEL was contracted by Battelle Memorial Institute to perform a modified Fish Short-Term Reproduction Assay (FSTRA) under EPA Test Guidelines OPPTS 890.1350 and OECD Guideline 229 using 2-ethylhexyl 4-hydroxybenzoate [2-EHHB] (test substance) as directed by USEPA Task Order (TO) 17 under USEPA/Battelle Memorial Institute contract EP-W-11-063 and sponsor-approved Quality Assurance Project Plan (QAPP) (1). This study was conducted in accordance with the USEPA FIFRA, Good Laboratory Practice (GLP) Standards, Final Rule, 40 CFR Part 160 with exceptions noted in Section 1 (2), EPA Test Guidelines OPPTS 890.1350 (3) and OECD Guideline 229 (4), FEL Quality Assurance Management Plan (QAMP) (5), relevant facility standard operating procedures (SOPs), and the following Study Protocol No. BATT01-10, prepared for FEL Study No. BATT01-00404. The in-life exposure phase of the study was initiated on February 6, 2017 and concluded February 27, 2017. 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 primary objective of the study was to evaluate the potential reproductive toxicity of 2-EHHB to Japanese medaka in accordance with the guidelines established in OPPTS 890.1350 Fish Short-Term Reproduction Assay (3) and OECD Guideline 229 (4) with modification to use medaka *in lieu* of fathead minnow. As directed by the sponsor, chemicals previously tested in fathead minnow were to be tested in other fish species, such as medaka and zebrafish. This will

allow the concordance and applicability of toxicity endpoints collected for all three species to be examined. The Fish Short-Term Reproduction Assay measures a suite of histological and biochemical endpoints that reflect effects associated with disturbance to the hypothalamus-pituitary-gonadal (HPG) endocrine axis (6,7). The assay is initiated with sexually mature male and female fathead minnows traditionally, but medaka were used as a modification in the present study (00404). During a 21-d in-life exposure phase, survival, reproductive behavior, secondary sexual characteristics, fecundity, and fertility were monitored. At the conclusion of the in-life exposure phase, measurements were made that were indicative of the status of the reproductive endocrine system, including secondary sex characteristics; gonado-somatic index (GSI); liver vitellogenin (VTG) mRNA; and gonad histopathology. These endpoints are generally inclusive of the traditional FSTRA endpoints (3,4), but also contained additional endpoints to provide supplemental data, namely genetic sex, anal fin papillae and liver and thyroid histopathology, with statistical analysis of the histopathology results.

7. STUDY PERSONNEL

- Dr. Vincent Brown, Battelle -Study Monitor
- Dr. Douglas Fort, FEL – Study Director
- Ms. Deanne Fort, FEL – Manager, In-life study facility
- Mr. Michael Mathis, FEL – QAU Manager
- Ms. Amanda Milligan, EAG Laboratories – PI, liver vitellogenin
- Dr. Tom Leak, EAG Laboratories, Inc. – PI, analytical chemistry
- Dr. Jeffrey Wolf, EPL – PI, Histopathology
- Mr. Kevin Todhunter, FEL – Technician
- Ms. Jennifer Staines, FEL – Technician
- Ms. Franchesca Rollerson, FEL – Technician
- Mr. Troy Fort, FEL – Technician

8. ANIMAL WELFARE COMPLIANCE

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

- The Sponsor signature on the protocol documents for the Study Director the Sponsor's assurance that the study described in the protocol did 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 before completion of the test interfered with study objectives. Upon completion of the test all distressed animals were painlessly euthanized in a timely manner.

- Methods of euthanasia used during this study were in conformance with the above referenced regulation and are consistent with EPA Test Guidelines OPPTS 890.1350 (3).

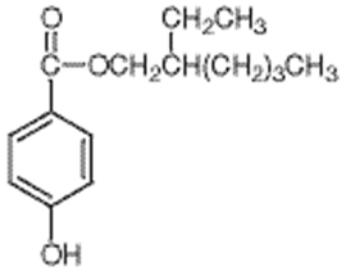
9. MATERIALS AND METHODS

9.1. TEST SUBSTANCE

2-EHHB (TCI America, Portland, OR, lot number 7CZZO, received October 15, 2015, 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 (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

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 used were determined by a range-finding study designed to identify the concentration of test chemical that causes lethality and morbidity.

9.1.1. LABORATORY WATER

Dechlorinated 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" 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. Water quality characteristics of the laboratory water are monitored bimonthly for pH, dissolved oxygen (DO), conductivity, hardness, alkalinity, ammonia, residual oxidants; and at study day (SD) 0 and 21 (in-life conclusion) for iodide (I-), polyaromatic hydrocarbons (PAHs), pesticides, and metals. The culture water was most recently analyzed for pesticides, PAHs, and metals on February 2, 2017 and all water quality measurements cited above met the U.S. EPA criteria for aquatic toxicity test culture/dilution water.

9.2. TEST SYSTEM

The test species was Japanese medaka (*Oryzias latipes*) (8). Medaka is a well-studied laboratory animal that exhibits ease of use in the laboratory, rapid development, and simplicity of observation during development and reproduction. Medaka is readily induced to breed in captivity, and published methods exist for its culture, as well as short-term lethality, early life-stage and full life-cycle tests. Medaka used were from in-house cultures at FEL, which were established from stock procured from Aquatic Research Organisms (ARO, Hampton, NH USA) and originate from stocks maintained at USEPA, Duluth, MN.

9.2.1. FEEDING

The fish were fed at least twice per day with brine shrimp *ad libidum* to promote active reproduction and maintain body condition. Food was withheld from the fish for 12 hours prior to the day of tissue sampling to aid in histology processing of small fish. Uneaten food and fecal material was removed from the test vessels at least twice weekly (e.g., by carefully cleaning the bottom of each tank using suction).

9.3. EXPOSURE SYSTEM

The route of 2-EHHB exposure was via aquatic exposure. A flow-through diluter system (Benoit Mini-Diluter; ECT, Superior, WI) was used in the performance of the Fish Short-Term Reproduction Assay. The system had water-contact components of glass (aquaria), stainless steel (diluter housing and water bath), and Teflon® (tubing responsible for test material delivery). Exposure tanks were 10 L glass aquaria (with approximate measurements of 40 x 20 x 20 cm) equipped with standpipes that result in an actual tank volume of 10.0 L and minimum water depth of 12 to 15 cm. The system was capable of supporting 4 exposure concentrations and a control, with up to 4 replicates per treatment. The flow rate to each tank was 2.7 L/hr (6.5 volume exchanges/d). Fluorescent lighting was used to provide a photoperiod of 16 hr light and 8 hr dark (2 hour light intensity transition period) at an intensity that ranges from 540 to 1,000

lux (lumens/m^2) at the water surface. Water temperature was maintained at 25º ± 1°C, pH maintained between 6.5 to 9.0, and the dissolved oxygen (DO) concentration ≥ 4.9 mg/L (>60% of the air saturation) in each test tank. This design was intended to randomize out the effects associated with the local environment (i.e., light and water) and possible trends associated with the diluter during testing. All fish were impartially assigned to tanks before pre-exposure. After spawning was established in the pre-exposure phase, tanks with acceptable spawning medaka were randomly assigned to treatments within a block in the diluter system to start the 21-d exposure phase. 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. Saturator columns were prepared on December 3, 2015 and the same columns were used for the entire pre-exposure and exposure periods. 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 was 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 two times per week to monitor diluter system performance (Table 1). Total hardness and alkalinity were measured in the control and one replicate of the highest concentration once per week. Iodide (I-) was measured in the dilution water on SD 0 and 21 (in-life conclusion). I- was measured in facility dilution and during the study using ion-selective electrode (ISE) analysis in accordance with facility SOP. Aeration for all test vessels was initiated immediately if DO reached 4.9 mg/L. The target temperature for the pre-exposure and exposure phases was 25° ± 1°C. After initial diluter calibration, a sample of test solution from each diluter concentration was sampled for chemical analysis during the diluter equilibration phase. 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). A laboratory blank was also collected with the batch of samples. At each sampling point, the 2-EHHB stock and diluter samples were preserved 1:1 (20 mL sample:20 mL methanol) and prepared for shipment to EAG. Duplicate samples and samples not shipped on the day of collection were stored at FEL at 4°C. Samples were shipped to EAG once per week. Due to the biodegradability of 2-EHHB in aqueous conditions, analyses were performed as soon as possible following collection (Appendix C).

Exposure tanks were siphoned daily to remove uneaten food and waste products. Care was taken to minimize stress and trauma to the animals, especially during movement, cleaning of

aquaria, and manipulation. Stressful conditions/activities were avoided such as loud and/or incessant noise, tapping on aquaria, vibrations in the aquaria, excessive activity in the laboratory, and rapid changes in environmental conditions (light availability, temperature, pH, DO, water flow rates, etc.).

9.4. CHEMICAL ANALYSIS

Test samples were 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) (9). Analysis was accomplished based on methods developed and validated at EAG Laboratories. At EAG Laboratories, samples were first diluted with 5 mL of methanol at a 1:1 ratio. Samples were further diluted, if necessary, with 50:50 methanol:HPLC-grade water to produce sample concentrations that fell within the calibration curve. At least two quality control (QC) fortification spikes were prepared in a similar manner at concentrations that bracket the expected high and low test substance treatment concentrations. The samples were 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 (Appendix C).

Instrument: UPLC system: Shimadzu Nexera
MS Spectrometer: ABSciex API-6500 Q-Trap

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

Ionization: Turbo Ion 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: 5 µL

Mass Spec. Scan Mode: MRM

Polarity: Negative

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

The above instrument parameters were modified as needed to optimize chromatography or to suit the needs of the instrument used.

9.5. STUDY DESIGN AND ADDITIONAL EXPERIMENTAL CONDITIONS

During a 21-day chemical exposure, survival, reproductive behavior, fecundity and fertilization success was monitored daily. At termination of the assay, measurements were made of several endpoints reflective of the status of the reproductive endocrine system. This was established during a "pre-exposure" period of 11 days (7-14 days per OECD Guideline 229 (4)). The pre-exposure observation period was used to monitor reproductive performance as described for the chemical exposure period. The pre-exposure observations occurred in the same system/tanks as was utilized for the chemical test. An overview of the test and relevant test conditions are provided in Table 2.

9.6. 21-DAY ASSAY INITIATION AND CONDUCT

9.6.1. SELECTION AND WEIGHING OF TEST FISH

In accordance with OECD Guideline 229 (4), medaka were 16±2 wpf, previously maintained in communal culture tanks. More specifically, fish used in the present study were 17 weeks post-fertilization (wpf) and 15 weeks post-hatch (wph). Individual female fish weights ranged from 0.2889-0.4102 g with an arithmetic mean of 0.3521 g. Individual male fish weights ranged from 0.2971-0.4119 g with an arithmetic mean of 0.3456 g. Thus, for the whole batch of fish used in the test, the range in individual weights by sex at the initiation of the pre-exposure phase were kept within ± 20% of the arithmetic mean weight of the same sex.

9.6.2. FEEDING

The fish were fed at least twice per day with brine shrimp *ad libidum* to promote active reproduction and maintain body condition. Food was withheld from the fish for 12 hours prior to the day of tissue sampling to aid in histology processing of small fish. Uneaten food and fecal material was removed from the test vessels daily.

9.6.3. PRE-EXPOSURE

The pre-exposure phase was 12 days per OECD guideline 229 (4), which specifies 7 to 14 days. The assay used fish that are approximately 16±2 wpf, previously maintained in

communal culture tanks. Five sexually mature females and five male medaka were randomly assigned to each replicate exposure chamber at each treatment concentration. Any specimens whose phenotypic sex was uncertain were excluded from the assay. Additional exposure chambers were set up for pre-exposure to account for a lack of spawning in some chambers and/or mortality during the pre-exposure phase. The pre-exposure phase of the assay was conducted under conditions (temperature, photo period, feeding, etc.) identical to those used during the chemical exposure phase. The animals were fed brine shrimp twice daily *ad libidum*. One 4 in. breeding mop head constructed from inert acrylic yarn was placed in each tank to collect eggs per facility SOP. FEL has experienced markedly more consistent and productive breeding with medaka using an artificial substrate that simulates rice grass. During the daily egg collection, eggs were assessed for fecundity. The mop heads were removed from the tank daily, replaced with a new mop head, and the eggs were cultured directly on the mop head in a separate brood chamber. For each assay, successful pre-exposure (suitability for testing) was established when regular spawning occurred in each replicate test chamber at least two times in the immediately preceding 7 days.

9.6.4. CHEMICAL EXPOSURE

After successful spawning was verified during pre-exposure as per the requirements of the assay, the chemical exposure was initiated and continued for 21 days. Five female and five male fish per replicate tank during exposure phases were used. One 4 in. breeding mop head constructed from inert acrylic yarn was placed in each tank as an artificial substrate to collect eggs. During the egg collection phases, eggs were cultured directly on the mop heads in a separate brood chamber. The assays were conducted at four chemical concentrations, as well as a dilution water control, with four experimental units (replicates) per treatment. The replicate tanks were assigned to treatment levels according to a randomized complete block design, with the four highest-spawning replicates assigned (one each) to one treatment level or control first, followed by the assignment of the next four highest-spawning replicates by distribution to the four test groups, until all groups were established. The test chemical was delivered to the exposure chambers using a continuous-flow diluter., or other appropriate delivery system. The exposure phase was conducted for 21 days, during which time the appearance, behavior, and reproductive fecundity of the fish were assessed daily. At termination of the exposure period, the endpoints described in Table 2 were recorded.

9.7. OBSERVATIONS AND MEASUREMENTS

9.7.1. ENDPOINTS

9.7.1.1. GENERAL

Several endpoints were assessed over the course of and/or at conclusion of the assay. A summary of measurement endpoints and associated study criteria is provided in Table 3. At termination, body weight, and digital photographs were taken for standard length measurement and examination of anal papillary processes of all 200 fish. Prior to processing, fish were anesthetized using 200 mg/L buffered MS-222 (pH 7). Subsequently, gonads were excised for GSI measurement and liver collected for VTG mRNA analyses from 2 male and 2 female fish per replicate (80 medaka total). Prior to preparing the remaining 120 medaka for histopathology, caudal fins were clipped in duplicate with one clip genotyped and the second stored at -20°C for

potential confirmatory genotyping. These remaining 3 male and 3 female medaka per replicate (120 medaka total) were fixed in modified Davidson's solution and preserved in 10% neutral buffered formalin (NBF) as whole intact specimens for thyroid, liver, and gonad histopathology. Histopathology for three target tissues followed a tiered approach described below in subsection 10.11.1.

9.7.1.2. ENDPOINTS MEASURED IN ALL STUDY FISH

- **Survival –** Daily assessment of survival was made to provide a basis for expression and interpretation of reproductive output, that is, number of eggs/female/day. Fish were examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) noted. Any mortality was recorded and the dead fish removed as soon as possible.
- **Sampling of fish –** At the conclusion of the exposure, the fish were anesthetized by transfer to an oxygenated solution of tricaine methanesulfonate (MS-222) (100 mg/L buffered with 200 mg sodium bicarbonate [$NaHCO_3$]/L) for sampling.
- **Body weights and length–** Whole body weight was recorded at test termination and measured to the nearest 0.1 mg. Standard length was measured from electronically-digitized digital photographs to the nearest 0.1 mm.
- **Behavior of adults –** All abnormal behavior in all replicates of all treatments and the controls including, but not limited to hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, or feeding abstinence was qualitatively noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, was also qualitatively noted.
- **Fecundity –** Egg production was determined daily. The spawning substrates were removed from the tanks to enumerate any eggs that were present. If no eggs were present, the substrate was left in the tank. New substrates were added to replace any that were removed. Fecundity was expressed on the basis of surviving females per reproductive (study) day per replicate. The cumulative eggs laid over the in-life exposure duration was also determined.
- **Fertilization success –** After the spawning substrate was removed from the tank, the embryos were carefully rolled off with a gentle circular motion of an index finger and visually inspected under appropriate magnification. If spawning occurred that morning, embryos typically were undergoing late cleavage, and determination of the fertility rate (number embryos/number of eggs x 100) were easily achieved. Infertile eggs were opaque or clear with a white dot where the yolk has precipitated. Viable embryos remained clear for 36 to 48 hours until reaching the eyed stage.
- **Appearance of adults and observation of secondary sex characteristics –** Secondary sexual characteristics are under endocrine control; therefore, observations of physical appearance of the fish were made over the course of the test and at conclusion of the study. The number of anal fin papillae were counted as an indication of external male phenotype per OECD guide 229, Annex 5B (4). External female phenotypes were based on anal fin anatomy.

9.7.1.3. ENDPOINTS MEASURED IN TWO MALE AND TWO FEMALE FISH PER REPLICATE

- **VTG –** The measurement of VTG mRNA in liver was performed using PCR (8).
- **Gonad size (gonadosomatic index, or GSI) -** The fixed gonads were removed and weighed (to the nearest 0.1 mg) to determine the GSI as described in section 10.10.2.
- **Blood sampling –** No blood sampling was performed in medaka.

9.7.1.4. ENDPOINTS MEASURED IN REMAINING THREE MALE AND THREE FEMALE FISH PER REPLICATE

- **Genotypic Sex** – During the necropsy, two fin clips were collected from each fish, and placed in separate labeled vials with one of the two fin clips analyzed following necropsy. Fin clips not immediately analyzed were stored frozen at -20ºC until analyzed or disposed of as directed by Sponsor at study termination.
- **Gonad, liver, and thyroid histopathology –** Immediately after anesthetization of an individual fish, length and weight were measured. Whole carcasses were then fixed in modified Davidson's solution and preserved in 10% (v/v) neutral buffered formalin (NBF).

9.7.2. LIVER COLLECTION AND DETERMINATION OF GSI – IN-LIFE LABORATORY (FEL)

VTG mRNA was analyzed in livers from the 2 male and 2 female fish per replicate that were not selected for histopathology (8) (Appendix D). The livers of sacrificed fish were removed and immediately placed into RNAlater™ at room temperature. When all samples were collected, they were placed in a refrigerator at 1 to 9°C overnight and then moved to the lab freezer and stored at -20 ± 10°C until analyzed.

Using micro-dissection scissors, the spermatic ducts or oviducts were severed proximal to the genital pore. Micro-dissection forceps were then applied to the spermatic ducts/oviducts. Using gentle traction, the gonads were dissected out of the abdominal cavity in a caudal to cranial direction, severing the mesorchial/mesovarial attachments as needed using the micro-dissection scissors. The gonads were removed and weighed (fixed weight to the nearest 0.1 mg) to determine the GSI (%) (GSI=[gonad wt/body wt]*100).

9.7.3. TISSUE FIXATION/PRESERVATION FOR HISTOPATHOLOGY – IN-LIFE LABORATORY (FEL)

Fish were fixed in modified Davidson's fixative for 48-96 hours. After this time, each carcass was rinsed thoroughly in dechlorinated culture water, and then placed in 10% neutral buffered formalin (NBF) for storage. The volume of fixative in the container was at least 20 times the approximated volume of the tissues. The fixative container was gently agitated for 5 seconds to dislodge air bubbles from the cassette. Fixed tissue samples were sent to EPL (Sterling, VA) for histological examination.

9.7.4. GONAD, LIVER, AND THYROID HISTOPATHOLOGY - EPL TEST SITE

EPL, under the direction of the Sponsor, performed the tissue preparation, histology, and histopathological interpretation in accordance with appropriate facility guidance documents (SOPs) and the relevant guidance documents on histopathology for the FSTRA and MEOGRT (3,4,8,10). Following the conclusion of exposure (SD 21), FEL sent preserved intact whole carcass specimens to EPL via overnight courier for histopathological processing and analyses.

9.7.4.1. HISTOPATHOLOGICAL PROCEDURE

Routine histological procedures were used to assess the condition of gonads, liver, and thyroid from the fish and were performed by a toxicological pathologist with experience with small fishes (Appendix D). Specifically, preserved whole intact carcasses were sent to EPL (Sterling, VA) for histological examination. The following step-down process was used:

- Preserved, intact animals (carcasses) were shipped from the in-life laboratory to the histopathology laboratory (120 fish per species)
- All 120 histopathology animals were processed for sagittal sectioning and slides are prepared. The purpose was to enable the pathologist to read all three target tissues (gonad, liver, thyroid) on one slide if possible.
- In the base effort, the pathologist read the slides and evaluated the animals from the high concentration and control groups (48 fish per species, Part A).
- If potential treatment-related effects were observed in these High or Control animals, then with concurrence of Study Director, the pathologist read the slides from the Mid-High concentration group (24 fish per species, Part B.1)
- If potential treatment-related effects were observed in these Mid-High animals, then with concurrence of Study Director, the pathologist read the slides from the Mid-Low and Low concentration groups (48 fish per species, Part B.2).
- The pathologist considered each target tissue individually. Once the pathologist reached a treatment level with no potential treatment-related effects in that tissue, then the pathologist stopped with that tissue and did not read that tissue on the slides from the animals in the next lower concentration group.

It was important to define the criteria for the read-down as the determination of "potential treatment-related findings." Many animals had findings or abnormalities, but it was only by comparing treatment groups that the potential for relationship to treatment could be established.

The trigger to proceed to the next lower tier for pathology depended, to some extent, on the commonality of the potential treatment-related finding. The decision was assessed case-by-case. Unless it was a type of finding that was not generally (or ever) observed spontaneously, treatment relationship among the high dose animals was determined on a group-wise basis as opposed to an individual animal basis. However, a fairly rare type of finding (e.g., ovarian spermatogenesis) in one or two individuals in the high treatment group, potentially triggered a read down, even if it did not register as statistically significant. If an obvious, statistically significant treatment-related finding occurred in the high treatment group relative to control, but that finding was observed to a lesser, non-significant, extent in the mid-treatment group, the pathologist evaluated the next lower treatment group, because it potentially revealed a treatment-

response pattern that provided supporting evidence for the legitimacy of the relationship to treatment. Treatment relationship, and the histopathology decision tree, did not rely only on statistical significance. Not all statistically significant results are toxicologically important, and not all toxicologically important results are statistically significant. The pathologist conferred with the Study Director to determine the scientific justification for proceeding to the next lower tier.

9.7.5. GENOTYPING - IN-LIFE LABORATORY (FEL)

Two fin clips were collected from each fish in the "histopathology" group. One was stored at -20ºC, and the other was used for genotype analysis.

9.7.6. GENOTYPING PROCEDURE

Once the DNA was extracted, a polymerase chain reaction (PCR) method was used to amplify the dmy gene, if present (8). The presence of the dmy gene was a definitive indication of an XY (male) individual, regardless of phenotype. The *dmy* gene information was used to establish XX – XY genotype regardless of external phenotype which may be altered by exposure to an endocrine-disrupting chemical.

9.7.7. VITELLOGENIN ANALYSIS – EAG LABORATORIES, EASTON, MD TEST SITE

Samples were shipped to EAG Laboratories - Easton for analyses via overnight courier (Appendix E).

9.8. DATA ANALYSIS

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 raw data (DEST), statistical report (Battelle), and range-finding data are provided in Appendices F-H, respectively. The IQR was determined by (Q1 – 1.5*IQD) and (Q3 + 1.5*IQD), where IQD was the interquartile difference defined as Q3 minus Q1. In the present study (BATT01-00404), no duplicate samples were analyzed and thus, the IQR represents analysis of the original samples only. The statistical unit for analysis was the replicate. Measurements made on a per-tank basis included fecundity, normalized to number of female reproductive days. Endpoints evaluated on a per-fish basis (e.g., papillary processes measures) were averaged per tank. For all continuous quantitative endpoints (fecundity, VTG, and GSI) consistent with a monotonic concentration-response, the Jonckheere-Terpstra test or equivalent were applied in step-down manner to establish a significant treatment effect. For continuous endpoints that are not consistent with a monotonic concentration-response, the data were assessed for normality (preferably using the Shapiro-Wilk or Anderson-Darling test) and variance homogeneity (preferably using the Levene test). If non-normal or heterogeneity in variance was detected, a normalizing, variance stabilizing transformation was used. If after transformation, the data were normally distributed with homogeneous variance, a significant treatment effect was assessed using Dunnett's test (or the equivalent). If after transformation, the data were normally distributed with heterogeneous variance, effects on a given treatment was determined using the Tamhane-Dunnett (T3 test) or the Mann-Whitney-Wilcoxon U test (or the equivalent). If no normalizing transformations were found, significant treatment effects were evaluated using the Mann-Whitney-Wilcoxon U test with a Bonferroni-

Holm adjustment (or the equivalent) to the p-values. Dunnett's test (or the equivalent) was applied independently of any ANOVA F-test and the Mann-Whitney test (or equivalent) was used independently of Kruskall-Wallis ANOVA on Ranks test.

Mortality was assessed from the step-down Cochran-Armitage test (or equivalent) when the data were monotonic, and otherwise from Fisher's Exact test with a Bonferroni-Holm adjustment (or equivalent). Effects on papillary processes score were determined from the step-down application of the Jonckheere-Terpstra test (or equivalent) applied to the replicate medians. The unit of analysis was the replicate, so the data consisted of replicate medians if the Jonckheere-Terpstra or Mann-Whitney U test with a Bonferroni-Holm adjustment (or equivalent) was used, or the replicate means if Dunnett's test was used. Concentration-response monotonicity was assessed visually from the replicate and treatment means or medians or from formal tests such as previously described by OECD (11). The statistical significance of all tests indicated was assessed at $p = 0.05$. At the conclusion of the analyses, No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC) values were reported. A test termed RSCABS (Rao-Scott Cochran Armitage by Slices) that used 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 (12).

Analysis of the biological data were conducted both with and without suspected outliers (3,4,11). Potential outliers were identified by values that exceed the median plus three times the interquartile range (i.e., the difference between the 75th and 25th percentiles). If an explanation could not be made for the divergence of data, then both analyses were presented, assuming that the results differ. If the outlier did not alter the results, then the analysis including the outliers was presented. Analysis of the data from this study report revealed seven potential outliers occurring in the following biological endpoints: Fertilization Success, Female GSI (n=3), Female Body Weight, Male Body Weight, and Female Gonad Weight (Appendix G). No biological endpoint outliers were removed from the analyses presented in the present report for study BATT01-00404.

10. RESULTS

10.1. RANGE-FINDING TEST

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

10.2. WATER QUALITY MEASUREMENTS AND TEST SYSTEM PERFORMANCE

Results of water quality measurements and test system performance are presented in Table 4. I- levels in the dilution water were measured on Study Day (SD) 0 and 21 (in-life conclusion) of the 00404 study and contained 8.9 (±0.04) and 9.3 (±0.15) µg/L I-, respectively,

which fell within the acceptable range. All physicochemical water quality parameters in study were within acceptable ranges (Table 3).

10.3. CONFIRMATION OF TEST CONCENTRATIONS

Nominal 2-EHHB concentrations selected for the modified FSTRA 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 (<0.212 µg/L [minimum quantitation level, MQL]) (Table 5 and Appendix C). 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) of the control and each treatment per facility SOP. IQRs determined for the 0.0 (control), 3.60, 10.9, 33.0, and 100 µg/L 2-EHHB treatments were 0.106-0.106, 1.14-4.73, 5.42-15.5, 21.5-48.8, and 61.1-146 µg/L 2-EHHB, respectively. Based on IQR analysis of the control and each treatment group, no outliers were identified. The corresponding mean measured concentrations in the definitive study were 2.98, 10.6, 34.1, and 105 µg/L 2-EHHB, respectively (Table 6). 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 3.60, 10.9, 33.0, and 100 µg/L treatments were 9%, 6%, 9%, and 5%, respectively; which were acceptable based on the criteria establish in the test guidance (3) and protocol BATT01-10 for study BATT01-00404 indicating that the mean measured concentrations were a reasonable estimation of exposure concentration.

10.4. MORTALITY

Test organism survival during the study is presented in Table 7. No mortality was observed during the study. Since all medaka survived during the study, no statistical analyses were performed.

10.5. FECUNDITY AND FERTILIZATION

The effects of 2-EHHB exposure on reproductive fecundity and fertilization are provided in Table 8. The mean number of eggs produced/female/day ranged from 13.4 in control to 4.0 in the 100 µg/L 2-EHHB treatment. The mean egg production in each of the 2-EHHB treatments was significantly different from the control (Jonckheere-Terpstra test, p=0.0433, 0.0021, <0.0001, and <0.0001, respectively). Fertilization success ranged from 95.4% in the control to 75.8% in the 100 µg/L 2-EHHB treatment. The mean fertilization in the 10.9, 33, and 100 µg/L 2-EHHB treatments was significantly less than the control (Jonckheere-Terpstra test, p=0.0034, <0.0001, and <0.0001, respectively).

10.6. SECONDARY SEXUAL CHARACTERISTICS

10.6.1. ANAL FIN PAPILLAE

The effects of 2-EHHB exposure on the presence of anal fin papillae are provided in Table 9. No anal fin papillae were present in female medaka. Anal fin papillae count in male medaka ranged from 83.0 in the control to 66.4 in the 100 µg/L 2-EHHB treatment. The mean papillae count in the 33.0 and 100 µg/L 2-EHHB treatments was significantly less than the control (Jonckheere-Terpstra test, p=0.0394 and 0.0012, respectively).

10.7. LIVER VITELLOGENIN (VTG) MRNA

The effects of 2-EHHB exposure on liver VTG mRNA expression are provided in Table 10. Female liver VTG expression ranged from 4.06 log10(copies VTG/ng total RNA) in the 100 µg/L 2-EHHB treatment to 5.03 in the 3.6 µg/L 2-EHHB treatment. Male liver VTG expression ranged from 1.74 log10(copies VTG/ng total RNA) in the control to 3.42 log10(copies VTG/ng total RNA) in the 3.6 µg/L 2-EHHB treatment. Male liver VTG mRNA expression was significantly greater in the 3.6 and 10.9 µg/L 2-EHHB treatments than the control expression (Dunnett's test, p=0.0017 and 0.0069, respectively). Male liver VTG mRNA expression represented a non-monotonic concentration-response which can occur in studies focusing on endocrine active compounds (13) and might be indicative of possible low-concentration effects associated with 2-EHHB exposure (14-17).

10.8. GONAD WEIGHT

The effects of 2-EHHB exposure on gonad weight are provided in Table 11. Mean ovary weight ranged from 19.5 mg in female medaka exposed to 100 µg/L 2-EHHB to 32.1 mg in medaka exposed to 3.6 µg/L 2-EHHB. Mean testes weight ranged from 2.6 mg in male medaka exposed to 33.0 µg/L 2-EHHB to 3.4 mg in medaka exposed to 3.6 µg/L 2-EHHB.

10.9. GONADOSOMATIC INDEX (GSI)

The effects of 2-EHHB exposure on the gonadosomatic indices are provided in Table 12. Mean GSI in female medaka ranged from 6.1% in the 100 µg/L 2-EHHB treatment to 9.1% in the 3.6 µg/L 2-EHHB treatment. Mean GSI in male medaka ranged from 0.8% in the 33.0 µg/L 2-EHHB treatment and control to 0.9% in the 3.6, 10.9, and 100 µg/L 2-EHHB treatment.

10.10. GROWTH

10.10.1. BODY WEIGHT

The effect of 2-EHHB exposure on body weight is provided in Table 13. Mean body weight, one of two measures of larval growth, ranged from 359.0 mg in female medaka exposed to 100 µg/L 2-EHHB to 422.0 mg in female medaka exposed to 33.0 µg/L 2-EHHB. Mean body weight in male medaka ranged from 357.9 mg in medaka from the 100 µg/L 2-EHHB treatment to 435.5 mg in medaka from the 3.6 µg/L 2-EHHB.

10.10.2. STANDARD LENGTH

The effect of 2-EHHB exposure on standard length is presented in Table 14. Mean standard length, the second of two measures of larval growth, ranged from 25.8 mm in control females to 27.4 mm in female medaka exposed to 33.0 µg/L 2-EHHB.

Mean standard length ranged from 26.4 mm in males exposed to 100 µg/L 2-EHHB to 28.1 mm in medaka exposed to 3.6 µg/L 2-EHHB.

10.11. EXTERNAL PHENOTYPIC, HISTOLOGICAL, AND GENETIC SEX

The effect of 2-EHHB on external phenotypic, histological, and genetic sex is provided in Table 15. External phenotypic and genetic sex were the same in the control and each of the 2-EHHB treatments. Histological sex was also the same as phenotypic and genetic sex in the control and the treatments included in the histopathology read-down. Per the study design, nominally only three males and three females per replicate had genetic sex determined by fin clip.

10.12. HISTOPATHOLOGY

The results of histopathological evaluation of the thyroid, liver, and gonads are provided in Tables 16-19. The histopathology report (EPL, Sterling, VA) is provided in Appendix D. In consultation with the study director the pathologist examined tissues of male fish from the following treatment groups: thyroid (control; 10.9; 33.0; 100.0 µg/L); liver (control; 100.0 µg/L); gonad (control; 100.0 µg/L). The pathologist examined tissues of female fish from the following treatment groups: thyroid (control; 100.0 µg/L); liver (control; 10.9; 33.0; 100.0 µg/L); and gonad (control; 10.9; 33.0; 100.0 µg/L).

10.12.1. THYROID

Modest increase in the severity of follicular cell hypertrophy (minimal to mild) in male medaka exposed to 100.0 µg/L 2-EHHB (4/11 had mild hypertrophy) compared to controls (2/12 had mild hypertrophy) was observed (Table 16). However, this difference was most likely attributable to inter-specimen variation. In addition, modest decreases in the severity of follicular cell hyperplasia in male medaka exposed to 10.9 and 33.0 µg/L 2-EHHB (7/12 and 8/12 had mild hyperplasia, respectively) relative to controls (10/12 had mild hyperplasia) which was most likely due to inter-animal variation since no concentration-response pattern was associated with this finding. The pathologist noted that the amount and appearance of thyroid tissue in male medaka can vary appreciably depending on the precise location of the sections evaluated, therefore, slight grading differences should be interpreted with caution.

Reduced presence of minimal hyaline degeneration (follicular cells) was observed in female medaka from the 33.0 and 100 µg/L 2-EHHB treatments relative to control (RSCABS, p=0.0374 and 0.0263, respectively) (Table 16). Although hyaline degeneration of follicular cells was decreased statistically in females of the 33.0 and 100.0 µg/L 2-EHHB treatment groups relative to controls; however, a statistical decrease in that particular finding, as is true of most degenerative-type changes, was not considered to be toxicologically relevant.

10.12.2. LIVER

Increased presence of mild cystic degeneration in male medaka exposed to 100 µg/L 2-EHHB (RSCABS, p=0.0315), but decreased presence of mild cystic degeneration in male medaka exposed to 33.0 and 10.9 µg/L 2-EHHB (RSCABS, p=0.0188 and 0.0343, respectively) compared to control were observed (Table 17). A reduced occurrence of minimal eosinophilic cytoplasmic inclusions was noted in male medaka from the 10.9, 33.0, and 100 µg/L 2-EHHB treatments compared to control (RSCABS, p=0.0142, 0.0047, and 0.0273, respectively). Increased minimal (RSCABS, p=0.0273), mild (RSCABS, p=0.0025), and moderate (RSCABS, p=0.0263) granulomatous inflammation in male medaka from the 100 µg/L 2-EHHB treatment relative to control was noted (RSCABS, p=0.0273). Although these findings were statistically significant, they were not considered to be toxicologically relevant by the pathologist.

Decreased occurrence of mild pigmented macrophage aggregates in female medaka exposed to 100 µg/L 2-EHHB (RSCABS, p=0.0307) compared to control was observed (Table 17). However, a statistical decrease in that particular finding is not likely to be toxicologically relevant based on information provided by the pathologist. In addition, increased presence of mild and moderate vacuolation in female medaka exposed to 33.0 and 100 µg/L 2-EHHB compared to control was observed (RSCABS, p=0.0148 and 0.0028) for mild and p=0.0278 and 0.228 for moderate, respectively). A treatment-related decrease in the occurrence of minimal, mild, and moderate basophilia was observed in liver from female medaka exposed to 33.0 and 100 µg/L 2-EHHB compared to control (RSCABS, p=0.0005 and 0.0438 [minimal], 0.0001 and 0.0008 [mild], and 0.0020 and 0.0195 [moderate], respectively). A slight decrease in basophilia in fish of the 10.9 µg/L treatment group relative to controls was insubstantial and not statistically significant. This occurred as decrease in the severity of this finding in the 100.0 µg/L dose group relative to controls, and a decrease of both the severity and prevalence of basophilia in the 33.0 µg/L dose group. On an individual fish basis, the presence of zero, minimal, or mild basophilia in females was often (but not always) associated with a low (i.e., Grade 1 or 2) ovarian stage score and/or oocyte atresia. An increase in the presence of minimal, mild, and moderate focus of cellular alteration was observed in the 100 µg/L 2-EHHB treatment relative to control (RSCABS, p=0.0307, 0.0263, and 0.0263 for minimal, mild, and moderated severity). Because of the low prevalence of this finding, its relationship to treatment is uncertain.

10.12.3. GONAD

No histopathological findings were noted in the testis of male medaka exposed to 2-EHHB compared to the control. An increase in mild oocyte atresia in female medaka exposed to 100 µg/L 2-EHHB compared to control (RSCABS, p=0.0075) (Table 18). Decreased occurrence of minimal and mild egg debris in oviduct in female medaka exposed to 33.0 and 100 µg/L 2-EHHB compared to control was observed (RSCABS, p=0.0374 and 0.0263, respectively [for both minimal and mild]. Mean ovarian stage scores were slightly greater than controls in females of the 100.0 mg/L dose group, but were lower than controls in 10.9 and 33.0 mg/L group females. Decreased occurrence of minimal and mild egg debris in oviduct in female medaka exposed to 33.0 and 100 µg/L 2-EHHB compared to control was observed (RSCABS, p=0.0374 and 0.0263, respectively [for both minimal and mild]; however, these decreases in that were not deemed likely to be toxicologically relevant by the pathologist.

There were six gonadal germ cell neoplasms observed in this study (Table 19), none of which were thought to be treatment related. Benign seminomas were found in the testes of one control male (replicate B) and two 100.0 µg/L treatment group males (replicates A and C). Malignant seminomas, with intra-coelomic metastasis (to the anterior coelomic wall in one case, and ventral liver capsule in another), were identified in the ovaries of one control female (replicate A) and one 100.0 µg/L treatment group female (replicate D). Interestingly, while the malignant seminomas of the ovaries were of the spermatocytic type (which is characteristic of most teleosts), the testicular seminomas were of the less common spermatogonial type. A benign dysgerminoma occurred in the testis of a single 100.0 µg/L treatment group male (replicate A). The labeling of benign versus malignant for these tumors was based solely on the presence or absence of metastasis, as the cytologic features were not generally a useful indicator of malignancy. The relatively high prevalence (for medaka) of these germ cell tumors and their presence in controls suggests that this is likely a strain-related background issue.

In the current study, gonadal germ cell tumors were observed in four male and two female medaka. Although the occurrence of these neoplasms was not considered treatment-related, two germ cell neoplasms were identified in the control and four in the 100 µg/L 2-EHHB treatment group. Overall, the recording of gonadal tumors in six of 72 examined medaka (control - 2.8% and 8% overall) was somewhat unusual, based on the experience of the pathologist and the results of at least one published report (18), in which germ cell neoplasms were detected in only 26 of approximately 10,000 medaka (0.3%). The investigators (18) reported that the tumors were most likely associated with age rather than chemical exposure suggesting that the development of the neoplasms was most likely attributable to culturing techniques, genetic factors, or a virus, based on the tendency for multiple occurrences to cluster in the fish of the same cohort. This finding is perplexing as culturing practices for medaka have been consistently maintained with a low background rate of occurrence similar to that reported (18). The age of the fish used in the present study (15.5 wpf) and the breeders used to produce the fish used in the study are controlled to eliminate age as a confounding factor. The genotype of the fish affected with the neoplasms matched the phenotype identified. Most importantly for the present study, the occurrence of the six germ cell neoplasms was distributed among control and high dose groups from both sexes, and thus their presence did not affect the interpretation of the study.

10.13. CLINICAL SIGNS OF TOXICITY

Clinical signs of toxicity were not observed during the conduct of the present study.

11. DISCUSSION

11.1. PERFORMANCE CRITERIA AND VALIDITY

Performance of the present study and the relationship to the performance criteria and test validity established in protocol BATT01-10 and study guidance document (3) are provided in Table 20. 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 control spawn produced >10 eggs/female/day and the spawn frequency were at least every 4 days. Control egg fertilization was >80%. 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 25±1ºC. 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 890.1350 Fish Short-Term Reproduction Assay (FSTRA) (3) and OECD Guideline 229 (4) (Table 20).

11.2. ASSESSMENT OF THE FISH SHORT-TERM REPRODUCTION ASSAY RESULTS

Results of present study met the performance criteria established for the OPPTS 890.1350 Fish Short-Term Reproduction Assay (FSTRA) (3) and were considered valid (Table 20). Following Ankley and Jensen (19), the following results were considered as a basis for concluding if 2-EHHB has the potential to interact with the endocrine system.

- The mean measured concentrations for the 0.0 (control), 3.6, 10.9, 33.0, and 100 µg/L 2-EHHB treatments (nominal) were (<0.212 µg/L [minimum quantitation level, MQL]), 2.98, 10.6, 34.1, and 105 µg/L 2-EHHB (see Table 6).

- Survival rates in the control and 2-EHHB treatments were both 100%.

- The mean egg production in all of the 2-EHHB treatments was significantly less than the control (Jonckheere-Terpstra test, p=0.0433, 0.0021, <0.0001, and <0.0001 for the 3.6, 10.9, 33.0 and 100 µg/L treatments, respectively). The mean fertilization in the 10.9, 33, and 100 µg/L 2-EHHB treatments was significantly less than the control (Jonckheere-Terpstra test, p=0.0034, <0.0001, and <0.0001, respectively). It is plausible that only the effects on reproductive fecundity observed in the 33.0 and 100 µg/L treatments were toxicologically-relevant based on the magnitude and longevity of effect during the study.

- The mean anal fin papillae count in the 33.0 and 100 µg/L 2-EHHB treatments was significantly less than the control (Jonckheere-Terpstra test, p=0.0394 and 0.0012, respectively). No papillae were found in control or 2-EHHB-treated female fish. A reduction in mean male anal fin papillae counts in response to treatment can be considered indicative of endocrine activity.

- Male liver VTG mRNA expression was significantly greater in the 3.6 and 10.9 µg/L 2-EHHB treatments than the control expression (Dunnett's test, p=0.0017 and 0.0069, respectively). Male liver VTG mRNA expression represented a non-monotonic concentration-response which can occur in studies focusing on endocrine active compounds (13) and might be indicative of possible low-concentration effects associated with 2-EHHB exposure (14-17). Further, these results suggested that 2-EHHB may induce VTG expression at low concentrations indicative of a weak estrogen. Exposure to 2-EHHB did not affect female liver VTG mRNA expression. Variability in the data obtained, however, may have affected data interpretation.

- Exposure to 2-EHHB did not affect gonad weight or GSI in male or female medaka relative to the control.

- Exposure to 2-EHHB did not affect body weight or standard length in male or female medaka relative to the control.

- **THYROID:** Modest increase in the severity of follicular cell hypertrophy (minimal to mild) in male medaka exposed to 100.0 µg/L 2-EHHB (4/11 had mild hypertrophy) compared to controls (2/12 had mild hypertrophy) was observed. However, this difference was most likely attributable to inter-specimen variation. In addition, modest decreases in the severity of follicular cell hyperplasia in male medaka exposed to 10.9 and 33.0 µg/L 2-EHHB (7/12 and 8/12 had mild hyperplasia, respectively) relative to controls (10/12 had mild hyperplasia) which was most likely due to inter-specimen variation since no concentration-response pattern was associated with this finding. Reduced presence of minimal hyaline degeneration (follicular cells) was observed in in the thyroid of female medaka from the 33.0 and 100 µg/L 2-EHHB treatments relative to control (RSCABS, p=0.0374 and 0.0263, respectively). However, this particular finding, as is true of most degenerative-type changes, was not considered to be toxicologically relevant (see Appendix D).

- **MALE LIVER:** Increased presence of mild cystic degeneration in liver from male medaka exposed to 100 µg/L 2-EHHB (RSCABS, p=0.0315), but decreased presence of mild cystic degeneration in male medaka exposed to 33.0 and 10.9 µg/L 2-EHHB (RSCABS, p=0.0188 and 0.0343, respectively) compared to control were observed. A reduced occurrence of minimal eosinophilic cytoplasmic inclusions was noted in liver from male medaka from the 10.9, 33.0, and 100 µg/L 2-EHHB treatments compared to control (RSCABS, p=0.0142, 0.0047, and 0.0273, respectively). Increased minimal (RSCABS, p=0.0273), mild (RSCABS, p=0.0025), and moderate (RSCABS, p=0.0263) granulomatous inflammation in male medaka from the 100 µg/L 2-EHHB treatment relative to control was noted (RSCABS, p=0.0273). Although these findings were statistically significant, they were not considered to be toxicologically relevant by the pathologist (see Appendix D).

- **FEMALE LIVER:** Decreased occurrence of mild pigmented macrophage aggregates in liver from female medaka exposed to 100 µg/L 2-EHHB (RSCABS, p=0.0307) compared to control was observed. In addition, increased presence of mild and moderate vacuolation in liver from female medaka exposed to 33.0 and 100 100 µg/L 2-EHHB compared to control was observed (RSCABS, p=0.0148 and 0.0028) for mild and p=0.0278 and 0.228 for moderate, respectively). A treatment-related decrease in the occurrence of minimal, mild, and moderate basophilia was observed in liver from female medaka exposed to 33.0 and 100 µg/L 2-EHHB compared to control (RSCABS, p=0.0005 and 0.0438 [minimal], 0.0001 and 0.0008 [mild], and 0.0020 and 0.0195 [moderate], respectively). A slight decrease in basophilia in fish of the 10.9 µg/L treatment group relative to

controls was insubstantial and not statistically significant. This occurred as decrease in the severity of this finding in the 100.0 µg/L dose group relative to controls, and a decrease of both the severity and prevalence of basophilia in the 33.0 µg/L dose group. On an individual fish basis, the presence of zero, minimal, or mild basophilia in females was often (but not always) associated with a low (i.e., Grade 1 or 2) ovarian stage score and/or oocyte atresia. An increase in the presence of minimal, mild, and moderate focus of cellular alteration was observed in liver from female medaka exposed to the 100 µg/L 2-EHHB treatment relative to control (RSCABS, p=0.0307, 0.0263, and 0.0263 for minimal, mild, and moderated severity). Although these findings were statistically significant, they were not considered to be toxicologically relevant by the pathologist (see Appendix D).

- **TESTIS:** No histopathological findings were noted in the testis of male medaka exposed to 2-EHHB compared to the control.

- **OVARY:** An increase in mild oocyte atresia in female medaka exposed to 100 µg/L 2-EHHB compared to control (RSCABS, p=0.0075). Decreased occurrence of minimal and mild egg debris in oviduct in female medaka exposed to 33.0 and 100 µg/L 2-EHHB compared to control was observed (RSCABS, p=0.0374 and 0.0263, respectively [for both minimal and mild]. Mean ovarian stage scores were slightly greater than controls in females of the 100.0 mg/L dose group, but were lower than controls in 10.9 and 33.0 µg/L group females. Especially in the 33.0 µg/L dose group, the high prevalence of oocyte atresia appeared to contribute to the low stage scores, as atresia appeared to affect the more mature vitellogenic follicles preferentially. Decreased occurrence of minimal and mild egg debris in oviduct in female medaka exposed to 33.0 and 100 µg/L 2-EHHB compared to control was observed (RSCABS, p=0.0374 and 0.0263, respectively [for both minimal and mild]; however, these decreases in egg debris were not deemed likely to be toxicologically relevant by the pathologist.

- **OTHER FINDINGS – GONADS:** There were six gonadal germ cell neoplasms observed in this study (Table 19), none of which were thought to be treatment related. Benign seminomas were found in the testes of one control male (replicate B) and two 100.0 µg/L treatment group males (replicates A and C). Malignant seminomas, with intra-coelomic metastasis (to the anterior coelomic wall in one case, and ventral liver capsule in another), were identified in the ovaries of one control female (replicate A) and one 100.0 µg/L treatment group female (replicate D). Interestingly, while the malignant seminomas of the ovaries were of the spermatocytic type (which is characteristic of most teleosts), the testicular seminomas were of the less common spermatogonial type. A benign dysgerminoma occurred in the testis of a single 100.0 µg/L treatment group male (replicate A). The labeling of benign versus malignant for these tumors was based solely on the presence or absence of metastasis, as the cytologic features were not generally a useful indicator of malignancy. The relatively high prevalence (for medaka) of these germ cell tumors and their presence in controls

suggests that this is likely a strain-related background issue. In the current study, gonadal germ cell tumors were observed in four male and two female medaka. Although the occurrence of these neoplasms was not considered treatment-related, two germ cell neoplasms were identified in the control and four in the 100 µg/L 2-EHHB treatment group.

Overall, the recording of gonadal tumors in six of 72 examined medaka (control - 2.8% and 8% overall) was somewhat unusual, based on the experience of the pathologist and the results of at least one published report (18), in which germ cell neoplasms were detected in only 26 of approximately 10,000 medaka (0.3%). The investigators (18) reported that the tumors were most likely associated with age rather than chemical exposure suggesting that the development of the neoplasms was most likely attributable to culturing techniques, genetic factors, or a virus, based on the tendency for multiple occurrences to cluster in the fish of the same cohort. Overall, this finding is perplexing as culturing practices for medaka have been consistently maintained with a low background rate of occurrence similar to that reported (18). The age of the fish used in the present study (15.5 wpf) and the breeders used to produce the fish used in the study are controlled to eliminate age as a confounding factor. The genotype of the fish affected with the neoplasms matched the phenotype identified. Most importantly for the present study, the occurrence of the six germ cell neoplasms was distributed among control and high dose groups from both sexes, and thus their presence did not affect the interpretation of the study.

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

12. CONCLUSIONS

In conclusion, survival rates in the control and 2-EHHB treatments were both 100%. Exposure to 2-EHHB did not affect body weight or standard length in male or female medaka relative to the control. Exposure to 2-EHHB increased cystic degeneration, eosinophilic cytoplasmic inclusions, and granulomatous inflammation of the liver in male medaka, and decreased occurrence of pigmented macrophage aggregates, vacuolation, basophilia, and increased focus of cellular alteration in the liver of female medaka exposed to 2-EHHB. Although these findings were statistically significant, they were not considered to be toxicologically relevant by the pathologist with the potential exception of basophilia. In this case, a slight decrease in basophilia in fish of the 10.9 µg/L treatment group relative to controls was insubstantial and not statistically significant. This occurred as decrease in the severity of this finding in the 100.0 µg/L dose group relative to controls, and a decrease of both the severity and prevalence of basophilia in the 33.0 µg/L dose group. On an individual fish basis, the presence of zero, minimal, or mild basophilia in females was often (but not always) associated with a low (i.e., Grade 1 or 2) ovarian stage score and/or oocyte atresia.

Results from the present study indicated that 2-EHHB reduced reproductive fecundity, including both egg production and fertility. No significant differences were found in gonad weight or GSI in male or female medaka relative to the control. Exposure to 2-EHHB reduced

anal fin papillae counts in male medaka compared to control. No anal fin papillae were found in female medaka. The reduction in mean male anal fin papillae counts suggested a decrease in male sexual characteristics. Male liver VTG mRNA expression was increased relative to the control in the 3.6 and 10.9 µg/L 2-EHHB treatments. Male liver VTG mRNA expression represented a non-monotonic concentration-response which can occur in studies focusing on endocrine active compounds (13) and might be indicative of possible low-concentration effects associated with 2-EHHB exposure (14-17). However, care in interpreting potential low-concentration effects should be tempered based on variability in the data set. Further, these results suggested that 2-EHHB may induce VTG expression at low concentrations indicative of a weak estrogen. Exposure to 2-EHHB did not affect female liver VTG mRNA expression.

A modest increase in the severity of follicular cell hypertrophy (minimal to mild) in male medaka exposed to 100.0 µg/L 2-EHHB compared to controls was observed. In addition, modest decreases in the severity of follicular cell hyperplasia in male medaka exposed to 10.9 and 33.0 µg/L 2-EHHB relative to controls which was most likely due to inter-animal variation since no concentration-response pattern was associated with this finding. Reduced presence of minimal hyaline degeneration (follicular cells) was observed in in the thyroid of female medaka from the 33.0 and 100 µg/L 2-EHHB treatments relative to control. However, this particular finding, as is true of most degenerative-type changes, was not considered to be toxicologically relevant. No histopathological findings were noted in the testis of male medaka exposed to 2-EHHB compared to the control. Increased mild oocyte atresia in female medaka exposed to 2-EHHB compared to control and decreased occurrence of egg debris in oviduct in female medaka exposed to 2-EHHB compared to control was observed. However, these decreases in egg debris were not deemed likely to be toxicologically relevant by the pathologist. Mean ovarian stage scores were slightly greater than controls in females of the 100.0 mg/L dose group, but were lower than controls in 10.9 and 33.0 mg/L group females.

Six gonadal germ cell neoplasms were observed in this study, none of which were thought to be treatment related. Benign seminomas were found in the testes of one control male (replicate B) and two 100.0 µg/L treatment group males (replicates A and C). Malignant seminomas, with intra-coelomic metastasis (to the anterior coelomic wall in one case, and ventral liver capsule in another), were identified in the ovaries of one control female (replicate A) and one 100.0 µg/L treatment group female (replicate D). Interestingly, while the malignant seminomas of the ovaries were of the spermatocytic type (which is characteristic of most teleosts), the testicular seminomas were of the less common spermatogonial type. A benign dysgerminoma occurred in the testis of a single 100.0 µg/L treatment group male (replicate A). Although the occurrence of these neoplasms was not considered treatment-related, two germ cell neoplasms were identified in the control and four in the 100 µg/L 2-EHHB treatment group. Overall, the recording of gonadal tumors in six of 72 examined medaka (control - 2.8% and 8% overall) was somewhat unusual, based on the experience of the pathologist and the results of at least one published report (18), in which germ cell neoplasms were detected in only 26 of approximately 10,000 medaka (0.3%). The investigators (18) reported that the tumors were most likely associated with age rather than chemical exposure suggesting that the development of the neoplasms was most likely attributable to culturing techniques, genetic factors, or a virus, based on the tendency for multiple occurrences to cluster in the fish of the same cohort. This finding is perplexing as culturing practices for medaka have been consistently maintained with a

low background rate of occurrence similar to that reported (18). The age of the fish used in the present study (15.5 wpf) and the breeders used to produce the fish used in the study are controlled to eliminate age as a confounding factor. The genotype of the fish affected with the neoplasms matched the phenotype identified. Most importantly for the present study, the occurrence of the six germ cell neoplasms was distributed among control and high dose groups from both sexes, and thus their presence did not affect the interpretation of the study. Based on endpoints measured in the present modified FSTRA study and the decision criteria in the FSTRA test guideline (OCSPP 890.1350) (3) and OECD Guideline 229 (4), 2-EHHB appeared to interfere with the function of the endocrine system in medaka.

13. SAMPLE HANDLING AND CUSTODY

All samples received, generated during the course of testing, and submitted to EAG Laboratories (Columbia, MO and Easton, MD) 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 as described in protocol section 10.5 and preserved as described by EAG Laboratories were shipped to EAG Laboratories (Columbia, MO) 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.

14. RECORD MAINTENANCE AND ARCHIVAL

Test facility-related records (personnel training, equipment calibration and maintenance, storage temperature records, etc.) were retained at the Test Facility per facility SOP. 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 were transferred to compact discs (CDs) and verified as exact copies of the original. Copies of the electronic disc and the Final Study Report were 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 were maintained in the archive at FEL until shipped to the archive location below in accordance with facility SOP. In addition, all EPL-generated histology data records were shipped to the Sponsor for archiving. Original raw analytical data and original analytical reports from EAG Laboratories were sent to the Sponsor for archiving. The archive location was:

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

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16. 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 SOP. Surplus test substance was disposed of in accordance with those requirements provided in the facility SOPs.

17. STUDY PROTOCOL ADHERENCE

The study was performed in accordance with Study Protocol No. BATT01-2 (Appendix A). There were twelve amendments to and one deviation from the Study Protocol (Appendix A). The following protocol deviation was noted:

- "Analytical balance capable of measuring to 3 decimal places (mg)" and "Body weights and length– Whole body weight will be recorded at test termination and measured to the nearest 10 mg." An analytical balance (Ohaus E10640) with 0.1 mg sensitivity was used to weigh fish and gonads. Body weight was recorded to the nearest 0.1 mg rather than 10 mg. Since medaka are general smaller fish than fathead minnows, greater sensitivity in measurement was desired, and thus no impact.

18. REFERENCES

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TABLES

Table 1. Summary of Study BATT01-00404 Endpoint Results[1]

Endpoint	Female/Male NOEC (µg 2-EHHB/L)	Female/Male LOEC (µg 2-EHHB/L)
Clinical signs of disease and/or general toxicities	100/100	>100/>100
Survival	100/100	>100/>100
Body Weight	100/100	>100/>100
Standard Body Length	100/100	>100/>100
Liver Histopathology	3.6/10.9	10.9/33.0
Liver VTG mRNA	100/100[2]	>100/>100[3]
Anal Fin Papillae	NA[3]/10.9	NA[2]/33.0
Thyroid Histopathology	10.9/100	33.0/100
Gonad Weight	100/100	>100/>100
GSI	100/100	>100/>100
Gonad Histopathology	10.9/100	33/>100
Genotype/Phenotype Comparison[4]	100/100	>100/>100
Reproductive Fecundity	<0.212	3.6
Fertilization	3.60	10.9
Clinical signs of disease and/or general toxicities	100/100	>100/>100

[1] Nominal concentrations.

[2] Significant reduction was noted in male medaka in the 3.60 and 10.9 µg/L treatments.

[3] Not applicable, no papillae were found in female medaka.

[4] Based on inference, no statistical analyses performed.

Table 2. Experimental Conditions for In-Life Study

Parameter	Conditions
Test species	Reproductively active Japanese medaka (*Oryzias latipes*)
Pre-exposure phase duration	12 d; Data collected: fecundity (daily)
Exposure phase duration	21 d
Dilution water	Dechlorinated ($DeCl_2$) tap water
Test chemical; Supplier, Lot No., Expiration, Purity	2-ethylhexyl 4-hydroxybenzoate, CAS No. 5153-25-3, lot number H0506, expiration date and re-test date not provided, 98.0% (w/w) pure [w/w]
Test chamber size	10 L glass aquarium (tank)
Test media volume	10 L
Media flow rate	2.7 L / h
Rate of media exchange	ca. 6.5 tank volumes / d
Chemical Stock Preparation	Prepared using solid-liquid phase saturator columns
Test concentrations	100.0, 33.0, 10.9, 3.6, and 0 (control) µg/L
Replicates / treatment	4
Fish / replicate chamber Fish / treatment concentration Fish / test	10 (5 females and 5 male) 40 (20 females and 20 males) 200 (100 females and 100 males)
Feeding regime	Frozen brine shrimp, twice daily
Photo period	16 h light:8 h dark
Media temperature	25° ± 1°C
Light intensity	540 – 1000 lux
Aeration	Only if needed (DO <4.9 mg/L)
Dissolved oxygen (DO)	≥ 4.9 mg/L
pH	6.5 - 9.0
Hardness and alkalinity	Measure once / week
Biological endpoints	See Table 2
Analytical Chemistry Sample Schedule	Equilibration phase and 4 Events (d 0, d 21, and 2 events between d 0 and d 21)
Test validity criteria	D.O. ≥ 4.9 mg/L (≥ 60% saturation); mean temp 25° ± 1°C; 90% survival and successful egg production in controls (≥10 eggs/female/day; spawning occurs at least every 4 d in each control replicate; Fertility >80%. Mean measured (replicate mean) treatment exposure concentration CV < 20% for all replicates.

Table 3. Summary of Endpoints – Modified FSTRA for Medaka

Endpoints	Fish Examined/Replicate	Study Day	Description
Survival	All	Daily	Daily assessment of survival should be made to provide a basis for expression and interpretation of reproductive output.
Appearance of Adults	All	Daily	The external appearance of the adults should be assessed as part of the daily observations, and any unusual changes should be noted. These observations are especially important for assessing endocrine active agents that are androgenic.
Fecundity	All	Daily	Egg production should be determined daily, but only during the late morning.
Fertilization Success	All	Daily	If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate is easily achieved.
Secondary Sex Characteristics 10.	All	21	Japanese medaka: count number of papillary processes None for zebrafish.
Body Weights and Total Fish Lengths	All	21	Measured at necropsy.
Fin clip (genotyping):	All	21	Fin clip will be collected and preserved at -20°C for genotypic assessment. Genotyping in all fish
Liver Vitellogenin (VTG):	2 ♂ & 2♀	21	Liver will be excised and preserved in RNAlater™ frozen at -20°C.
Gonad Size (GSI)	2 ♂ & 2♀	21	Following liver excision, the fixed gonads should be removed and weighed (to the nearest 0.1 mg) to determine the GSI (GSI=100% x gonad wt/body wt).
Thyroid, Liver, Gonad Histopathology	Remaining 3 ♂ & 3♀	21	Whole animal sagittal sectioning will be performed and slides evaluated by a toxicologic pathologist with experience with small fish (EPL). Pathology evaluation using a tiered approach, depending on pathology findings in high concentration groups.

Table 4. Summary of Water Quality Characteristics in the Test System

Parameter	Nominal Concentration (µg/L)	Replicate	Minimum	Maximum	Measurement Interval
pH (s.u.)	0.0	A	7.3	7.7	2x weekly
		B	7.3	7.7	2x weekly
		C	7.3	7.7	2x weekly
		D	7.3	7.7	2x weekly
	3.6	A	7.3	7.9	2x weekly
		B	7.4	7.8	2x weekly
		C	7.3	7.8	2x weekly
		D	7.3	7.8	2x weekly
	10.9	A	7.3	7.7	2x weekly
		B	7.3	7.7	2x weekly
		C	7.3	7.8	2x weekly
		D	7.3	7.8	2x weekly
	33	A	7.4	7.8	2x weekly
		B	7.3	7.8	2x weekly
		C	7.3	7.8	2x weekly
		D	7.3	7.8	2x weekly
	100	A	7.4	7.8	2x weekly
		B	7.3	7.8	2x weekly
		C	7.3	7.8	2x weekly
		D	7.3	7.8	2x weekly
Dissolved oxygen (mg/L)	0.0	A	6.9	7.6	2x weekly
		B	6.6	7.6	2x weekly
		C	6.7	7.3	2x weekly
		D	6.9	7.5	2x weekly
	3.6	A	6.9	7.4	2x weekly
		B	6.8	7.4	2x weekly
		C	6.5	7.4	2x weekly
		D	6.9	7.6	2x weekly
	10.9	A	6.9	7.5	2x weekly
		B	6.8	7.6	2x weekly
		C	7.0	7.4	2x weekly
		D	7.0	7.4	2x weekly
	33	A	6.9	7.4	2x weekly
		B	6.8	7.5	2x weekly
		C	6.8	7.4	2x weekly
		D	6.7	7.4	2x weekly
	100	A	6.9	7.4	2x weekly
		B	6.7	7.5	2x weekly
		C	6.7	7.6	2x weekly
		D	6.8	7.6	2x weekly

Table 4. (continued)

Summary of Water Quality Characteristics in the Test System

Parameter	Nominal Concentration (µg/L)	Replicate	Minimum	Maximum	Measurement Interval
Temperature (ºC)	0.0	A	25.4	25.5	Daily
		B	25.4	25.6	Daily
		C	25.4	25.6	Daily
		D	25.3	25.6	Daily
	3.6	A	25.3	25.5	Daily
		B	25.4	25.5	Daily
		C	25.4	25.5	Daily
		D	25.3	25.6	Daily
	10.9	A	25.4	25.5	Daily
		B	25.4	25.5	Daily
		C	25.4	25.6	Daily
		D	25.3	25.5	Daily
	33	A	25.3	25.5	Daily
		B	25.3	25.6	Daily
		C	25.4	25.5	Daily
		D	25.4	25.5	Daily
	100	A	25.3	25.5	Daily
		B	25.4	25.5	Daily
		C	25.3	25.6	Daily
		D	25.4	25.5	Daily
Light Intensity[1] (lux)	0.0	A	660	972	2x weekly
		B	630	999	2x weekly
		C	697	984	2x weekly
		D	710	929	2x weekly
	3.6	A	596	967	2x weekly
		B	612	971	2x weekly
		C	748	980	2x weekly
		D	701	961	2x weekly
	10.9	A	794	948	2x weekly
		B	810	995	2x weekly
		C	763	911	2x weekly
		D	756	984	2x weekly
	33	A	620	980	2x weekly
		B	731	942	2x weekly
		C	720	935	2x weekly
		D	698	979	2x weekly
	100	A	644	987	2x weekly
		B	703	977	2x weekly
		C	786	952	2x weekly
		D	665	883	2x weekly

[1] Measured at water level.

Table 4 (*continued*)
Summary of Water Quality Characteristics in the Test System

Parameter	Nominal Concentration (µg/L)	Minimum	Maximum	Measurement Interval
Total Hardness (mg/L as CaCO3)	0.0	132	140	1x Weekly
	100	132	140	1x Weekly
Alkalinity (mg/L as CaCO3)	0.0	44	52	1x Weekly
	100	44	48	1x Weekly
Iodide (µg/L)	N/A	8.9	9.3	2x during study
Ammonia[1] (µg/L)	N/A	<0.06	<0.06	1x during study
Chlorine (µg/L)	N/A	<0.05	<0.05	1x during study

[1] Expressed as nitrogen.

Table 5. Summary of Treatment Concentrations in Modified FSTRA with 2-EHHB

Test Day	Nominal Concentration (µg/L)	Replicate	Measured Concentration[1] (µg/L)	CV[2] (%)
0	0.0	A	<MQL	---
		B	<MQL	
		C	<MQL	
		D	<MQL	
	3.6	A	4.38	12.0
		B	4.05	
		C	3.47	
		D	3.44	
	10.9	A	12.3	12.6
		B	11.4	
		C	10.9	
		D	14.4	
	33	A	42.8	9.1
		B	38.8	
		C	34.2	
		D	39.7	
	100	A	130	6.0
		B	114	
		C	128	
		D	120	
7	0.0	A	<MQL	---
		B	<MQL	
		C	<MQL	
		D	<MQL	
	3.6	A	2.66	7.5
		B	2.41	
		C	2.26	
		D	2.30	
	10.9	A	10.0	4.3
		B	9.32	
		C	9.26	
		D	9.09	
	33	A	29.5	6.8
		B	25.6	
		C	26.2	
		D	25.9	
	100	A	99.1	5.0
		B	92.7	
		C	92.4	
		D	87.9	

[1] Minimum Quantitation Level (MQL) = 0.212 µg/L

[2] Coefficient of variation = (Standard deviation / mean)100. Represents inter-replicate CV at each sampling point based on mean and standard deviation of each replicate (A-D) for each test concentration at each time points measured.

Table 5 *(cont.)* Summary of Treatment Concentrations in Modified FSTRA with 2-EHHB

Test Day	Nominal Concentration (µg/L)	Replicate	Measured Concentration[1] (µg/L)	CV[2] (%)
14	0.0	A	<MQL	---
		B	<MQL	
		C	<MQL	
		D	<MQL	
	3.6	A	3.36	7.0
		B	3.15	
		C	2.88	
		D	2.95	
	10.9	A	13.1	6.3
		B	11.6	
		C	11.4	
		D	12.2	
	33	A	38.5	8.0
		B	33.0	
		C	33.5	
		D	32.6	
	100	A	106	6.8
		B	95.6	
		C	93.1	
		D	91.4	
21	0.0	A	<MQL	---
		B	<MQL	
		C	<MQL	
		D	<MQL	
	3.6	A	2.87	7.3
		B	2.55	
		C	2.49	
		D	2.46	
	10.9	A	9.70	7.4
		B	8.17	
		C	8.61	
		D	8.61	
	33	A	43.9	13.9
		B	32.5	
		C	34.2	
		D	35.4	
	100	A	104	8.6
		B	93.9	
		C	115	
		D	110	

[1] Minimum Quantitation Level (MQL) = 0.212 µg/L

[2] Coefficient of variation = (Standard deviation / mean)100. Represents inter-replicate CV at each sampling point based on mean and standard deviation of each replicate (A-D) for each test concentration at each time points measured.

Table 6. Summary of Mean Measured Concentrations in in Modified FSTRA with 2-EHHB

Test Day	Nominal Concentration (µg/L)	Mean Measured Concentration (µg/L) [1]	CV[2] (%)
Mean Measured Concentrations	0.0	<MQL[3]	---
	3.6	2.98	9
	10.9	10.6	6
	33.0	34.1	9
	100	105	5

[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) = 0.212 µg/L.

Table 7. Effect of 2-EHHB Exposure on 21-Day Survival Rate[1]

		Female Survival (%)					Male Survival (%)					Overall Survival (%)				
Treatment (µg/L)	Replicate	N	Percent	Mean	SEM	CV (%)	N	Percent	Mean	SEM	CV (%)	N	Percent	Mean	SEM	CV (%)
0.0	A	5	100.0	100.0	0.0	0.0	5	100.0	100.0	0.0	0.0	10	100.0	100.0	0.0	0.0
	B	5	100.0				5	100.0				10	100.0			
	C	5	100.0				5	100.0				10	100.0			
	D	5	100.0				5	100.0				10	100.0			
3.6	A	5	100.0	100.0	0.0	0.0	5	100.0	100.0	0.0	0.0	10	100.0	100.0	0.0	0.0
	B	5	100.0				5	100.0				10	100.0			
	C	5	100.0				5	100.0				10	100.0			
	D	5	100.0				5	100.0				10	100.0			
10.9	A	5	100.0	100.0	0.0	0.0	5	100.0	100.0	0.0	0.0	10	100.0	100.0	0.0	0.0
	B	5	100.0				5	100.0				10	100.0			
	C	5	100.0				5	100.0				10	100.0			
	D	5	100.0				5	100.0				10	100.0			
33	A	5	100.0	100.0	0.0	0.0	5	100.0	100.0	0.0	0.0	10	100.0	100.0	0.0	0.0
	B	5	100.0				5	100.0				10	100.0			
	C	5	100.0				5	100.0				10	100.0			
	D	5	100.0				5	100.0				10	100.0			
100	A	5	100.0	100.0	0.0	0.0	5	100.0	100.0	0.0	0.0	10	100.0	100.0	0.0	0.0
	B	5	100.0				5	100.0				10	100.0			
	C	5	100.0				5	100.0				10	100.0			
	D	5	100.0				5	100.0				10	100.0			

[1] SEM=Standard error of the mean. CV(%)=Coefficient of variation = (standard deviation / mean) × 100. Mean, SEM, and CV (%) calculated from the percent survival estimate for each replicate within treatment (i.e., N=4). CV represents inter-replicate CV at each sampling point based on mean and standard deviation of each replicate (A-D) for each test concentration.

Table 8. Effect of 2-EHHB Exposure on Fecundity and Fertilization Success[1]

		Fecundity (Number of Eggs per Female per Day)					Fertilization Success (%)				
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	SEM	CV (%)	N	Replicate Mean	Mean	SEM	CV (%)
0.0	A	5	13.66	13.43	0.13	1.87	5	95.12	95.35	0.14	0.29
	B	5	13.56				5	95.51			
	C	5	13.40				5	95.66			
	D	5	13.09				5	95.12			
3.6	A	5	12.90	12.88[2]	0.11	1.74	5	95.13	95.08	0.05	0.11
	B	5	12.69				5	95.20			
	C	5	12.73				5	95.06			
	D	5	13.18				5	94.94			
10.9	A	5	11.91	12.25[3]	0.19	3.16	5	94.88	94.54[4]	0.12	0.25
	B	5	12.15				5	94.36			
	C	5	12.13				5	94.43			
	D	5	12.81				5	94.50			
33	A	5	6.88	6.29[5]	0.33	10.48	5	87.53	86.33[6]	0.49	1.12
	B	5	6.08				5	86.52			
	C	5	6.76				5	85.21			
	D	5	5.46				5	86.04			
100	A	5	4.25	3.96[7]	0.20	9.98	5	80.27	75.77[8]	1.62	4.26
	B	5	4.23				5	73.20			
	C	5	3.96				5	75.96			
	D	5	3.40				5	73.67			

[1] SEM=Standard error of the mean. CV(%)=Coefficient of variation = (standard deviation / mean) × 100. Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4). CV represents inter-replicate CV at each sampling point based on mean and standard deviation of each replicate (A-D) for each test concentration.

[2] Significantly less than control (Jonckheere-Terpstra test, p=0.0433).

[3] Significantly less than control (Jonckheere-Terpstra test, p=0.0021).

[4] Significantly less than control (Jonckheere-Terpstra test, p=0.0034).

[5] Significantly less than control (Jonckheere-Terpstra test, p<0.0001).

[6] Significantly less than control (Jonckheere-Terpstra test, p<0.0001).

[7] Significantly less than control (Jonckheere-Terpstra test, p<0.0001).

[8] Significantly less than control (Jonckheere-Terpstra test, p<0.0001).

Table 9. Effect of 2-EHHB Exposure on Papillary Processes Score by Sex[1]

		Female Papillary Processes Score					Male Papillary Processes Score				
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	SEM	CV (%)	N	Replicate Mean	Mean	SEM	CV (%)
0.0	A	5	0.00	0.00	0.00	.	5	79.2	83.0	1.3	3.3
	B	5	0.00				5	85.6			
	C	5	0.00				5	83.8			
	D	5	0.00				5	83.2			
3.6	A	5	0.00	0.00	0.00	.	5	91.4	82.2	3.9	9.4
	B	5	0.00				5	84.4			
	C	5	0.00				5	73.0			
	D	5	0.00				5	80.0			
10.9	A	5	0.00	0.00	0.00	.	5	87.2	79.5	3.3	8.3
	B	5	0.00				5	81.6			
	C	5	0.00				5	77.6			
	D	5	0.00				5	71.6			
33	A	5	0.00	0.00	0.00	.	5	64.2	71.1[2]	4.9	13.7
	B	5	0.00				5	81.6			
	C	5	0.00				5	61.6			
	D	5	0.00				5	77.0			
100	A	5	0.00	0.00	0.00	.	5	71.4	66.4[3]	4.4	13.2
	B	5	0.00				5	59.0			
	C	5	0.00				5	59.0			
	D	5	0.00				5	76.2			

[1] SEM=Standard error of the mean. CV(%)=Coefficient of variation = (standard deviation / mean) × 100. Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4). CV represents inter-replicate CV at each sampling point based on mean and standard deviation of each replicate (A-D) for each test concentration. All female papillary processes scores were 0.

[2] Significantly less than control (Jonckheere-Terpstra test, p=0.0394).

[3] Significantly less than control (Jonckheere-Terpstra test, p=0.0012).

Table 10. Effect of 2-EHHB Exposure on Liver Vitellogenin (VTG) mRNA Expression by Sex[1]

		Female VTG[Log 10(copies VTG/ng total RNA)]					Male VTG[Log 10(copies VTG/ng total RNA)]				
Treatment (µg/L)	**Replicate**	**N**	**Replicate Mean**	**Mean**	**SEM**	**CV (%)**	**N**	**Replicate Mean**	**Mean**	**SEM**	**CV (%)**
0.0	A	2	3.940	4.500	0.325	14.456	2	1.500	1.735	0.080	9.195
	B	2	3.985				2	1.800			
	C	2	5.275				2	1.785			
	D	2	4.800				2	1.855			
3.6	A	2	4.735	5.031	0.160	6.364	2	2.925	3.418[2]	0.266	15.595
	B	2	4.775				2	2.990			
	C	2	5.335				2	3.920			
	D	2	5.280				2	3.835			
10.9	A	2	4.895	4.976	0.137	5.512	2	3.585	3.150[3]	0.260	16.487
	B	2	4.860				2	3.485			
	C	2	5.380				2	2.440			
	D	2	4.770				2	3.090			
33	A	2	4.230	4.283	0.385	17.958	2	1.560	2.259	0.404	35.961
	B	2	5.160				2	2.345			
	C	2	3.295				2	3.370			
	D	2	4.445				2	1.760			
100	A	2	3.510	4.058	0.337	16.598	2	2.230	1.958	0.223	22.809
	B	2	3.530				2	1.290			
	C	2	4.915				2	2.145			
	D	2	4.275				2	2.165			

[1] SEM=Standard error of the mean. CV(%)=Coefficient of variation = (standard deviation / mean) × 100. Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4). CV represents inter-replicate CV at each sampling point based on mean and standard deviation of each replicate (A-D) for each test concentration.

[2] Significantly greater than control (Dunnett's test, p=0.0017).

[3] Significantly greater than control (Dunnett's test, p=0.0069).

Table 11. Effect of 2-EHHB Exposure on Gonad Weight by Sex[1]

		Female Gonad Weight (mg)					Male Gonad Weight (mg)				
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	SEM	CV (%)	N	Replicate Mean	Mean	SEM	CV (%)
0.0	A	2	22.2	23.9	3.5	29.2	2	2.0	2.9	0.6	42.3
	B	2	30.6				2	4.6			
	C	2	14.9				2	3.0			
	D	2	28.1				2	2.0			
3.6	A	2	24.9	32.1	4.3	26.7	2	2.3	3.4	0.5	31.8
	B	2	31.1				2	3.2			
	C	2	44.4				2	4.9			
	D	2	28.1				2	3.3			
10.9	A	2	20.4	25.1	3.3	26.5	2	2.1	3.2	0.4	26.9
	B	2	34.8				2	3.6			
	C	2	23.7				2	3.0			
	D	2	21.4				2	4.1			
33	A	2	30.0	28.5	2.4	16.7	2	2.4	2.6	0.4	31.2
	B	2	34.5				2	3.7			
	C	2	23.7				2	2.8			
	D	2	25.9				2	1.7			
100	A	2	19.9	19.5	3.0	30.2	2	2.7	3.0	0.2	15.2
	B	2	15.9				2	3.4			
	C	2	27.7				2	2.5			
	D	2	14.6				2	3.4			

[1] SEM=Standard error of the mean. CV(%)=Coefficient of variation = (standard deviation / mean) × 100. Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4). CV represents inter-replicate CV at each sampling point based on mean and standard deviation of each replicate (A-D) for each test concentration.

Table 12. Effect of 2-EHHB Exposure on Gonadosomatic Indices (GSI) by Sex[1]

		Female GSI (%)					Male GSI (%)				
Treatment (µg/L)	**Replicate**	**N**	**Replicate Mean**	**Mean**	**SEM**	**CV (%)**	**N**	**Replicate Mean**	**Mean**	**SEM**	**CV (%)**
0.0	A	2	6.37	7.05	0.99	28.22	2	0.55	0.82	0.17	42.28
	B	2	9.33				2	1.31			
	C	2	4.68				2	0.79			
	D	2	7.81				2	0.62			
3.6	A	2	7.79	9.06	0.93	20.58	2	0.63	0.89	0.13	29.31
	B	2	8.96				2	0.91			
	C	2	11.73				2	1.24			
	D	2	7.77				2	0.79			
10.9	A	2	6.65	7.67	0.96	24.95	2	0.60	0.91	0.11	25.20
	B	2	10.53				2	1.15			
	C	2	6.96				2	0.94			
	D	2	6.55				2	0.95			
33	A	2	8.84	8.55	0.57	13.38	2	0.61	0.78	0.14	35.51
	B	2	10.03				2	1.14			
	C	2	7.45				2	0.86			
	D	2	7.89				2	0.53			
100	A	2	6.31	6.12	0.89	29.21	2	0.84	0.93	0.07	14.48
	B	2	5.15				2	1.04			
	C	2	8.54				2	0.80			
	D	2	4.47				2	1.06			

[1] SEM=Standard error of the mean. CV(%)=Coefficient of variation = (standard deviation / mean) × 100. Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4). CV represents inter-replicate CV at each sampling point based on mean and standard deviation of each replicate (A-D) for each test concentration.

Table 13. Effect of 2-EHHB Exposure on Body Weight by Sex[1]

		Female Body Weight (mg)					Male Body Weight (mg)				
Treatment (µg/L)	**Replicate**	**N**	**Replicate Mean**	**Mean**	**SEM**	**CV (%)**	**N**	**Replicate Mean**	**Mean**	**SEM**	**CV (%)**
0.0	A	5	388.3	365.9	10.8	5.9	5	370.2	389.0	10.9	5.6
	B	5	371.4				5	388.1			
	C	5	367.5				5	425.4			
	D	5	336.3				5	437.1			
3.6	A	5	334.1	379.0	16.3	8.6	5	435.5	435.5	16.0	7.4
	B	5	376.9				5	390.6			
	C	5	396.3				5	368.5			
	D	5	408.7				5	362.8			
10.9	A	5	363.5	371.0	3.5	1.9	5	416.4	416.4	24.1	11.6
	B	5	377.4				5	376.7			
	C	5	376.5				5	409.7			
	D	5	366.7				5	381.1			
33	A	5	371.7	422.0	25.0	11.9	5	402.7	402.7	12.4	6.2
	B	5	453.1				5	374.7			
	C	5	387.8				5	414.7			
	D	5	475.3				5	424.6			
100	A	5	347.1	359.0	9.0	5.0	5	357.9	357.9	7.0	3.9
	B	5	345.0				5	387.4			
	C	5	359.6				5	380.3			
	D	5	384.2				5	369.1			

[1] SEM=Standard error of the mean. CV(%)=Coefficient of variation = (standard deviation / mean) × 100. Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4). CV represents inter-replicate CV at each sampling point based on mean and standard deviation of each replicate (A-D) for each test concentration.

Table 14. Effect of 2-EHHB Exposure on Body Length by Sex[1]

		Female Body Length (mm)					Male Body Length (mm)				
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	SEM	CV (%)	N	Replicate Mean	Mean	SEM	CV (%)
0.0	A	5	26.0	25.8	0.2	1.6	5	26.7	26.7	0.3	2.4
	B	5	25.9				5	27.6			
	C	5	26.1				5	26.3			
	D	5	25.2				5	26.1			
3.6	A	5	24.8	26.2	0.7	5.5	5	27.6	28.1	0.4	2.6
	B	5	25.1				5	27.5			
	C	5	27.3				5	28.0			
	D	5	27.5				5	29.1			
10.9	A	5	26.6	26.4	0.1	0.8	5	28.8	28.0	0.4	3.1
	B	5	26.3				5	27.6			
	C	5	26.4				5	26.9			
	D	5	26.1				5	28.6			
33	A	5	27.2	27.4	0.8	5.9	5	28.3	27.3	0.5	3.6
	B	5	28.0				5	27.5			
	C	5	25.3				5	26.0			
	D	5	29.1				5	27.7			
100	A	5	26.8	26.5	0.3	2.5	5	27.2	26.4	0.5	3.5
	B	5	25.5				5	25.1			
	C	5	26.8				5	27.0			
	D	5	26.8				5	26.3			

[1] SEM=Standard error of the mean. CV(%)=Coefficient of variation = (standard deviation / mean) × 100. Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4). CV represents inter-replicate CV at each sampling point based on mean and standard deviation of each replicate (A-D) for each test concentration.

Table 15. External Phenotypic, Histological, and Genetic Sex in Medaka at Study Termination

Treatment Group (µg/L)	External Phenotypic Sex[1]		Histological Sex[2]		Genetic Sex[3]	
	Female (No.)	Male (No.)	Female (No.)	Male (No.)	Female (No.)	Male (No.)
0.0	12	12	12	12	12	12
3.6	12	12	NE[4]	NE	12	12
10.9	12	12	12	NE	12	12
33.0	12	12	12	NE	12	12
100	12	12	12	12	12	12

[1] Determined by in-life laboratory (FEL) during pre-exposure phase of study.
[2] Determined by histopathologist (EPL, Sterling VA).
[3] Determined from fin clip and analysis of *dmy* gene expression by in-life laboratory (FEL).
[4] NE=Not evaluated, no read-down.

Table 16. Effect of 2-EHHB Exposure on Thyroid Histopathological Findings

Treatment Group (µg/L)	Males					Females				
	0.0	3.6	10.9	33.0	100.0	0.0	3.6	10.9	33.0	100.0
Number Examined[1]	12	12	12	12	12	12	12	12	12	12
Follicular Cell Hyperplasia	12	NE[2]	12	12	12	0	NE	NE	NE	1
minimal	2	NE	5	4	2	-	NE	NE	NE	1
mild	10	NE	7	8	10	-	NE	NE	NE	-
Follicular Cell Hypertrophy	12	NE	12	11	11	0	NE	NE	NE	0
minimal	10	NE	11	10	7	-	NE	NE	NE	-
mild	2	NE	1	1	4	-	NE	NE	NE	-
Hyaline Degeneration	9	NE	8	5	5	2	NE	NE	NE	0
minimal	7	NE	6	2	5	1	NE	NE	NE	-
mild	2	NE	2	3	-	1	NE	NE	NE	-

[1] Reduced presence of minimal hyaline degeneration (follicular cells) was observed in female medaka from the 33.0 and 100 µg/L 2-EHHB treatments relative to control (RSCABS, p=0.0374 and 0.0263, respectively). However, the pathologist's comments regarding this finding were

[2] Not evaluated, no read-down.

Table 17. Effect of 2-EHHB Exposure on Liver Histopathological Findings

Treatment Group (µg/L)	Males					Females				
	0.0	3.6	10.9	33.0	100.0	0.0	3.6	10.9	33.0	100.0
Number Examined[1]	12	12	12	12	12	12	12	12	12	12
Basophilia	0	NE[2]	NE	NE	1	12	NE	12	6	11
minimal	-	NE	NE	NE	1	-	NE	1	2[3]	3[4]
mild	-	NE	NE	NE	-	2	NE	3	1[5]	-[6]
moderate	-	NE	NE	NE	-	10	NE	8	3[7]	8[8]
Focus of Cellular Alteration[9]	0	NE	NE	NE	0	0	NE	0	1	2
minimal	-	NE	NE	NE	-	-	NE	-	1	-[10]
moderate	-	NE	NE	NE	-	-	NE	-	-	2[11]
Vacuolation	8	NE	NE	NE	7	10	NE	11	11	11
minimal	8	NE	NE	NE	6	9	NE	9	3	3
mild	-	NE	NE	NE	1	-	NE	2	2[12]	4[13]
moderate	-	NE	NE	NE	-	1	NE	-	6[14]	4[15]

[1]Decreased occurrence of mild pigmented macrophage aggregates in female medaka exposed to 100 µg/L 2-EHHB (RSCABS, p=0.0307) compared to control was observed. Increased presence of mild cystic degeneration in male medaka exposed to 100 µg/L 2-EHHB (RSCABS, p=0.0315), but decreased presence of mild cystic degeneration in male medaka exposed to 33.0 and 10.9 µg/L 2-EHHB (RSCABS, -p=0.0188 and p=0.0343, respectively) compared to control were observed. A reduced occurrence of minimal eosinophilic cytoplasmic inclusions was noted in male medaka from the 10.9, 33.0, and 100 µg/L 2-EHHB treatments compared to control (RSCABS, p=0.0142, 0.0047, and 0.0273, respectively). Increased minimal (RSCABS, p=0.0273), mild (RSCABS, p=0.0025), and moderate (RSCABS, p=0.0263) granulomatous inflammation in male medaka from the 100 µg/L 2-EHHB treatment relative to control was noted (RSCABS, p=0.0273).

[2] Not evaluated, no read-down.

[3] Significantly less than control (RSCABS, p=0.0005).

[4] Significantly less than control (RSCABS, p=0.0438).

[5] Significantly less than control (RSCABS, p=0.0001).

[6] Significantly less than control (RSCABS, p=0.0008).

[7] Significantly less than control (RSCABS, p=0.0020).

[8] Significantly less than control (RSCABS, p=0.0195).

[9] Focus of cellular alteration of mild severity was significantly greater than control (RSCABS, p=0.0263).

[10] Significantly greater than control (RSCABS, p=0.0307).

[11] Significantly greater than control (RSCABS, p=0.0263).

[12] Significantly greater than control (RSCABS, p=0.0148).

[13] Significantly greater than control (RSCABS, p=0.0028).

[14] Significantly greater than control (RSCABS, p=0.0278).

[15] Significantly greater than control (RSCABS, p=0.0228).

Table 18. Effect of 2-EHHB Exposure on Ovary Histopathological Findings

Treatment Group (µg/L)	0.0	3.6	10.9	33.0	100.0
Number Examined[1]	12	12	12	12	12
Oocyte Atresia	5	NE[2]	5	9	8
minimal	5	NE	3	7	3
mild	-	NE	2	-	4[3]
moderate	-	NE	-	1	1
severe	-	NE	-	1	-
Ovary Stage		NE			
Stage 1	1	NE	4	5	3
Stage 2	4	NE	2	3	6
Stage 3	5	NE	6	1	9
Stage 4	2	NE	-	1	12
Mean Stage Score	2.7	NE	2.2	1.8	2.5
Unable to Stage	-	NE	-	2	-

Table 19. Prevalence of Gonadal Germ Cell Tumors

Sex	Males					Females				
2-EHHB Treatment Group (µg/L)	0.0	3.6	10.9	33.0	100.0	0.0	3.6	10.9	33.0	100.0
Number Examined	12	12	12	12	12	12	12	12	12	12
Dysgerminoma, Benign	0	NE	NE	NE	1	0	NE	0	0	0
Seminoma, Benign	1	NE	NE	NE	2	0	NE	0	0	0
Seminoma, Malignant	0	NE	NE	NE	0	1	NE	0	0	1

[1] Decreased occurrence of minimal and mild egg debris in oviduct in female medaka exposed to 33.0 and 100 µg/L 2-EHHB (RSCABS, p=0.0183) compared to control was observed (RSCABS, p=0.0374 and 0.0263, respectively [for both minimal and mild].

[2] Not evaluated, no read-down.

[3] Significantly greater than control (RSCABS, p=0.0075).

Table 20. 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	√
Control Spawn	≥10 eggs/female/day, with spawning occurring at least every 4 days	√
Control fertilization	>80%	√
DO	≥ 4.9 mg/L	√
pH	6.5 – 8.5	√
Water temperature	25 ± 1°C	√
Test concentrations without overt toxicity (excluding control)	≥ 2	√

[1] Based on modified Fish Short-Term Reproduction Assay (FSTRA) under EPA Test Guidelines OPPTS 890.1350 (3) and OECD Guideline 229 (4).
[2] CVs of the intra-replicate means of the measured test concentrations.

Appendix A. Protocol, Protocol Amendments, and Protocol Deviations

FEL Protocol No.:	**BATT01-10**
GLP Study Title:	**Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka**
Test Guideline:	**U.S. EPA, OPPTS 890.1350 (October 2009), modified and OECD guideline 229 (October 2012), modified**
FEL Study No.:	**BATT01-00404**
Test System:	***Oryzias latipes***
Test Substance:	**2-Ethylhexyl 4-Hydroxybenzoate**
USEPA Task Order:	**TO 17**
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)	**EAG Laboratories 7200 East ABC Lane Columbia, MO 65202**
PI Support Site: (Vitellogenin)	**EAG Laboratories 8598 Commerce Drive Easton, MD 21601**
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.		1/10/2017
SPONSOR REPRESENTATIVE:[1] Vincent J. Brown, Ph.D.		10 JAN 2017

[1]Study Monitor.

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2. GOOD LABORATORY PRACTICE (GLP)

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

- United States Environmental Protection Agency, Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), Good Laboratory Practice Standards, Title 40 Code of Federal Regulations Part 160 (effective October 16, 1989) (2) 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, BATT01-00404, 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 will be conducted following standard validated methods, this exception will not be expected to impact 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 Quality Assurance Unit (QAU) is present at the test facility (FEL), each Principal Investigator (PI) support facility (EAG 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 a modified Fish Short-Term Reproduction Assay (FSTRA) under EPA Test Guidelines OPPTS 890.1350 (3) and OECD Guideline 229 (4) using 2-ethylhexyl 4-hydroxybenzoate [2-EHHB] (test substance) as directed by USEPA Task Order (TO) 17 under USEPA/Battelle Memorial Institute contract EP-W-11-063. The modification will utilize the Japanese medaka as the test system. This study will be conducted in accordance with the FEL Quality Assurance Management Plan (QAMP) (5), relevant facility standard operating procedures (SOPs), and the following Study Protocol No. BATT01-10, prepared for FEL Study No. BATT01-00404.

5. OBJECTIVE

The primary objective of the study will be to evaluate the potential reproductive toxicity of 2-EHHB to Japanese medaka in accordance with the guidelines established in OPPTS 890.1350 Fish Short-Term Reproduction Assay (3) and OECD Guideline 229 (4) with modification to use medaka *in lieu* of fathead minnow. The Fish Short-Term Reproduction Assay measures a suite of histological and biochemical endpoints that reflect effects associated with disturbance to the hypothalamus-pituitary-gonadal (HPG) endocrine axis (6,7). The assay is initiated with sexually mature male and female fathead minnows traditionally, but will use medaka as a modification in the present study (00404). During a 21-d in-life exposure phase, survival, reproductive behavior, secondary sexual characteristics, fecundity, and fertility are monitored. At the conclusion of the in-life exposure phase, measurements are made that are indicative of the status of the reproductive endocrine system, including secondary sex characteristics; genetic sex; gonado-somatic index (GSI); and liver vitellogenin (VTG) mRNA gonad, liver, and thyroid histopathology. These endpoints are generally inclusive of the traditional FSTRA endpoints, but will also contain additional endpoints relevant to medaka.

6. TESTING FACILITY

The in-life portion of the study, to include the pre-exposure and exposure phases of the modified FSTRA, 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 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.

Vitellogenin mRNA analyses in liver will be performed at EAG Laboratories, Inc., Biochemistry Department, 8598 Commerce Drive, Easton, MD 21601. Mr. Matthew Rhodes, PI of the planned analyses, will serve as study contact for EAG Laboratories and may be reached at 410.822.8600 (x244) or mrhodes@eag.com.

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, ext. 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 fish 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 890.1350 (3).

9. STUDY SCHEDULE

The experimental study is proposed to start in January 2017 after Sponsor approval of the Final Study Protocol. The actual in-life test duration will include a range-finding study, a 7 to 14-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.

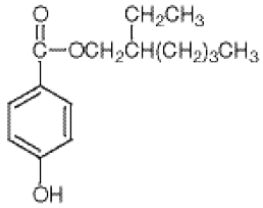
10. EXPERIMENTAL DESIGN

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-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 will be used to maximize 2-EHHB concentrations in water and will be used to prepare a master stock solution that will ultimately be used to prepare each test concentration. 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 (3,4). 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) 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.

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 study day (SD) 0 and 21 (in-life conclusion) 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. The culture water at FEL was analyzed most recently on November 15, 2016 and contained 6.6 (±0.06) µg/L I-. The next scheduled facility water analysis for polyaromatic hydrocarbons (PAHs), pesticides, and metals is scheduled for January or February, 2017. 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 BATT01-00404. Any departures from recommended values will be promptly brought to the attention of the Study Sponsor/Monitor.

10.3. Test System

The test species will be medaka (*Oryzias latipes*) (8). Medaka is a well-studied laboratory animal that exhibits ease of use in the laboratory, rapid development, and simplicity of

observation during development and reproduction. Medaka is readily induced to breed in captivity, and published methods exist for its culture, as well as short-term lethality, early life-stage and full life-cycle tests.

10.4. EXPOSURE SYSTEM

The route of 2-EHHB exposure will be abiotic via culture media. A flow-through diluter system (Benoit Mini-Diluter; ECT, Superior, WI) will be used in the performance of the Fish Short-Term Reproduction Assay. 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 10 L glass aquaria (with approximate measurements of 40 x 20 x 20 cm) equipped with standpipes that result in an actual tank volume of 10.0 L and minimum water depth of 12 to 15 cm. The system will be capable of supporting 4 exposure concentrations and a control, with up to 4 replicates per treatment. The flow rate to each tank will be 2.7 L/hr (6.5 volume exchanges/d). Fluorescent lighting will be used to provide a photoperiod of 16 hr light and 8 hr dark (2-hour light intensity transition period) at an intensity that ranges from 540 to 1,000 lux (lumens/m^2) at the water surface. Water temperature will be maintained at 25° ± 1°C, pH maintained between 6.5 to 9.0, and the dissolved oxygen (DO) concentration ≥ 4.9 mg/L (> 60% of the air saturation) in each test tank. This design is intended to randomize out the effects associated with the local environment (i.e., light and water) and possible trends associated with the diluter during testing. All fish will be impartially assigned to tanks before pre-exposure. After spawning is established in the pre-exposure phase, tanks with acceptable spawning medaka will be randomly assigned to treatments within a block in the diluter system to start the 21-d exposure phase.

Temperature will be measured daily; and pH, DO, and light intensity (lux) will be measured two times per week to monitor diluter system performance (Table 1). Total hardness and alkalinity will be measured in the control and one replicate of the highest concentration once per week. I- will be measured in the dilution water on SD 0 and 21 (in-life conclusion).

10.5. TEST SUBSTANCE ANALYSES - EAG LABORATORIES, COLUMBIA, MO TEST SITE

Test substance analysis of the diluter test solutions and stock solutions will be performed by EAG 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 EAG Laboratories and in accordance with method validation performed at EAG Laboratories previously for studies BATT01-00388 (AMA) and BATT01-00390 (LAGDA) (9) attached. 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:

- Glass aquaria;
- Aquarium heaters (adjustable to 25°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;
- Glassware (19-L carboy, beakers, volumetric flasks, Erlenmeyer flasks, graduated cylinders, etc.);
- Adjustable pipetters (10 to 5,000 µL) or assorted pipettes of equivalent sizes;
- Well plates;
- Well plate densitomer;
- ELISA kits (VTG); and
- Water baths

10.7. TEST ANIMAL SELECTION

All fish will be maintained on a 16-h light:8 h dark photoperiod. To maximize medaka reproduction within the modified FSTRA, the test aquaria will be visually isolated from the general laboratory environment by shielded sliding glass doors on the diluter system. A record of observations and any prophylactic and therapeutic disease treatments during the culture period preceding the test will be maintained. The exposure phase will be initiated with sexually dimorphic adult fish from a laboratory supply of reproductively mature animals cultured at 25 ± 1°C. The fish will be actively spawning. For the whole group of fish used in the test, the range in individual weights by sex at the start of the test will be maintained within ± 20% of the arithmetic mean weight of the same sex. A sub-sample of fish will be weighed before the in-life test is initiated to estimate the mean weight. The fish selected will be the same age within the range of 16±2 weeks post fertilization (wpf) [0.35 g ± 20%]. Test fish will be selected from a single laboratory stock which has been acclimated for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test. Test fish will be obtained from an in-house culture, as shipping of adult fish is stressful and will interfere with reliable spawning. Mortalities in the culture fish will be recorded and the following criteria applied: 1) Mortalities of greater than 10% of culture population in seven days preceding transfer to the test system: reject the entire batch; 2) Mortalities of between 5% and 10% of population: acclimation for seven additional days; if more than 5% mortality during second seven days, reject the entire batch; and, 3) Mortalities of less than 5% of population in seven days preceding transfer: accept the batch. Fish will not receive treatment for disease in the two-week acclimation period preceding the test, during pre-exposure, or during the exposure period. Moribund fish or fish with clinical signs of disease will not be used.

10.8. TEST METHOD

During a 21-day chemical exposure, survival, reproductive behavior, fecundity and fertilization success will be monitored daily. At termination of the assay, measurements will be made of several endpoints reflective of the status of the reproductive endocrine system. This will

be established during a "pre-exposure" period of 7-14 days. The pre-exposure observation period will be used to monitor reproductive performance as described for the chemical exposure period. The pre-exposure observations will occur in the same system/tanks as will be utilized for the chemical test. An overview of the test and relevant test conditions are provided in Table 1.

10.9. 21-Day Assay Initiation and Conduct

10.9.1. Selection and Weighing of Test Fish

Variation in weight of the fish at the initiation of the pre-exposure phase of the study will be controlled using the following process. For the whole batch of fish used in the test, the range in individual weights by sex at the initiation of the pre-exposure phase will be kept, if possible, within ± 20% of the arithmetic mean weight of the same sex. A sub-sample of fish will be weighed prior to the initiation of the pre-exposure phase in order to estimate the mean weight.

10.9.2. Conditions of Exposure

The pre-exposure phase a minimum of 7 days and a maximum of 14 days. The in-life exposure duration will be 21 days.

10.9.3. Feeding

The fish will be fed at least twice per day with brine shrimp *ad libidum* to promote active reproduction and maintain body condition. Food will be withheld from the fish for 12 hours prior to the day of tissue sampling to aid in histology processing of small fish. Uneaten food and fecal material will be removed from the test vessels at least twice weekly (e.g., by carefully cleaning the bottom of each tank using suction).

10.9.4. Pre-exposure

The pre-exposure phase will last 7-14 days. The assay will use fish that are approximately 16±2 wpf, previously maintained in communal culture tanks. Five sexually mature females and five male medaka will be randomly assigned to each replicate exposure chamber at each treatment concentration. Additional exposure chambers will be set up for pre-exposure to account for a lack of spawning in some chambers and/or mortality during the pre-exposure phase. Any specimens whose sex cannot be identified will be excluded from the assay. The pre-exposure phase of the assay will be conducted under conditions (temperature, photo period, feeding, etc.) identical to those used during the chemical exposure. The animals will be fed brine shrimp twice daily *ad libidum*. One 4 in. breeding mop head constructed from inert acrylic yarn will be placed in each tank to collect eggs. During the egg collection phases, eggs will be cultured directly on the mop head in a separate brood chamber. Fecundity data will be collected daily. For each assay, successful pre-exposure (suitability for testing) is established when regular spawning occurs in each replicate test chamber at least two times in the immediately preceding 7 days.

10.9.5. Chemical exposure

After successful spawning is verified during pre-exposure as per the requirements of the assay, the chemical exposure will be initiated and continued for 21 days. Five female and five male fish per replicate tank during exposure phases will be used. One 4 in. breeding mop head constructed from inert acrylic yarn will be placed in each tank as an artificial substrate to collect eggs. During the egg collection phases, eggs will be cultured directly on the mop heads in a separate brood chamber. The assays will be conducted at four chemical concentrations, as well as a dilution water control, with four experimental units (replicates) per treatment. The replicate tanks will be assigned to treatment levels according to randomized complete block design, with the four highest-spawning replicates assigned (one each) to one treatment level or control first, followed by the assignment of the next four highest-spawning replicates by distribution to the four test groups, until all groups are established. The test chemical will be delivered to the exposure chambers using a continuous-flow diluter, or other appropriate delivery system. The exposure phase will be conducted for 21 days, during which time the appearance, behavior, and reproductive fecundity of the fish will be assessed daily. At termination of the exposure period, the endpoints described in Table 2 will be recorded.

All exposure condition parameters will be analyzed at pre-exposure phase d 0, in-life exposure phase d 0, and in-life test termination. Temperature and flow rates will be measured daily in all tanks; DO, pH, and light intensity will be measured two times per week in all tanks; and hardness and alkalinity in the control and one replicate of the high test concentration will be monitored once per week to assess diluter system performance. Aeration for all test vessels should be started immediately if DO reaches 4.9 mg/L. The target temperature for the pre-exposure and exposure phases will be 25° ± 1°C. If aquaria temperatures reach 24°C, submersible heaters will be placed in the water bath.

Table 1. Experimental Conditions for In-Life Study

Parameter	Conditions
Test species	Reproductively active Japanese medaka (*Oryzias latipes*)
Pre-exposure phase duration	7- 14 d; Data collected: fecundity (daily)
Exposure phase duration	21 d
Dilution water	Dechlorinated ($DeCl_2$) tap water
Test chemical; Supplier, Lot No., Expiration, Purity	2-ethylhexyl hydroxybenzoate, CAS No. 5153-25-3, lot number H0506, expiration date and re-test date not provided, 98.0% (w/w) pure [w/w]
Test chamber size	10 L glass aquarium (tank)
Test media volume	10 L
Media flow rate	2.7 L / h
Rate of media exchange	ca. 6.5 tank volumes / d
Chemical Stock Concentration	ca. 6 mg/L prepared using solid-liquid phase saturator columns
Test concentrations	100.0, 33.0, 10.9, 3.6, and 0 (control) µg/L
Replicates / treatment	4
Fish / replicate chamber Fish / treatment concentration Fish / test	10 (5 females and 5 male) 40 (20 females and 20 males) 200 (100 females and 100 males)
Feeding regime	Frozen brine shrimp, twice daily
Photo period	16 h light:8 h dark
Media temperature	25° ± 1°C
Light intensity	540 – 1000 lux
Aeration	Only if needed (DO <4.9 mg/L)
Dissolved oxygen (DO)	≥ 4.9 mg/L
pH	6.5 - 9.0
Hardness and alkalinity	Measure once / week
Biological endpoints	See Table 2
Analytical Chemistry Sample Schedule	Equilibration phase and 4 Events (d 0, d 21, and 2 events between d 0 and d 21)
Test validity criteria	D.O. ≥ 4.9 mg/L (≥ 60% saturation); mean temp 25° ± 1°C; 90% survival and successful egg production in controls; spawning occurs at least every 4 d in each control replicate; Fertility > 95%; fertility > 95%. Measured treatment exposure concentration CV < 20% for all replicates.

10.10. Observations and Measurements

10.10.1. Endpoints

10.10.1.1. General

Several endpoints will be assessed over the course of and/or at conclusion of the assay. A summary of measurement endpoints and associated study criteria is provided in Table 2. At termination, body weight, and digital photographs will be taken for standard length measurement and examination of anal papillary processes of all 200 fish. Prior to processing, fish will be anesthetized using 200 mg/L buffered MS-222 (pH 7). Subsequently, gonads will be excised for GSI measurement and liver collected for VTG mRNA analyses from 2 male and 2 female fish per replicate (80 medaka). Prior to preparing the remaining 120 medaka for histopathology, caudal fin will be clipped and stored at -20°C for possible genotyping will follow a tiered approach described below in subsection 10.12. These remaining 3 male and 3 female medaka (120 medaka) will be fixed in modified Davidson's solution and preserved in 10% neutral buffered formalin (NBF) as whole intact specimens for thyroid, liver and gonad histopathology. Histopathology for three target tissues will follow a tiered approach described below in subsection 10.11.1.

10.10.1.2. Endpoints Measured in All Study Fish

- **Survival** – Daily assessment of survival will be made to provide a basis for expression and interpretation of reproductive output, that is, number of eggs/female/day. Fish will be examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) noted. Any mortality will be recorded and the dead fish removed as soon as possible.
- **Sampling of fish** – At the conclusion of the exposure, the fish will be anesthetized by transfer to an oxygenated solution of tricaine methanesulfonate (MS-222) (100 mg/L buffered with 200 mg sodium bicarbonate [$NaHCO_3$]/L) for sampling.
- **Body weights and length**– Whole body weight will be recorded at test termination and measured to the nearest 10 mg. Standard length will be measured from digitized digital photographs to the nearest 0.1 mm.
- **Behavior of adults** – Abnormal behavior (relative to controls) such as hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, or feeding abstinence will be noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, will also be noted.
- **Fecundity** – Egg production will be determined daily. The spawning substrates will be removed from the tanks to enumerate any eggs that are present. If no eggs are present, the substrate will be left in the tank; new substrates should be added to replace any that are removed. Fecundity will be expressed on the basis of surviving females per reproductive (test) day per replicate. The cumulative eggs laid over the in-life exposure duration will also be determined.
- **Fertilization success** – After the spawning substrate has been removed from the tank, the embryos will be carefully rolled off with a gentle circular motion of an index finger and

visually inspected under appropriate magnification. If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate (number embryos/number of eggs x 100%) will be easily achieved. Infertile eggs are opaque or clear with a white dot where the yolk has precipitated. Viable embryos remain clear for 36 to 48 hours until reaching the eyed stage.

- **Appearance of adults and observation of secondary sex characteristics** – Secondary sexual characteristics are under endocrine control; therefore, observations of physical appearance of the fish should be made over the course of the test and at conclusion of the study. Because some aspects of appearance (primarily color) can change quickly with handling, it is important that qualitative observations be made prior to removal of animals from the test system. The number of anal fin papillae will be counted as an indication of male phenotype per OECD guide 229, Annex 5B (4). Female phenotypes will be based on anal fin anatomy.

10.10.1.3. Endpoints Measured in Two Male and Two Female Fish per Replicate

- **VTG** – The measurement of VTG mRNA in liver will be performed using PCR (8).
- **Gonad size (GSI)** - The fixed gonads will be removed and weighed (to the nearest 0.1 mg) to determine the GSI as described in section 10.10.2.
- **Blood sampling –** No blood sampling will be performed in medaka.

10.10.1.4. Endpoints Measured in Remaining Three Male and Three Female Fish per Replicate

- **Genotypic Sex** – During the necropsy, a fin clip will be collected from each fish, placed in a labeled vial and frozen at -20°C until analyzed.
- **Gonad, liver and thyroid histopathology** – Immediately after anesthetization of an individual fish, length and weight will be measured. Whole carcasses will then be fixed in modified Davidson's solution and preserved in 10% (v/v) neutral buffered formalin (NBF).

10.10.2. Liver Collection and Determination of GSI – In-Life Laboratory (FEL)

VTG mRNA will be analyzed in livers from the 2 male and 2 female fish per replicate that are not selected for histopathology (8). The livers of sacrificed fish will be removed and immediately placed into RNAlater at room temperature. When all samples have been collected, they will be placed in a refrigerator at 1 to 9°C overnight and then moved to the lab freezer and stored at -20 ± 10°C until analyzed.

Using micro-dissection scissors, the spermatic ducts or oviducts will be severed proximal to the genital pore. Micro-dissection forceps will then be applied to the spermatic ducts/oviducts. Using gentle traction, the gonads will be dissected out of the abdominal cavity in a caudal to cranial direction, severing the mesorchial/mesovarial attachments as needed using the micro-dissection scissors. The left and right gonads may be excised individually or they may be

excised simultaneously and subsequently divided at their caudal attachment. The gonads will be removed and weighed (fixed weight to the nearest 0.1 mg) to determine the GSI (GSI=100% x gonad wt/body wt).

10.10.3. Tissue Fixation/Preservation for Histopathology – In-Life Laboratory (FEL)

Fish will be fixed in modified Davidson's fixative for 48-96 hours. After this time, each carcass will be rinsed thoroughly in dechlorinated culture water, and then placed in 10% neutral buffered formalin (NBF) for storage. The volume of fixative in the container will be at least 20 times the approximated volume of the tissues. The fixative container will be gently agitated for 5 seconds to dislodge air bubbles from the cassette. Fixed tissue samples will be sent to EPL (Sterling, VA) for histological examination.

10.11. Gonad, Liver and 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 FSTRA and MEOGRT (3,4,8,10). Following the conclusion of exposure (SD 21), FEL will send preserved intact whole carcass specimens to EPL via overnight courier for histopathological processing and analyses.

10.11.1. Histopathological Procedure

Routine histological procedures will be used to assess the condition of gonads, liver, and thyroid from the fish and will be performed by a toxicological pathologist with experience with small fishes. Specifically, preserved whole intact carcasses will be sent to EPL (Sterling, VA) for histological examination. The following step-down process will be used:

- Preserved, intact animals (carcasses) are shipped from the in-life laboratory to the histopathology laboratory (120 fish per species)
- All 120 histopathology animals are processed for sagittal sectioning and slides are prepared. The purpose is to enable the pathologist to read all three target tissues (gonad, liver, thyroid) on one slide if possible.
- In the base effort, the pathologist reads the slides and evaluates the animals from the high concentration and control groups (48 fish per species, Part A).
- If potential treatment-related effects are observed in these High or Control animals, then with concurrence of Study Director, the pathologist reads the slides from the Mid-High concentration group (24 fish per species, Part B.1)
- If potential treatment-related effects are observed in these Mid-High animals, then with concurrence of Study Director, the pathologist reads the slides from the Mid-Low and Low concentration groups (48 fish per species, Part B.2).
- The pathologist considers each target tissue individually. Once the pathologist reaches a treatment level with no potential treatment-related effects in that tissue, then the pathologist stops with that tissue and does not read that tissue on the slides from the animals in the next lower concentration group.

It is important to define the criteria for the read-down as the determination of "potential treatment-related findings." Many animals may have findings or abnormalities, but it is only by comparing dose groups that the potential for relationship to treatment can be established.

The trigger to proceed to the next lower tier for pathology should depend, to some extent, on the commonality of the potential treatment-related finding. The decision should be assessed case-by-case. Unless it is a type of finding that is not generally (or ever) observed spontaneously, treatment relationship among the high dose animals is determined on a group-wise basis as opposed to an individual animal basis. However, if a fairly rare type of finding (e.g., ovarian spermatogenesis) were to occur in one or two individuals in the high dose group, that could possibly trigger a read down, even if it does not register as significant by RSCABS. If an obvious, statistically significant treatment-related finding occurs in the high dose group relative to control, but that finding is observed to a lesser, non-significant, extent in the mid-dose group, the pathologist should evaluate the next lower dose group, because it could potentially reveal a dose-response pattern that would provide supporting evidence for the legitimacy of the relationship to treatment. Treatment relationship, and the histopathology decision tree, should not rely only on RSCABs significance. Not all statistically significant results are toxicologically important, and not all toxicologically important results are statistically significant. The pathologist should confer with the Study Director, and if needed the Prime Contractor and Sponsor, to determine the scientific justification for proceeding to the next lower tier.

10.12. GENOTYPING - IN-LIFE LABORATORY (FEL)

The following step-down process will be used to assess genotype:

- Fin clips will be taken from each of the designated "histopathology" animals (=120) and stored.
- The in-life lab/Study Director will collaborate with the designated pathologist to learn the results of the gonad pathology evaluations.
- Genotyping (genetic sex determination by biochemistry evaluation) will be triggered by assessment of gonad tissue only. If the pathologist finds evidence of ovotestes or testes-ovo in some fish, genotyping using the fin clips collected will be performed to elucidate phenotype/genotype mismatches related to test substance exposure. This will assist in determining if the changes are normal or are treatment-related.
- More specifically, if treatment-related gonadal pathology findings are observed in the highest test concentration or control fish, genotyping will be performed on the clips from these same fish (N=48).
- If treatment-related gonadal pathology findings are observed in the mid-highest treatment group, genotyping will be performed on the clips from these same animals (N=24).
- If treatment-related gonadal pathology findings are seen in the mid-low or low treatment group animals, genotyping will be performed on the clips from these same animals (N=48).

10.12.1. Genotyping Procedure

Either the dorsal or ventral tip of the caudal fin of each fish will be clipped. Once the DNA is extracted, a polymerase chain reaction (PCR) method will be used to amplify the *dmy* gene, if present (3). The presence of the *dmy* gene is a definitive indication of an XY (male) individual, regardless of phenotype. The *dmy* gene information will be used to establish XX – XY breeding pairs (in both F0 and F1 generation adults) regardless of external phenotype which may be altered by exposure to an endocrine-disrupting chemical.

10.13. Vitellogenin Analysis – EAG Laboratories, Easton, MD Test Site

Samples will be shipped to EAG Laboratories - Easton for analyses via overnight courier.

10.13. 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 890.1350 test guideline (3) and OECD Guideline 229 (4), the TO 17 QAPP (1), and will generally follow procedures described in the document Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application (11). The statistical unit for analysis will be the replicate. Measurements made on a per-tank basis include fecundity, normalized to number of female reproductive days. Endpoints evaluated on a per-fish basis (e.g., papillary processes measures) will be averaged per tank. For all continuous quantitative endpoints (fecundity, VTG, and GSI) consistent with a monotonic concentration-response, the Jonckheere-Terpstra test or equivalent will be applied in step-down manner to establish a significant treatment effect. For continuous endpoints that are not consistent with a monotonic concentration-response, the data will be assessed for normality (preferably using the Shapiro-Wilk or Anderson-Darling test) and variance homogeneity (preferably using the Levene test). If non-normal or heterogeneity in variance is detected, a normalizing, variance stabilizing transformation will be used. If after transformation, the data are normally distributed with homogeneous variance, a significant treatment effect will be assessed using Dunnett's test (or the equivalent). If after transformation, the data are normally distributed with heterogeneous variance, effects on a given treatment will be determined using the Tamhane-Dunnett (T3 test) or the Mann-Whitney-Wilcoxon U test (or the equivalent). If no normalizing transformations are found, significant treatment effects will be evaluated using the Mann-Whitney-Wilcoxon U test with a Bonferroni-Holm adjustment (or the equivalent) to the p-values. Dunnett's test (or the equivalent) will be applied independently of any ANOVA F-test and the Mann-Whitney test (or equivalent) will be used independently of Kruskall-Wallis ANOVA on Ranks test.

Mortality will be assessed from the step-down Cochran-Armitage test (or equivalent) when the data are monotonic, and otherwise from Fisher's Exact test with a Bonferroni-Holm adjustment (or equivalent). Effects on papillary processes score will be determined from the step-down application of the Jonckheere-Terpstra test (or equivalent) applied to the replicate medians. The unit of analysis will be the replicate so the data consist of replicate medians if the

Jonckheere-Terpstra or Mann-Whitney U test is used, or the replicate means if Dunnett's test is used. Concentration-response monotonicity can be assessed visually from the replicate and treatment means or medians or from formal tests such as previously described by OECD (7). The statistical significance of all tests indicated will be assessed at $p = 0.05$. At the conclusion of the analyses, No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC) values will be reported.

Analysis will be conducted both with and without suspected outliers (3,4, 11). Potential outliers will be identified by values that exceed the median plus three times the interquartile range (i.e., the difference between the 75th and 25th percentiles). If an explanation cannot be made for the divergence of data, then both analyses will be presented, assuming that the results differ. If the outlier does not alter the results, then the analysis including the outliers will be presented. If differences occur, then the implications of removing the outliers will be carefully documented.

10.13.1. 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 clean water control group.

10.14. Performance Criteria and Test Validity

The following criteria will be maintained throughout the study (3,4).

- Water quality characteristics will remain within the limits of tolerance described in Table 1 (water temperature did not differ by more than ± 1°C between test vessels at any one time during the exposure period and was maintained within a range of 1°C of the 25°C temperature specified for the Japanese medaka).
- There will be more than 90% survival of control animals over the duration of the chemical exposure.
- Evidence of spawning will be present in all replicates prior to chemical exposure. Control fish in each replicate will spawn, at a minimum, every 4 days and there will be an average of at least 15 eggs/female/day/test chamber. There will be greater than 95% fertility of eggs from the control animals during the exposure.

10.15. Contingencies

The three problems most likely to be encountered are related to insufficient numbers of tanks with correct sex ratios and successfully demonstrated spawning, unplanned mortality in

control or exposure tanks, and low or high measured concentrations relative to the nominal level of the test chemicals. These problems should be dealt with in the following manner:

- For the present study, extra tanks (up to 8 additional tanks) will be established to ensure an adequate supply of spawning Japanese medaka in groups with the correct sex ratio.
- If there is excessive mortality in any tank during pre-exposure or in control tanks during exposure, the cause should be investigated and the experiment may be reinitiated, in consultation with the Sponsor Monitor and the Sponsor.
- Prior to initiation of the chemical exposure, each diluter should be equilibrated for at least 1 week for its ability to maintain the desired concentration. If, during the exposures the measured concentration becomes unacceptably low or high, adjustments will be made to the diluter to correct the problem. If discrepancies arise between measured test concentrations and respective nominal concentrations, determination of theoretical exposure concentrations will be performed using the measured stock solutions. Time-weighted exposure concentrations will also be determined.

11. SAMPLE HANDLING AND CUSTODY

All samples received, generated during the course of testing, and submitted to EAG Laboratories (9) and EPL (10) in this study will be accompanied with an appropriately signed chain of custody and handled in accordance with facility SOPs (12,13). 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 and preserved as described by EAG Laboratories (9) will be shipped to EAG 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 Draft Final Report will be provided to the Sponsor Monitor 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:

EXECUTIVE SUMMARY

I. MATERIALS AND METHODS

A. Test Material
B. Test Organism
C. Exposure System
D. Study Design and Additional Experimental Conditions
E. Observations

II. RESULTS AND DISCUSSION

A. Results [as applicable]
Mortality
Development
Growth
Fecundity and Fertilization
Gross Pathology and Histopathology
Clinical Signs
Other Endpoints

B. Analysis and Conclusions
C. Study Deficiencies

III. REFERENCES

[Attachments and Other Supporting Data]

Appendices containing raw data, statistical reports, analytical reports, histology reports etc. will be included in the final report. Support laboratories will prepare QA audited 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 and Sponsor review and receipt of their comments by the Study Director, 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.) (14) 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 (15), study guidance documents, correspondence, and draft and final reports will be electronically maintained at the in-life test facility in accordance with facility SOPs (14) 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 (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 EAG 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. SPECIMEN ARCHIVAL

The preserved test specimens will be labeled and stored at FEL until study finalization in accordance with facility SOPs (17). 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
matten.sharlene@epa.gov

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) Testing Model Chemicals for Endocrine Effects in Two Fish Species, Zebrafish (*Danio rerio*) and Japanese Medaka (*Oryzia latipes*) using the Fish Short-Term Reproduction Assay EP-W-11-063 Task Order 17, version 1, October 7, 2016.

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. 890.1350 Fish Short-Term Reproduction Assay, United States Environmental Protection Agency, Washington DC, EPA 740-C-09-007, October 2009.

4. OECD, *Test No. 229: Fish Short Term Reproduction Assay*, OECD Publishing, Paris. DOI: http://dx.doi.org/10.1787/9789264076211-en, 2 October 2012.

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

6. Ankley G.T., Jensen K.J., Kahl M.D., Korte J.J., and Makynen E.A. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*)." Environ. Toxicol. Chem. 20:1276-1290, 2001.

7. Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Henry, T.R., Denny, J.S., Leino, R.L., Wilson, V.S., Cardon, M.C., Hartig, P.C., and Gray, L.E. Effects of the androgenic growth promoter 17-B-trenbolone on fecundity and reproductive endocrinology of the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 22:1350-1360, 2003.

8. U.S. Environmental Protection Agency (USEPA). OCSPP 890:2200: Medaka Extended One Generation Reproduction Test (MEOGRT), EPA No. 740-C-15-002, July 2015.

9. ABC Laboratories [EAG Laboratories]. (December 8, 2015). Validated Analytical Chemistry Test Method for 2-ethylhexyl 4-Hydroxybenzoate in Freshwater. Under subcontract with Battelle for U.S. EPA Prime Contract No. EP-W-11-063, Task Order 14.

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

11. 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.

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

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

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

15. SOP 4.9, QAU Inspection Reports, Audit Reports, and Report Routing, 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.

Table 2. Summary of Endpoints – Modified FSTRA for Medaka

Endpoints	Fish Examined/Replicate	Study Day	Description
Survival	All	Daily	Daily assessment of survival should be made to provide a basis for expression and interpretation of reproductive output.
Appearance of Adults	All	Daily	The external appearance of the adults should be assessed as part of the daily observations, and any unusual changes should be noted. These observations are especially important for assessing endocrine active agents that are androgenic.
Fecundity	All	Daily	Egg production should be determined daily, but only during the late morning.
Fertilization Success	All	Daily	If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate is easily achieved.
Secondary Sex Characteristics	All	21	Japanese medaka: count number of papillary processes None for zebrafish.
Body Weights and Total Fish Lengths	All	21	Measured at necropsy.
Fin clip (genotyping):	All	21	Fin clip will be collected and preserved at -20°C for genotypic assessment. Genotyping using a tiered approach, depending on pathology findings.
Liver Vitellogenin (VTG):	2 ♂ & 2♀	21	Liver will be excised and preserved in RNAlater frozen at -20°C.
Gonad Size (GSI)	2 ♂ & 2♀	21	Following liver excision, the fixed gonads should be removed and weighed (to the nearest 0.1 mg) to determine the GSI (GSI=100% x gonad wt/body wt).
Thyroid, Liver, Gonad Histopathology	Remaining 3 ♂ & 3♀	21	Whole animal sagittal sectioning will be performed and slides evaluated by a toxicologic pathologist with experience with small fish (EPL). Pathology evaluation using a tiered approach, depending on pathology findings in high concentration groups.

Attachment – 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

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.

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DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka	
Amendment Number: 01	**Document ID Number:** BATT01-10 (00404)
Submitted By: Douglas J. Fort	**Date:** 1/26/2017

Amendment Relating To: BATT01-00404

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

[ ] Other (describe):

Original Specifications:

1. Page 15, Section 10.10.1.4
   "...individual fish, length..."
2. Page 25, Table 2
   Endpoint "Body Weights and Total Fish Lengths"

Changed To:

1. "...individual fish, standard length..."
2. "Body Weights and Standard Fish Lengths"

Reason for Change: To clarify that standard fish lengths will be used in length endpoint measurements.

Approval:

Study Director: _____ Date: 2/1/2017

Sponsor Representative: Vincent J. Brown Date: 1 FEB 2017

Page **1** of **1**

FEL

DOCUMENT AMENDMENT FORM

Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

Amendment Number: 02 | **Document ID Number**: BATT01-10 (00404)

Submitted By: Douglas J. Fort | **Date**: 2/6/2017

Amendment Relating To: BATT01-00404

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

[ ] Other (describe):

Original Specifications:

1. Page 13, Table 1

"D.O. ≥ 4.9 mg/L (≥ 60% saturation); mean temp 25° ± 1°C; 90% survival and successful egg production in controls; spawning occurs at least every 4 d in each control replicate; Fertility > 95%; fertility > 95%. Measured treatment exposure concentration CV < 20% for all replicates."

2. Page 19, Section 10.15. Performance Criteria and Test Validity

The following criteria will be maintained throughout the study (3,4).

- Water quality characteristics will remain within the limits of tolerance described in Table 1 (water temperature did not differ by more than ± 1°C between test vessels at any one time during the exposure period and was maintained within a range of 1°C of the 25°C temperature specified for the Japanese medaka).
- There will be more than 90% survival of control animals over the duration of the chemical exposure.
- Evidence of spawning will be present in all replicates prior to chemical exposure. Control fish in each replicate will spawn, at a minimum, every 4 days and there will be an average of at least 15 eggs/female/day/test chamber. There will be greater than 95% fertility of eggs from the control animals during the exposure.

Changed To:

1. D.O. ≥ 4.9 mg/L (≥ 60% saturation); mean temp 25° ± 1°C; 90% survival and successful egg production in controls; spawning occurs at least every 4 d in each control replicate; Fertility >80%; Measured treatment exposure concentration CV < 20% for all replicates.

2. The following criteria will be maintained throughout the study (3,4).

- Water quality characteristics will remain within the limits of tolerance described in Table 1 (water temperature did not differ by more than ± 1°C between test vessels at any one time during the

FEL

exposure period and was maintained within a range of 1°C of the 25°C temperature specified for the Japanese medaka). • There will be more than 90% survival of control animals over the duration of the chemical exposure. • Evidence of spawning will be present in all replicates prior to chemical exposure. Control fish in each replicate will spawn, at a minimum, every 4 days and there will be an average of at least 10 eggs/female/day/test chamber (4). There will be greater than 80% fertility of eggs from the control animals during the exposure (3).
Reason for Change: To harmonize the criteria between the participating labs and provide realistic expectation of control performance. More specifically, while 95% fertility rate is reasonable for fathead minnow, use the 80% criterion that is cited in the MEOGRT guideline (3) and specific to medaka is more realistic. Similarly, there is clear support in OECD 229 (4) for minimum egg production of 10 eggs/female/day/test chamber.

Approval:

Study Director: _____ Date: 2/7/2017

Sponsor Representative: _____ Date: 7 FEB 2017

FEL

DOCUMENT AMENDMENT FORM

Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

Amendment Number: 03 | **Document ID Number:** BATT01-10 (00404)

Submitted By: Douglas J. Fort | **Date:** 2/6/2017

Amendment Relating To: BATT01-00404

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

[ ] Other (describe):

Original Specifications:

1. Page 8, Section 10.2, Dilution and Laboratory Control Water

"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. The culture water at FEL was analyzed most recently on November 15, 2016 and contained 6.6 (±0.06) µg/L l-. The next scheduled facility water analysis for polyaromatic hydrocarbons (PAHs), pesticides, and metals is scheduled for January or February, 2017. 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 BATT01-00404. Any departures from recommended values will be promptly brought to the attention of the Study Sponsor/Monitor."

Changed To:

1. Results of dilution and laboratory control water analysis for pesticides, PAHs, and metals are attached.

Reason for Change: Provide results of annual facility water quality analysis.

Approval:

Study Director: _____ Date: 2/7/2017

Sponsor Representative: _____ Date: 7 FEB 2017

Page 1 of 2

FEL

FEL

Attachment: Results of Dilution / Laboratory Control Water Analyses

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 #: FEL01-00000
Project Name:

Date Received: 1/17/2017
Report Date: 2/2/2017

Lab Number	Sample Identification	Date Sampled	Analysis Date	Analyzed Time	By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201700231	2017-001 DeCl2 WATER	1/16/2017	1/23/2017	13:19	SV	TPH DRO Extraction		Start				SPE	11180
		1/16/2017	1/24/2017	13:08	MY	TPH-DRO	U	BDL	mg/l	0.1	0.1	OK8000/81	11192
													11178
201700232	2017-001 DeCl2 WATER					TPH-G-W:							11178
		1/16/2017	1/20/2017	14:56	SV	TPH-GRO	U	BDL	mg/l	0.02	0.02	OK8020/80	11178
		1/16/2017	1/20/2017		SV	TFT (Surr)		93	%Rec	70	70	OK8020/80	11178
		1/16/2017	1/20/2017		SV	BFB (Surr)		94	%Rec	70	70	OK8020/80	11178
													11179
201700233	2017-001 DeCl2 WATER	1/16/2017	1/23/2017	10:30	SV	Pesticide Extraction		Start				EPA_3510	11179
													11285
201700233	2017-001 DeCl2 WATER					PEST-OC-608:							11285
		1/16/2017	2/1/2017	14:09	MY	Alpha-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Gamma-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Beta-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Delta-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Heptachlor	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Aldrin	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Heptachlor Epoxide	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	g-Chlordane	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	a-Chlordane	U	BDL	ug/l	0.05	0.05	EPA_608	11285

Susie Southwell

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Environmental Laboratory and Consulting Company

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Certificate of Analysis

ODEQ ID # 9953

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

Project #: FEL01-00000
Project Name:

Date Received: 1/17/2017
Report Date: 2/2/2017

Lab Number	Sample Identification	Date Sampled	Analysis Date	Time	Analyzed By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201700233	2017-001 DeCl2 WATER	1/16/2017	2/1/2017		MY	Endosulfan I	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	4,4-DDE	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Dieldrin	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Endrin	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	4,4-DDD	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Endosulfan II	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Endrin Aldehyde	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	4,4-DDT	U	BDL	ug/l	0.1	0.1	EPA_608	11285
		1/16/2017	2/1/2017		MY	Endosulfan Sulfate	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Methoxyclor	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Endrin Ketone	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Toxaphene	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	TCMX (Surr)		30	%Rec	30	30	EPA_608	11285
		1/16/2017	2/1/2017		MY	1,2-dcbp (Surr)		85	%Rec	66	66	EPA_608	11285
													11167
201700234	2017-001 DeCl2 WATER	1/16/2017	1/20/2017	14:01	JL	Arsenic	U	BDL	mg/l	0.01	0.01	200.7/6010	11167
		1/16/2017	1/20/2017	14:01	JL	Cadmium	U	BDL	mg/l	.0015	0.0015	200.7/6010	11169
		1/16/2017	1/20/2017	14:01	JL	Chromium	U	BDL	mg/l	0.002	0.002	200.7/6010	11165
		1/16/2017	1/20/2017	14:01	JL	Copper	U	BDL	mg/l	.0015	0.0015	200.7/6010	11161
		1/16/2017	1/20/2017	14:01	JL	Iron	U	BDL	mg/l	0.009	0.009	200.7/6010	11164
		1/16/2017	1/20/2017	14:01	JL	Lead	U	BDL	mg/l	0.005	0.005	200.7/6010	11166

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Date Received: 1/17/2017
Report Date: 2/2/2017

Lab Number	Sample Identification	Date Sampled	Analysis Date	Analyzed Time	By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201700234	2017-001 DeCl2 WATER	1/16/2017	1/20/2017	16:06	SV	Mercury	U	BDL	mg/l	.0002	0.0002	EPA_245.2	11176
		1/16/2017	1/20/2017	14:01	JL	Nickel	U	BDL	mg/l	0.006	0.006	200.7/6010	11163
		1/16/2017	1/20/2017	14:01	JL	Zinc	U	BDL	mg/l	0.002	0.002	200.7/6010	11162
201700235		1/16/2017	1/18/2017	11:28	SV	Semi-Volatile Extraction		Start				EPA_3510	11112
													11160
201700235	2017-001 DeCl2 WATER					PAH-625-W:							11160
		1/16/2017	1/20/2017	14:09	MY	Naphthalene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	2-Methylnaphthalene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	2-Chloronaphthalene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Acenaphthylene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Acenaphthene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Fluorene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Phenanthrene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Anthracene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Fluoranthene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Pyrene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Benzo(a)anthracene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Chrysene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Benzo(b)fluoranthene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Benzo(k)fluoranthene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Benzo(a)pyrene	U	BDL	ug/l	5	5	EPA_625	11160

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Date Received: 1/17/2017
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Lab Number	Sample Identification	Date Sampled	Analysis Date	Time	Analyzed By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201700235	2017-001 DeCl2 WATER	1/16/2017	1/20/2017		MY	Indeno(1,2,3-Cd)pyrene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Dibenz(a,h)anthracene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Benzo(g,h,i)perylene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	NB-d5 (Surr)		49	%Rec	25	25	EPA_625	11160
		1/16/2017	1/20/2017		MY	2-FBP (Surr)		34	%Rec	12	12	EPA_625	11160
		1/16/2017	1/20/2017		MY	2,4,6-TBP (Surr)		45	%Rec	14	14	EPA_625	11160

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.

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FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

Amendment Number: 04 | **Document ID Number**: BATT01-10 (00404)

Submitted By: Douglas J. Fort | **Date**: 2/9/2017

Amendment Relating To: BATT01-00404

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

[ ] Other (describe):

Original Specifications:

1. Page 8-9, Section 10.3, Test System

   "The test species will be medaka (*Oryzias latipes*) (8). Medaka is a well-studied laboratory animal that exhibits ease of use in the laboratory, rapid development, and simplicity of observation during development and reproduction. Medaka is readily induced to breed in captivity, and published methods exist for its culture, as well as short-term lethality, early life-stage and full life-cycle tests."

2. Page 11, Section 10.9.4. Pre-exposure

   "Any specimens whose sex cannot be identified will be excluded from the assay."

3. Page 14, Section 10.10.1.2. Endpoints Measured in All Study Fish

   "Behavior of adults – Abnormal behavior (relative to controls) such as hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, or feeding abstinence will be noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, will also be noted."

4. Page 15, Section 10.10.1.4. Endpoints Measured in Remaining Three Male and Three Female Fish per Replicate

   "Genotypic Sex – During the necropsy, a fin clip will be collected from each fish, placed in a labeled vial and frozen at -20ºC until analyzed."

5. Page 17, Section 10.12. Genoptyping – In-Life Laboratory (FEL)

   Entire section.

FEL

Changed To:

1. Add sentence to end of paragraph. "Medaka used will be from in-house cultures at FEL, which were established from stock procured from Aquatic Research Organisms (ARO, Hampton, NH USA) and originate from stocks maintained at USEPA, Duluth, MN."

2. "Any specimens whose phenotypic sex cannot be identified will be excluded from the assay."

3. **"Behavior of adults –** All abnormal behavior in all replicates of all treatments and the controls including, but not limited to hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, or feeding abstinence will be qualitatively noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, will also be qualitatively noted."

4. **"Genotypic Sex** – During the necropsy, two fin clips will be collected from each fish, and placed in separate labeled vials unless one of the two fin clips are analyzed immediately following necropsy. Fin clips not immediately analyzed will be stored frozen at -20°C until analyzed or disposed of as directed by Sponsor at study termination."

5. 10.12. GENOTYPING - IN-LIFE LABORATORY (FEL)
Two fin clips will be collected from each fish in the "histopathology" group. One will be stored at -20°C, and the other will be used for genotype analysis.

   10.12.1. Genotyping Procedure
   Once the DNA is extracted, a polymerase chain reaction (PCR) method will be used to amplify the dmy gene, if present (3). The presence of the dmy gene is a definitive indication of an XY (male) individual, regardless of phenotype. The *dmy* gene information will be used to establish XX – XY genotype regardless of external phenotype which may be altered by exposure to an endocrine-disrupting chemical.

FEL

Reason for Change:

1. Provide source of test system.
2. Clarify that sex determination at this prior the initiation of pre-exposure is based only on phenotype.
3. Indicate that behavior will be observed and noted in all replicates of the control and treatments and that assessment is qualitative.
4. Two fin clips will be collected during necropsy so that one can be stored as backup to be analyzed if necessary as determined by the sponsor.
5. A change in process was requested changing the original tiered approach of genetic sexing determined by the histopathologist and study director to analysis of all 120 fish. Also, corrects a typographical error; there are no F0 or F1 fish present in this study.

Approval:

Study Director: _____ Date: 2/14/2017

Sponsor Representative: Vincent J. Bloom Date: 14 FEB 2017

BATT01-00404
Protocol Amendment 05

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

Amendment Number: 05 | **Document ID Number**: BATT01-10 (00404)

Submitted By: Douglas J. Fort | **Date**: 2/21/2017

Amendment Relating To: BATT01-00404

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

[ ] Other (describe):

Original Specifications:

1. Pages 3, 18 and 19, Section 10.13. Data Analysis and Statistics, 10.13.1 Use of Compromised Treatment Levels, 10.14. Performance Criteria and Test Validity, 10.15. Contingencies.

2. Page 18, Section 10.13. Data Analysis and Statistics

   "Mortality will be assessed from the step-down Cochran-Armitage test (or equivalent) when the data are monotonic, and otherwise from Fisher's Exact test with a Bonferroni-Holm adjustment (or equivalent). Effects on papillary processes score will be determined from the step-down application of the Jonckheere-Terpstra test (or equivalent) applied to the replicate medians. The unit of analysis will be the replicate so the data consist of replicate medians if the Jonckheere-Terpstra or Mann-Whitney U test is used, or the replicate means if Dunnett's test is used. Concentration-response monotonicity can be assessed visually from the replicate and treatment means or medians or from formal tests such as previously described by OECD (7). The statistical significance of all tests indicated will be assessed at $p = 0.05$. At the conclusion of the analyses, No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC) values will be reported."

3. Page 24, Section 17, References

BATT01-00404 FEL
Protocol Amendment 05

Changed To:

1. 10.14. Data Analysis and Statistics, 10.14.1 Use of Compromised Treatment Levels, 10.15. Performance Criteria and Test Validity, 10.16. Contingencies.

2. Mortality will be assessed from the step-down Cochran-Armitage test (or equivalent) when the data are monotonic, and otherwise from Fisher's Exact test with a Bonferroni-Holm adjustment (or equivalent). Effects on papillary processes score will be determined from the step-down application of the Jonckheere-Terpstra test (or equivalent) applied to the replicate medians. The unit of analysis will be the replicate so the data consist of replicate medians if the Jonckheere-Terpstra or Mann-Whitney U test with a Bonferroni-Holm adjustment (or equivalent) is used, or the replicate means if Dunnett's test is used. Concentration-response monotonicity can be assessed visually from the replicate and treatment means or medians or from formal tests such as previously described by OECD (4). The statistical significance of all tests indicated will be assessed at $p = 0.05$. At the conclusion of the analyses, No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC) values will be reported. 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 (19)."

3. Add 19. 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 for Change:

1. Correct for two sections numbered 10.13.

2. Adds RSCABS statistical analyses of histopathology data and appropriate reference for analysis. Provides a more current approach to evaluating histopathology results. Also, corrects reference number.

3. Adds reference citation for RSCABS analyses.

Approval:

Study Director: _____ Date: 2/28/2017

Sponsor Representative: _____ Date: 28FEB2017

BATT01-00404
Protocol Amendment 06

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka	
Amendment Number: 06	**Document ID Number:** BATT01-10 (00404)
Submitted By: Douglas J. Fort	**Date:** 3/14/2017
Amendment Relating To: BATT01-00404	
[X] Protocol [ ] Study Plan [ ] QAPP [ ] QAMP [ ] SOP	
[ ] Other (describe):	

Original Specifications:

1. Pages 6, Section 7. Testing Sites

   Vitellogenin mRNA analyses in liver will be performed at EAG Laboratories, Inc., Biochemistry Department, 8598 Commerce Drive, Easton, MD 21601. Mr. Matthew Rhodes, PI of the planned analyses, will serve as study contact for EAG Laboratories and may be reached at 410.822.8600 (x244) or mrhodes@eag.com.

Changed To:

1. Amanda Milligan, amilligan@eag.com, 410.822.8600 will replace Matthew Rhodes as PI.

Reason for Change:

1. New PI for vitellogenin analyses at EAG Laboratories has been assigned due to the departure of Matthew Rhodes.

Approval:

Study Director: _____ Date: 3/23/2017

Sponsor Representative: _____ Date: 3/23/2017

Page 1 of 1

BATT01-00404 FEL
Protocol Amendment 07

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka	
Amendment Number: 07	**Document ID Number:** BATT01-10 (00404)
Submitted By: Douglas J. Fort	**Date:** 4/7/2017

Amendment Relating To: BATT01-00404

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

[ ] Other (describe):

Original Specifications:

1. Page 8, Section 10.2, Dilution and Laboratory Control Water

   "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. The culture water at FEL was analyzed most recently on November 15, 2016 and contained 6.6 (±0.06) µg/L l-. The next scheduled facility water analysis for polyaromatic hydrocarbons (PAHs), pesticides, and metals is scheduled for January or February, 2017. 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 BATT01-00404. Any departures from recommended values will be promptly brought to the attention of the Study Sponsor/Monitor."

Changed To:

1. Results of dilution and laboratory control water analysis for pesticides, PAHs, and metals are attached.

Reason for Change:

1. Provide results of annual facility water quality analysis.

Approval:

Study Director: _____ Date: 4/12/17

Sponsor Representative: _____ Date: 12 APR 2017

BATT01-00404 FEL
Protocol Amendment 07

Attachment: Results of Dilution / Laboratory Control Water Analyses

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Certificate of Analysis

ODEQ ID # 9953

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

Project #: FEL01-00000
Project Name:

Date Received: 1/17/2017
Report Date: 2/2/2017

Lab Number	Sample Identification	Date Sampled	Analysis Date	Analyzed Time	By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201700231	2017-001 DeCl2 WATER	1/16/2017	1/23/2017	13:19	SV	TPH DRO Extraction		Start				SPE	11180
		1/16/2017	1/24/2017	13:08	MY	TPH-DRO	U	BDL	mg/l	0.1	0.1	OK8000/81	11192
													11178
201700232	2017-001 DeCl2 WATER					TPH-G-W:							11178
		1/16/2017	1/20/2017	14:56	SV	TPH-GRO	U	BDL	mg/l	0.02	0.02	OK8020/80	11178
		1/16/2017	1/20/2017		SV	TFT (Surr)		93	%Rec	70	70	OK8020/80	11178
		1/16/2017	1/20/2017		SV	BFB (Surr)		94	%Rec	70	70	OK8020/80	11178
													11179
201700233	2017-001 DeCl2 WATER	1/16/2017	1/23/2017	10:30	SV	Pesticide Extraction		Start				EPA_3510	11179
													11285
201700233	2017-001 DeCl2 WATER					PEST-OC-608:							11285
		1/16/2017	2/1/2017	14:09	MY	Alpha-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Gamma-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Beta-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Delta-BHC	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Heptachlor	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Aldrin	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Heptachlor Epoxide	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	g-Chlordane	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	a-Chlordane	U	BDL	ug/l	0.05	0.05	EPA_608	11285

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Lab Number	Sample Identification	Date Sampled	Analysis Date	Time	Analyzed By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201700233	2017-001 DeCl2 WATER	1/16/2017	2/1/2017		MY	Endosulfan I	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	4,4-DDE	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Dieldrin	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Endrin	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	4,4-DDD	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Endosulfan II	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Endrin Aldehyde	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	4,4-DDT	U	BDL	ug/l	0.1	0.1	EPA_608	11285
		1/16/2017	2/1/2017		MY	Endosulfan Sulfate	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Methoxyclor	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Endrin Ketone	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	Toxaphene	U	BDL	ug/l	0.05	0.05	EPA_608	11285
		1/16/2017	2/1/2017		MY	TCMX (Surr)		30	%Rec	30	30	EPA_608	11285
		1/16/2017	2/1/2017		MY	1,2-dcbp (Surr)		85	%Rec	66	66	EPA_608	11285
													11167
201700234	2017-001 DeCl2 WATER	1/16/2017	1/20/2017	14:01	JL	Arsenic	U	BDL	mg/l	0.01	0.01	200.7/6010	11167
		1/16/2017	1/20/2017	14:01	JL	Cadmium	U	BDL	mg/l	.0015	0.0015	200.7/6010	11169
		1/16/2017	1/20/2017	14:01	JL	Chromium	U	BDL	mg/l	0.002	0.002	200.7/6010	11165
		1/16/2017	1/20/2017	14:01	JL	Copper	U	BDL	mg/l	.0015	0.0015	200.7/6010	11161
		1/16/2017	1/20/2017	14:01	JL	Iron	U	BDL	mg/l	0.009	0.009	200.7/6010	11164
		1/16/2017	1/20/2017	14:01	JL	Lead	U	BDL	mg/l	0.005	0.005	200.7/6010	11166

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Certificate of Analysis

ODEQ ID # 9953

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

Project #: FEL01-00000
Project Name:

Date Received: 1/17/2017
Report Date: 2/2/2017

Lab Number	Sample Identification	Date Sampled	Analysis Date	Analyzed Time	By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201700234	2017-001 DeCl2 WATER	1/16/2017	1/20/2017	16:06	SV	Mercury	U	BDL	mg/l	.0002	0.0002	EPA_245.2	11176
		1/16/2017	1/20/2017	14:01	JL	Nickel	U	BDL	mg/l	0.006	0.006	200.7/6010	11163
		1/16/2017	1/20/2017	14:01	JL	Zinc	U	BDL	mg/l	0.002	0.002	200.7/6010	11162
201700235		1/16/2017	1/18/2017	11:28	SV	Semi-Volatile Extraction		Start				EPA_3510	11112
													11160
201700235	2017-001 DeCl2 WATER					PAH-625-W:							11160
		1/16/2017	1/20/2017	14:09	MY	Naphthalene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	2-Methylnaphthalene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	2-Chloronaphthalene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Acenaphthylene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Acenaphthene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Fluorene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Phenanthrene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Anthracene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Fluoranthene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Pyrene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Benzo(a)anthracene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Chrysene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Benzo(b)fluoranthene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Benzo(k)fluoranthene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Benzo(a)pyrene	U	BDL	ug/l	5	5	EPA_625	11160

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Project #: FEL01-00000
Project Name:

Date Received: 1/17/2017
Report Date: 2/2/2017

Lab Number	Sample Identification	Date Sampled	Analysis Date	Time	Analyzed By	Parameter	Q	Results	Units	RL	SQL	Method	Batch
201700235	2017-001 DeCl2 WATER	1/16/2017	1/20/2017		MY	Indeno(1,2,3-Cd)pyrene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Dibenz(a,h)anthracene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	Benzo(g,h,i)perylene	U	BDL	ug/l	5	5	EPA_625	11160
		1/16/2017	1/20/2017		MY	NB-d5 (Surr)		49	%Rec	25	25	EPA_625	11160
		1/16/2017	1/20/2017		MY	2-FBP (Surr)		34	%Rec	12	12	EPA_625	11160
		1/16/2017	1/20/2017		MY	2,4,6-TBP (Surr)		45	%Rec	14	14	EPA_625	11160

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

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BATT01-00404
Protocol Amendment 08

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka	
Amendment Number: 08	**Document ID Number**: BATT01-10 (00404)
Submitted By: Douglas J. Fort	**Date**: 5/1/2017

Amendment Relating To: BATT01-00404

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

[ ] Other (describe):

Original Specifications:

1. Page 16, Section 10.11. Gonad, Liver and 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 FSTRA and MEOGRT (3,4,8,10). Following the conclusion of exposure (SD 21), FEL will send preserved intact whole carcass specimens to EPL via overnight courier for histopathological processing and analyses."

2. Page 20, Section 11. Sample Handling and Custody

   All samples received, generated during the course of testing, and submitted to EAG Laboratories (9) and EPL (10) in this study will be accompanied with an appropriately signed chain of custody and handled in accordance with facility SOPs (12,13). 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 and preserved as described by EAG Laboratories (9) will be shipped to EAG 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.

3. Page 23, Section 17. References

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

Changed To:

1. "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 new guidance document for medaka histopathology for modified FSTRAs (10 – attached) based on

BATT01-00404

FEL

Protocol Amendment 08

relevant procedures from the previous FSTRA (3,4) and MEOGRT (8) histopathology guidelines used for similar TO14 studies of small fish. The attached methodology (10) will be followed for study 00404. Following the conclusion of exposure (SD 21), FEL will send preserved intact whole carcass specimens to EPL via overnight courier for histopathological processing and analyses."

2. All samples generated during the course of testing by (FEL12,13) 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. 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 and preserved as described by EAG Laboratories will be shipped to EAG 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.

3. 10. Battelle. (2017). Histological Guidance for Modified Fish Short-Term Reproduction Assays (FSTRAs) in Zebrafish (*Danio rerio*) and Japanese Medaka (*Oryzias latipes*) with Liver and Thyroid Evaluation, approved by USEPA April 26, 2017 for TO 17, Battelle, Columbus, OH, 38 p. This document is provided as an attachment to amendment 8.

Reason for Change:

1. New guidance for 00404 study histopathology provided.
2. Provides clarification for the use of each facilities SOPs regarding sample handling and custody.
3. Replaces old guidance information with new guidance reference and provides the document.

Approval:

Study Director: _____ Date: 5/5/2017

Sponsor Representative: Vincent J. Brown Date: 5 MAY 2017

BATT01-00404 FEL
Protocol Amendment 08

Histological Guidance for Modified Fish Short-Term Reproduction Assays (FSTRAs) in Zebrafish (*Danio rerio*) and Japanese Medaka (*Oryzias latipes*) with Liver and Thyroid Evaluation (10)

**Histological Guidance for Modified Fish Short-Term Reproduction Assays (FSTRAs)
in Zebrafish (*Danio rerio*) and Japanese Medaka (*Oryzias latipes*)
with Liver and Thyroid Evaluation**

Prepared for
Endocrine Disruptor Screening Program
U.S. Environmental Protection Agency
Washington, DC

Prepared by
Battelle
Columbus, Ohio
Contract EP-W-11-063, Task Order 17

APPROVED

April 26, 2017

The purpose of this guidance is to document specific procedures for histopathological analysis in the Japanese medaka and zebrafish based on the general framework given in the U.S. EPA Task Order 17 Statement of Work (issued 8/8/2016) as modified by the Addendum for Task Order Modification 1 (dated 11/16/2016 and issued 12/2/2016). Reference is also made to the EPA OPPTS 890.1350 test guidelines (2009) and the OECD Guideline 229 (2012). This present guidance was prepared by Experimental Pathology Laboratories (EPL) and Fort Environmental Laboratories (FEL) under subcontract to Battelle, under the prime contract identified above.

This guidance consists of five parts:

- Summary of Assay and In-Life Laboratory Guidance (FEL)
- Consistent Coding of DEST "Severity Scoring Method" (DEST Column K; all labs)
- Histological Guidance for the Zebrafish FSTRA with Liver and Thyroid Evaluation (EPL)
- Histological Guidance for the Japanese Medaka FSTRA with Liver and Thyroid Evaluation (EPL)
- Attachment 1. Method Development Report (EPL).

The Method Development Report was prepared first, as a source for discussion with the EPA sponsors and others to develop the optimum procedures. This guidance was reviewed in draft form by EPL, FEL, Evans Analytical Group (EAG), and Integrated Laboratory Services (ILS).

Summary of Assay and In-Life Laboratory Guidance

This document is designed to incorporate the "Method Development to Determine the Optimum Approach for Sectioning of Thyroid Glands, Liver, and Gonads in Medaka and Zebrafish - DRAFT PATHOLOGY SUMMARY" prepared by Jeff Wolf, DVM, Ph.D. into an overall modified guidance for histopathology in the OECD 229 FSTRA for zebrafish and medaka. The experimental design and process of collecting, processing, and pathologically examining zebrafish and medaka from FSTRA studies is described in Table 1.

Table 1. Experimental Design and Process of Medaka and Zebrafish Collection, Processing, and Pathological Examination for FSTRA

Parameter	Process
Collection	Euthanize using 100 to 500 mg/L buffered MS-222 (pH 7) based on laboratory SOPs
Number of Treatments	4 concentrations and control
Number of Replicates	4
Number of Fish Processed	3 males and 3 females/replicate 12 males and 12 females/treatment and control 60 males and 60 females maximum per study
Method of Fixation	Modified Davidson's solution
Method of Preservation	10% neutral buffered formalin
Method of Sectioning	For **zebrafish,** follow methods established in "Histological Guidance for the Zebrafish FSTRA with Liver and Thyroid Evaluation" (J. Wolf, internal document, March 27, 2017). For **medaka**, the MEOGRT (USEPA, 2015) parasagittal sectioning method currently in use provides adequate amounts of thyroid gland, liver, and gonad tissue in orientations suitable for diagnostic evaluation
Examination[1,2]	First Step: Control, high concentration (treatment 4), up to 48 fish
	Second Step (if warranted from first step): Mid-High (treatment 3), up to 24 fish
	Third Step (if warranted from second step): Mid-Low (treatment 2) and Low (treatment 1), up to 48 fish

[1] Refer to Attachment 1 below: "Method Development to Determine the Optimum Approach for Sectioning of Thyroid Glands, Liver, and Gonads in Medaka and Zebrafish - DRAFT PATHOLOGY SUMMARY" prepared by Jeff Wolf, DVM, Ph.D.

[2] Step down movement determined by certified pathologist in conjunction with Study Director.

Consistent Coding of DEST "Severity Scoring Method" (DEST Column K)

The Instructions and the imbedded notes for the histopathology tabs (Male and Female) on the EPA-prescribed Data Entry Spreadsheet Template (DEST) contain the following guidance:

> "Tip: 1=relative to controls, 2=relative to normal. The two comparison methods and associated severity grading scale (0-4) are described beginning on page 15 of Appendix E in the test guideline."

This appears to coincide with the approach in the OECD No. 123 Guidance Document on the Diagnosis of Endocrine-Related Histopathology in Fish Gonads (2010, page 40), which leaves the scoring "at the discretion of the pathologist." We note that OECD No. 123 is cited as reference #22 in the histopathology discussion [pages 9–10, paragraph 48] of the OECD 229 FSTRA Guideline (2012).

In discussions with pathologists, these two comparison options did not seem to account for all of the anticipated findings, such as: 1) discrete features or changes; 2) multifocal or diffusely distributed features; or 3) tumors that are often scored as either present or absent. Also, the DEST is based on gonadal histopathology only, but the FSTRA study design for this task order has been modified to include thyroid and liver histopathology evaluations in both zebrafish and Japanese medaka species.

The three pathology laboratories recommend the following addition to the "Severity Scoring Method" as given in the FSTRA DEST:

Grading Method ID	Method
1	Comparison to control specimens
2	Comparison to normal histology as determined by the pathologist's experience
3	Count of occurrences per tissue section or percent of tissue in section involved (non-comparative determination)
4	Presence/Absence (*e.g.*, for tumors; non-comparative determination)
5	Other non-comparative determination (specify in study records and pathology report)

This change will require modifications to the layout of the DEST tabs for male and female histopathology, such that each observed lesion type or other histopathological finding will have an associated Grading Method ID. The labs will coordinate with each other and with the prime contractor to decide on a single Grading Method to be used for each unique lesion or other histopathological finding seen in the tissues being studied. The pathologists anticipate that, as with any attempt to use a set or unified vocabulary or system across laboratories and across test substances and species,

exceptional cases may arise, and that these will be documented in the study records as well as in the pathologist's report as appropriate.

With respect to the severity scores themselves (EPA 890.1350, Appendix E, pages 15–17; and OECD No. 123, pages 39–40), the labs recognize that when used, scores usually proceed from a low of 0 or 1 (least change seen) to a high of 4 (greatest change seen). However, when method 3 (count of occurrences or percent of tissue involved) is used to grade observed features, the grade may not be a measure of change relative to the control unless the feature is seldom observed in the controls. If the feature being scored is not rare in the control animals (*e.g.*, as may be the case for hepatocyte basophilia in the liver, post-ovulatory follicles in the ovaries, or tubular basophilia in kidneys), the score is an index of prevalence of the feature (a simple count or estimate of proportion of area of tissue involved), and is not a measure of change. This situation will typically be made obvious by relatively frequent non-zero grades for the feature in the control animals. The labs plan to use a score code of "P" for "Present" in the DEST by convention to distinguish this finding from other kinds of scores. The labs plan to confer as they receive and work on samples to verify the consistency in coding methods being used.

Histological Guidance for the Zebrafish OECD 229 FSTRA with Liver and Thyroid Evaluation

(a) Introduction. The goal of this document is to serve as general guidance for the collection, histological preparation of gonads, liver, and thyroid gland specimens from zebrafish (*Danio rerio*) in support of the Zebrafish FSTRA test. The Agency is providing histopathology guidance to help ensure that histological procedures and pathological evaluations are performed accurately and consistently. This histopathology guidance is based on procedures developed by the U.S. Environmental Protection Agency in consultation with other scientific experts. Specific products and/or equipment listed can be substituted with comparable materials. Laboratories may depart from some aspects of this guidance due to variability in some standard laboratory practices (*e.g.*, how much information is included on slide labels, etc.). This document is divided into two general sections: Necropsy Procedures and Histology Procedures.

(b) Necropsy Procedures. At the conclusion of the exposure, fish are anesthetized by transfer to an oxygenated solution of MS-222 (100 to 500 mg/L buffered with 200 mg $NaHCO_3$/L) for sampling. If potency of the solution is not adequate, additional MS-222 (<10 mg) may be added to strengthen effectiveness. If required, blood samples will be collected from fish sedated in this manner. Following collection of blood the fish will be euthanized as per Table 1. If other observations and measurements are to be made at this time (*e.g.*, length and body weight, blood collection, and evaluation of secondary sex characteristics), these tasks should be performed rapidly in order to avoid tissue autolysis which occurs rapidly in fish. To further avoid autolysis caused by delays in data collection, the number of fish

euthanized at any one time should be kept to a minimum. Prior to fixation, the tail is excised caudal to the abdominal cavity. Using a #11 blade or micro scissors, a slit incision approximately 3 mm in length is made carefully through the ventral abdominal wall to allow penetration of viscera by the fixative. Each fish is placed in an individual tissue cassette along with its corresponding identification label, and the cassette is placed in modified Davidson's solution for 24-48 hours. After the initial fixation period, the tissues are rinsed thoroughly in 70% ethanol, after which they may be stored in 10% neutral buffered formalin prior to histological processing.

Some protocols specify

- Davidson's for 48 to 96 hours
- The whole fish or fish carcass will be fixed/preserved in culture tubes of an appropriate size
- A water rinse is adequate when using Davidson's as the fixative (When Bouin's solution is used, tissues should be rinsed with 70% ethanol).
- Tissues are rinsed with D.I. water.

(c) Histology Procedures.

(1) Decalcification. Decalcification of the specimens is usually not required due to the presence of acetic acid in the modified Davidson's fixative. However, it is usually prudent to process and microtome a few control specimens ahead of the rest to ensure that decalcification is complete. If further decalcification is necessary, specimens may be immersed in a commercial formic acid/EDTA decalcifying solution for a short interval (*e.g.*, several hours or overnight) prior to processing.

(2) Processing and Embedding. Each whole fish specimen (*i.e.*, minus the tail) is processed in an automated tissue processor and infiltrated with paraffin according to routine methods (Figures 1 and 2).

(3) Microtomy. Section thickness is set at 4-5 microns. Each fish is step-sectioned in the parasagittal / sagittal plane at two distinct levels (Figures 1 and 2). Four sections will be acquired at each of the two levels, and each group of four sections will be placed on a single slide. At each level, the four sections are targeting the same tissue. Level 1 is targeting gonad, level 2 is targeting thyroid, and both are likely to contain liver. Four sections are acquired at each level to help ensure that testes or immature ovaries are captured at Level 1, and that thyroid is captured at Level 2. Additional slides may be used if needed to accommodate extra-large fish. A duplicate set of unstained sections is obtained at each of the two levels; these will be placed on two additional slides, for a total of four slides per fish (two stained and two unstained). Specific landmarks for each of the two levels are illustrated.

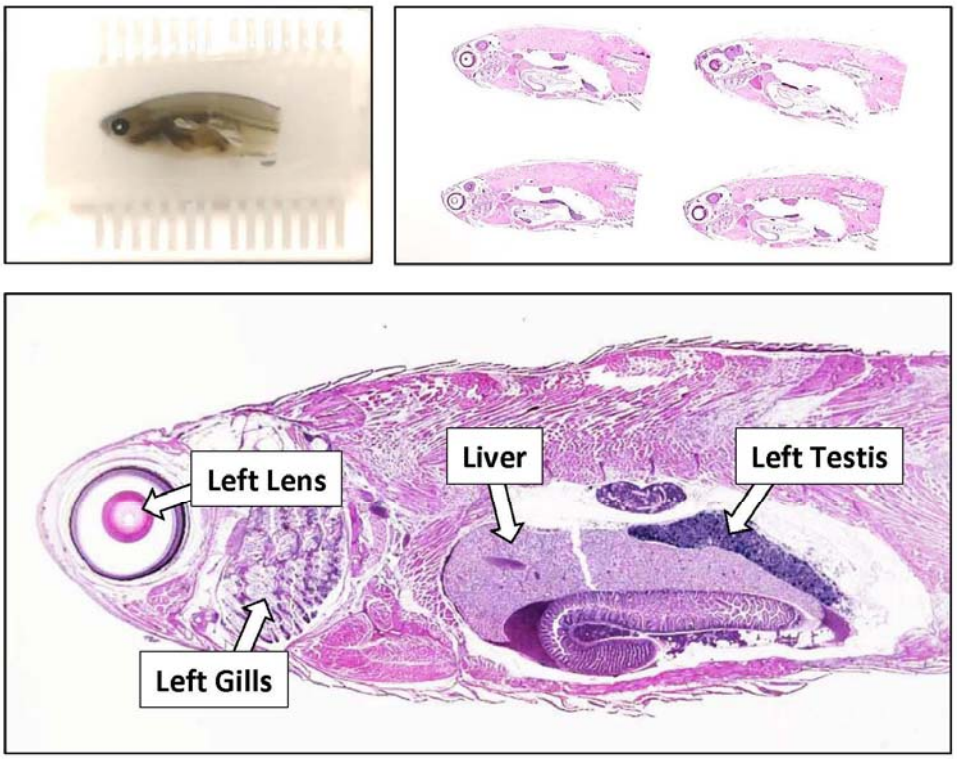


Figure 1. **Level 1**: The block is faced from the left toward the right side of the fish. Sections are trimmed away until the left eye is revealed and the body cavity is entered, and then trimming continues to the mid-portion of the lens. The lens will be visible as a solid sphere or ring within the eye, which can be seen in the sections and the block. The lens will be hard, the microtome blade will produce a scratch that can be heard and felt as the blade cuts through. Sections acquired at this level should reveal the visceral cavity, unless the fish was fixed with excessive body curvature. Four step sections (with duplicates to remain unstained) are acquired at intervals of approximately 20-40 microns depending on the size of the fish. The primary target at this level is the left gonad (testis or ovary). Obtaining sufficient amounts of mature testis (or immature gonad) consistently is more challenging than obtaining mature ovary. Liver tissue is likely to be present at both Levels 1 and 2.

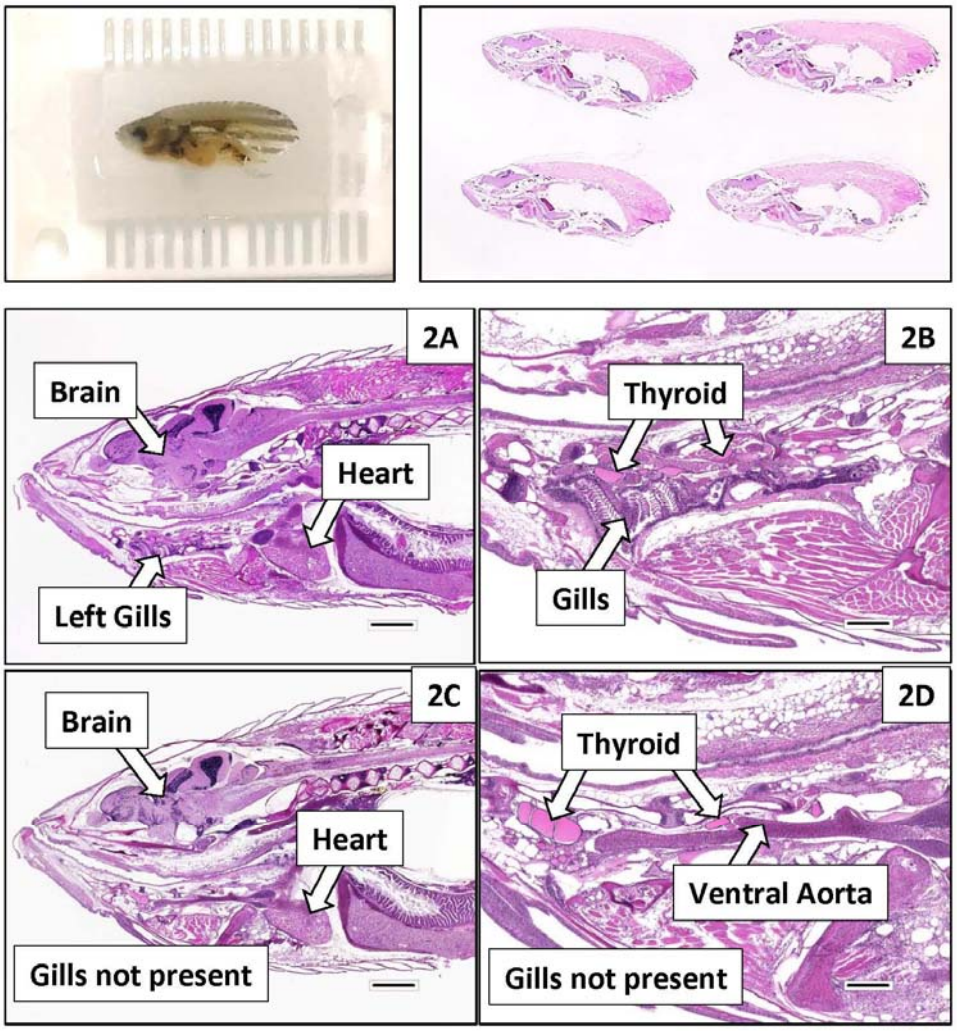


Figure 2. **Level 2**: Trimming is continued until the left gill chamber becomes smaller and almost disappears (2A and higher magnification 2B). In unstained sections, the gill chamber appears as a large clear area in the ventral head region. The left eye has completely disappeared at this level, and the brain and heart are large, although it may be necessary to examine unstained sections periodically under the microscope to visualize these landmarks. Four step sections (with duplicates to remain unstained) are obtained at approximately 10 micron intervals. In at least one of these sections, the ventral aorta should be evident (in unstained sections this structure can also be visualized as a dark horizontal stripe that projects rostrally from the heart) and the left gills

have disappeared (2C and higher magnification 2D). The primary target at this level is the thyroid gland tissue, variable amounts of which are loosely arranged circumferentially around the ventral aorta as it courses longitudinally through the mandibular region.

(4) **Staining and Coverslipping**. Slides destined for staining are stained with hematoxylin and eosin, and are covered with glass cover slips using an appropriate permanent mounting medium.

(5) **Labeling**. Slides can be labeled with the following information:

- Study number
- Name of the test chemical
- Generation and age of the specimen (*i.e.*, F1, 16 wpf)
- Treatment (concentration) or Control
- Individual animal identification number.

Histological Guidance for the Medaka OECD 229 FSTRA with Liver and Thyroid Evaluation

(a) Introduction. The goal of this document is to serve as general guidance for the collection, histological preparation of gonads, liver, and thyroid gland specimens from Japanese medaka (*Oryzias latipes*) in support of the Medaka FSTRA test. The Agency is providing histopathology guidance to help ensure that histological procedures and pathological evaluations are performed accurately and consistently. This histopathology guidance is based on procedures developed by the U.S. Environmental Protection Agency in consultation with other scientific experts. Specific products and/or equipment listed can be substituted with comparable materials. Laboratories may depart from some aspects of this guidance due to variability in some standard laboratory practices (*e.g.*, how much information is included on slide labels, etc.). This document is divided into two general sections: Necropsy Procedures and Histology Procedures.

(b) Necropsy Procedures. At the conclusion of the exposure, fish are anesthetized by transfer to an oxygenated solution of MS-222 (100 to 500 mg/L buffered with 200 mg NaHCO3/L) for sampling. If potency of the solution is not adequate, additional MS-222 (<10 mg) may be added to strengthen effectiveness. If other observations and measurements are to be made at this time (*e.g.*, length and body weight, blood collection, and evaluation of secondary sex characteristics), these tasks should be performed rapidly in order to avoid tissue autolysis which occurs rapidly in fish. To further avoid autolysis caused by delays in data collection, the number of fish euthanized at any one time should be kept to a minimum. Prior to fixation, the tail is excised caudal to the abdominal cavity. Using a #11 blade or micro scissors, a slit incision approximately 3 mm in length is made carefully through the ventral abdominal wall to allow penetration of viscera by the fixative. Each fish is placed in an individual tissue cassette along with its corresponding identification label, and the cassette is placed in modified

Davidson's solution for 24-48 hours. After the initial fixation period, the tissues are rinsed thoroughly in 70% ethanol, after which they may be stored in 10% neutral buffered formalin prior to histological processing.

Some protocols specify

- Davidson's for 48 to 96 hours
- The whole fish or fish carcass will be fixed/preserved in culture tubes of an appropriate size
- A water rinse is adequate when using Davidson's as the fixative (When Bouin's solution is used, tissues should be rinsed with 70% ethanol.)
- Tissues are rinsed with D.I. water.

(c) Histology Procedures. Histology procedures are identical to those described for the Medaka Extended One Generation Reproduction Test (MEOGRT, USEPA 2015). As part of initial efforts to improve on this method for the FSTRA evaluations, medaka of both sexes (a total of 42 fish) were embedded and sectioned in either the parasagittal, frontal, and transverse planes. Parasagittal sections were acquired entirely medial to the medial surfaces of the eyes, in order to maximize recovery of the gonad tissue. However, none of these procedures was found to be superior to the current MEOGRT protocol for recovery of the gonads, liver, and thyroid gland tissues.

(d) References.

(1) US Environmental Protection Agency (2015) OCSPP 890.2200: Medaka Extended One Generation Reproduction Test (MEOGRT). EPA No. 740-C-15-002, July, 2015. Available 3/11/16 at: http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPPT-2014-0766-0021.

Attachment 1

METHOD DEVELOPMENT TO DETERMINE OPTIMUM APPROACH FOR SECTIONING THYROID GLANDS IN ZEBRAFISH AND MEDAKA

DRAFT PATHOLOGY NARRATIVE

TO

BATTELLE MEMORIAL INSTITUTE

EPL PROJECT NUMBER 237-090

INTRODUCTION

EPA Task Order 17 concerns the investigation of effects of potential endocrine active compounds in the thyroid glands, liver, and gonads of two fish species, zebrafish *Danio rerio* and Japanese medaka *Oryzias latipes*. The goal of this project is to develop guidelines that describe histological methods for the optimal recovery of these tissues for pathological evaluation. The experimental design is presented in Table 1.

Table 1. Experimental Design

Species	Sectioning Plane	Males	Females	Undetermined
Medaka	Parasagittal	2	2	0
	Frontal	2	2	0
	Transverse	0	0	4
Zebrafish	Parasagittal	3	1	0
	Frontal	4	0	0
	Transverse	0	0	4

METHODS

Whole body samples of medaka and zebrafish were submitted to Experimental Pathology Laboratories, Inc. (EPL®), Sterling, Virginia from Fort Environmental Laboratories. At EPL, fish were decalcified using a commercially available formic acid/EDTA solution, and twelve fish per species were processed routinely for paraffin embedding. As listed in Table 1, four fish per species were embedded in each of the three sectioning planes: parasagittal (sagittal), frontal, and transverse. Males and females were incorporated into the design based on availability. All fish were embedded so that the left side was microtomed first. Step sections for smaller fish were acquired at approximately 40-50 µm for relatively smaller fish and 80-100 µm for larger fish. Specific microtomy instructions for each sectioning plane and species were as follows:

1. Parasagittal Sections (vertical longitudinal): step sections were acquired from the point at which the medial surface of left eye disappeared to the point at which the medial surface of the right eye appeared (resulted in 12-16 sections).
2. Frontal Sections (horizontal longitudinal):
    a. *Medaka*: step sections were acquired at levels that approximated the dorsal to the ventral extent of the ocular lens (resulted in 11-18 sections).
    b. *Zebrafish*: step sections were acquired through the gills and lower jaw (resulted in 10-15 sections).
3. Transverse Sections:
    a. *Medaka*: following excision of the head, step sections were obtained beginning at the caudal cut surface of the head and extending to the gill chamber (21 sections obtained per fish).
    b. *Zebrafish*: step sections were obtained through the head from a point at least 2-3 mm caudal to the eyes to anterior to the eyes.

All sections were placed on glass slides, stained with hematoxylin and eosin as per routine, and mounted using a commercial mounting medium and glass coverslips.

The pathologist scored each slide for the amount of diagnostically useful tissue of each of the three tissue types (thyroid gland, liver, and gonad) as follows: + = minimal amount of tissue present, ++ = moderate amount of tissue present, and +++ = abundant tissue present. For each animal and tissue type, the number of sections representing each score was tabulated on a spreadsheet. Sections that contained ++ or +++ amounts of tissue were considered to be suitable for diagnostic purposes, whereas sections with no tissue or only + amount of tissue were of zero or limited diagnostic value, respectively.

RESULTS AND DISCUSSION

SCORING OF SECTIONS

Section scoring results by species, sex, and sectioning plane are presented in Appendix B. Useful values to compare are the mean number of ++ and +++ sections for each tissue type.

As anticipated, parasagittal and frontal sections tended to provide the greatest amounts of tissue appropriate for diagnostic purposes as compared to transverse sections. Transverse sections were of limited diagnostic value for tissues other than the liver in medaka. For medaka, thyroid tissue was not recovered in any of the scored transverse sections, because the region sampled in all instances was slightly too far caudal. However, thyroid tissue was recovered subsequently in transverse sections from other medaka that had not been selected for scoring.

Overall, the most challenging tissues to collect consistently in histologic sections, in amounts and orientations suitable for toxicologic study, were the thyroid glands and testes of zebrafish, and the testes of medaka to a lesser extent. Recovery of zebrafish intestines in parasagittal sections would likely improve greatly if sections were acquired lateral to those specified by the methods used in this project (see Anatomic Considerations).

Anatomic considerations by organ and species, and the relative suitability of parasagittal and frontal sections for recovering suitable amounts of diagnostically relevant tissue, are discussed below. Photomicrographic figure images are in Appendix A.

ANATOMIC CONSIDERATIONS

Thyroid Glands. In **medaka**, the thyroid glands consist of unencapsulated loose aggregates of individual follicles that are located just caudal to the branchial cavity, and follow its caudal edge, extending laterally from the midline to the bones of the pectoral girdle, dorsally to just below the caudal oropharynx, and caudally to the anterior edge of the transverse septum. In control females, follicles are usually lined by single layers of flattened to cuboidal cells, whereas in control males the follicular epithelium typically consists of low to medium tall columnar cells. Follicles in males are often more densely packed and extend further rostrally, dorsally and ventrally than those of females, and some follicles may exhibit minor papillary infolding. Although small amounts of thyroid gland are usually evident at the sagittal midline, the tissue tends to be most abundant approximately 1/3 of the distance from the midline to the lateral surfaces of the fish. For medaka, recovery of thyroid tissue in diagnostic amounts was slightly better in frontal versus parasagittal sections, although the results were generally comparable.

Compared to medaka, **zebrafish** typically have far less thyroid tissue proportional to their body size. The scattered and variably-sized follicles are usually limited to a region at or very near the midline in the lower jaw, circumferentially surrounding the ventral aorta from its most anterior extent caudally to the level of the anterior bulbus arteriosus, with small amounts extending laterally into the gill arches.

In control fish, follicles are typically lined by a single layer of flattened to cuboidal epithelium, and there is no obvious evidence of sexual dimorphism. Optimal sagittal sections for visualizing thyroid gland in zebrafish are midline sections located between the left and right branchial chambers. In zebrafish, recovery of diagnostic amount of thyroid tissue was nearly identical in parasagittal and frontal sections.

Liver. In **medaka**, the liver is a chunky, essentially single-lobed organ, whose convex anterior surface is aligned with the curving posterior of the transverse septum and anterior coelomic cavity. Because the medaka liver is a comparatively large structure that extends almost the full width and height of the coelomic cavity, diagnostically sufficient quantities are readily captured in both parasagittal and frontal sections.

In contrast, the **zebrafish** liver is a slender, flattened, trilobed structure that abuts the transverse membrane anteriorly, and projects sinuous arms of tissue caudally along loops of intestinal tract in the mid to ventral coelomic cavity. Rather than tapering, these arms of liver tissue often widen terminally where the contours of the visceral mass permit. The widespread presence of this organ throughout the anterior to mid coelomic cavity usually guarantees that sufficient amounts of liver will be recovered in routine parasagittal and frontal sections.

Gonads. In **medaka**, the left and right gonads are conjoined and thus form a single tubular structure for much of the gonad length, although left and right accessory lobes branch off anteriolaterally at the caudal extent (creating a tri-lobed appearance in frontal sections). The main central lobe of the adult ovary is ventrally concave and typically located near the midline, although it may be pushed to the left of midline by the viscera below and swim bladder above. The more slender testis is commonly displaced to the left side of the coelomic cavity. Both the testis and ovary angle dorsally from their caudal to cranial aspects; consequently, the greatest lengths of gonad tissue are typically captured in either left parasagittal or frontal oblique (also called transverse oblique) sections. The mature ovary is typically much more easily recovered in sections as compared to the mature testis or immature (or regressed) gonads of either sex.

The gonads of **zebrafish** are not conjoined except near their caudal most aspect. From that point, the testes project anteriorly, laterally, and slightly dorsally. The right and left testes tend to hug the right and left coelomic walls, respectively, and there is typically a large dorsomedial bulge at the point where the anterior and posterior lobes of the swim bladder intersect. The ovaries exhibit a similar orientation; however, when mature, this tissue may occupy a substantial portion of the mid to caudal coelomic cavity, so that the left and right ovaries (each of which is axially bilobed) meet near the midline with a ventral concavity. Consequently, ovarian tissue is usually recovered in multiple parasagittal or frontal sections. However, because there is more separation between the right and left testes, diagnostically appropriate quantities can only be visualized in parasagittal sections acquired near the left and right coelomic walls. The two testes may be visualized in the same frontal (or frontal oblique) section, but because one testis is often positioned slightly more dorsal than the other, a single frontal section will often contain a much greater proportion of one testis relative to the other. Additionally, due to the medial location of the thyroid glands in zebrafish, it is difficult to capture adequate quantities

of testis and thyroid follicles in the same parasagittal section, although some parasagittal sections may contain the dorsomedial bulge of testis and a few small follicles in the gill arches.

CONCLUSION AND RECOMMENDATIONS

Overall, the most challenging tissues to collect consistently in histologic sections, in amounts and orientations suitable for toxicologic study, are the thyroid glands and testes of zebrafish, and the testes of medaka.

For **medaka**, the MEOGRT (USEPA, 2015) parasagittal sectioning method currently in use provides adequate amounts of thyroid gland, liver, and gonad tissue in orientations suitable for diagnostic evaluation. The procedure used in the current project, in which sections were acquired somewhat more medially than prescribed by the MEOGRT guidelines, did not yield appreciably greater amounts of thyroid gland, liver, or gonad tissue.

For **zebrafish**, adaptation of the MEOGRT procedure without modification would potentially yield inadequate amounts of thyroid gland for males and females, and recovery of testis in males may be inconsistent. Therefore, three viable options are proposed:

OPTION 1. Section zebrafish parasagittally from the left to right side. Acquire three sections near the left side of the fish to obtain the left gonad (especially testis), three sections near the midline to acquire thyroid, and three sections near the right side of the fish to obtain the right gonad. Liver would likely be present at all three locations. All in all, this would be somewhat time-consuming, and there would be no chance to return to the block if needed since it will be nearly exhausted.

OPTION 2. Section zebrafish parasagittally, but only acquire sections of the left gonad routinely, plus the midline sections for thyroid. Sections of the right gonad could still be obtained if needed. This would be less time-consuming for all involved.

OPTION 3. Section zebrafish in the frontal plane. Obtaining thyroid, liver, and gonad in the frontal plane is feasible. However, other tissues typically available in sagittal sections, such as the kidney, would not be present. Also, it may be technically more challenging for some laboratories to consistently obtain sections at the appropriate levels, and embed the fish so that they are consistently level in both the anterior-posterior and left-right axes.

REFERENCES

US Environmental Protection Agency (2015) OCSPP 890.2200: Medaka Extended One Generation Reproduction Test (MEOGRT). EPA No. 740-C-15-002, July, 2015. Available 3/11/16 at: http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPPT-2014-0766-0021.

APPENDIX A

PHOTOS AND LEGENDS

FIGURE

Figure 1. Female medaka thyroid (arrow), parasagittal section, Bar = 250 microns.

Figure 2. Male medaka thyroid (arrows), frontal section, Bar = 500 microns.

Figure 3. Male medaka thyroid (arrows), frontal section, Bar = 250 microns.

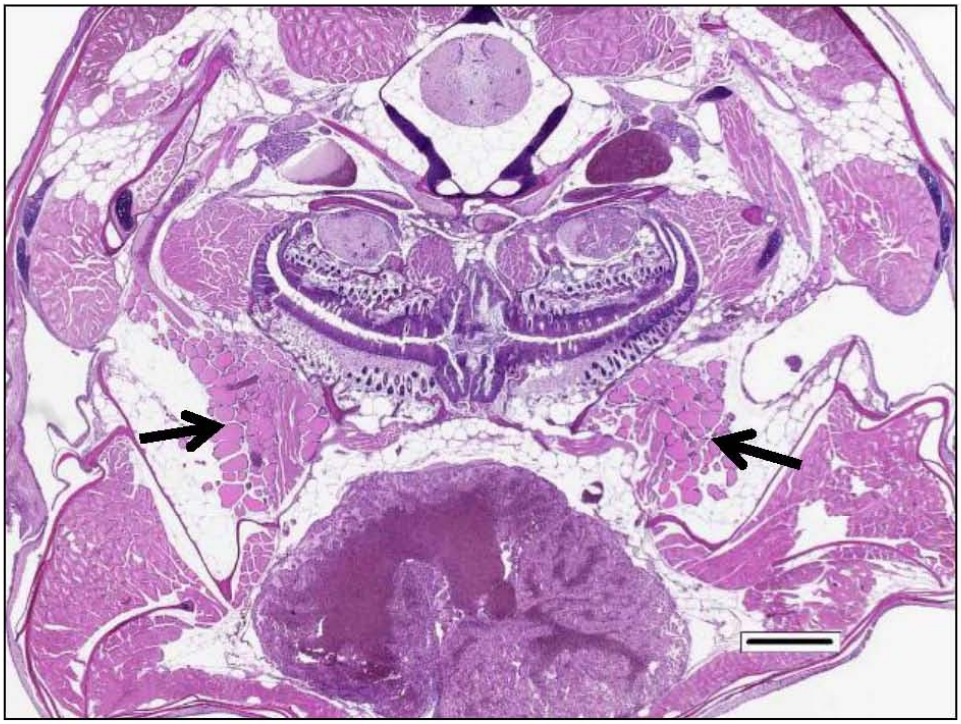


Figure 4. Male medaka thyroid (arrows), transverse section, Bar = 250 microns.

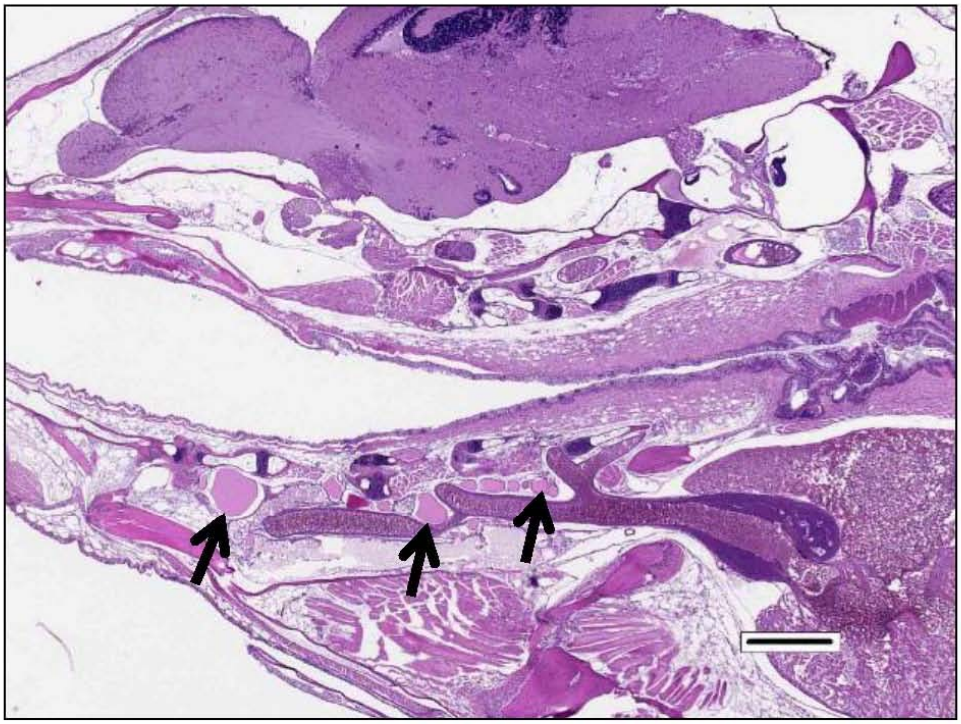


Figure 5. Zebrafish thyroid (arrows), parasagittal section, Bar = 500 microns.

Figure 6. Zebrafish thyroid, parasagittal section, Bar = 250 microns.

Figure 7. Zebrafish thyroid, parasagittal section, Bar = 250 microns.

Figure 8. Zebrafish thyroid follicle (arrow), parasagittal section, Bar = 100 microns.

Figure 9. Zebrafish thyroid (arrows), frontal section, Bar = 500 microns.

Figure 10. Zebrafish thyroid, frontal section, Bar = 250 microns.

Figure 11. Zebrafish thyroid (arrows), transverse section, Bar = 250 microns.

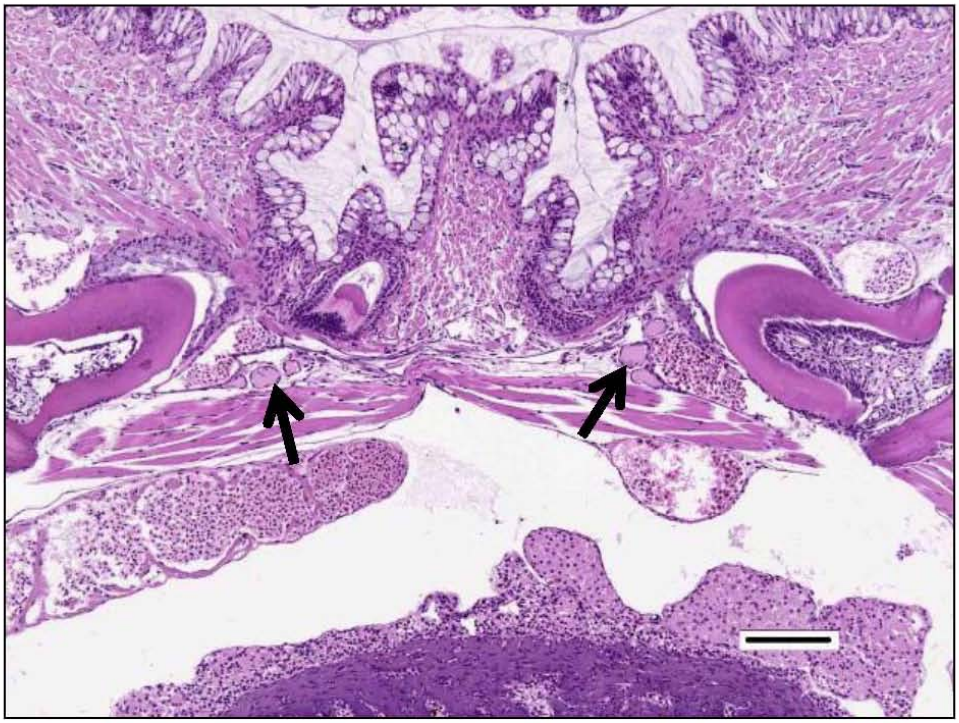


Figure 12. Zebrafish thyroid (arrows), transverse section, Bar = 100 microns.

Figure 13. Medaka thyroid (arrow), liver (L), and testis (T), parasagittal section, Bar = 500 microns.

Figure 14. Medaka liver (L) and ovary (O), parasagittal section, Bar = 800 microns.

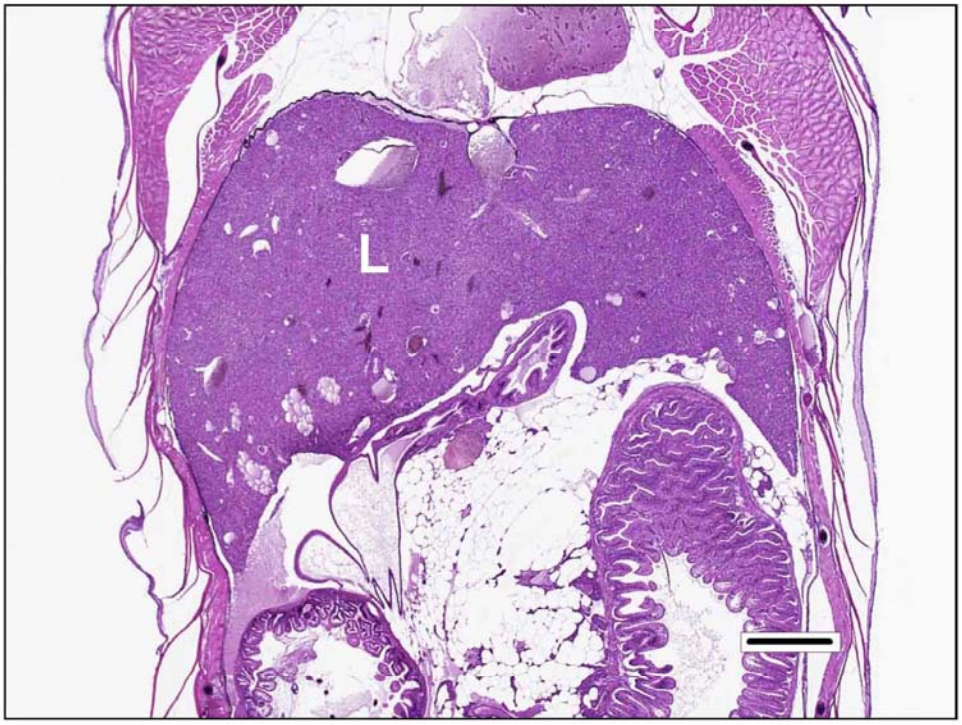


Figure 15. Female medaka liver (L), frontal section, Bar = 500 microns.

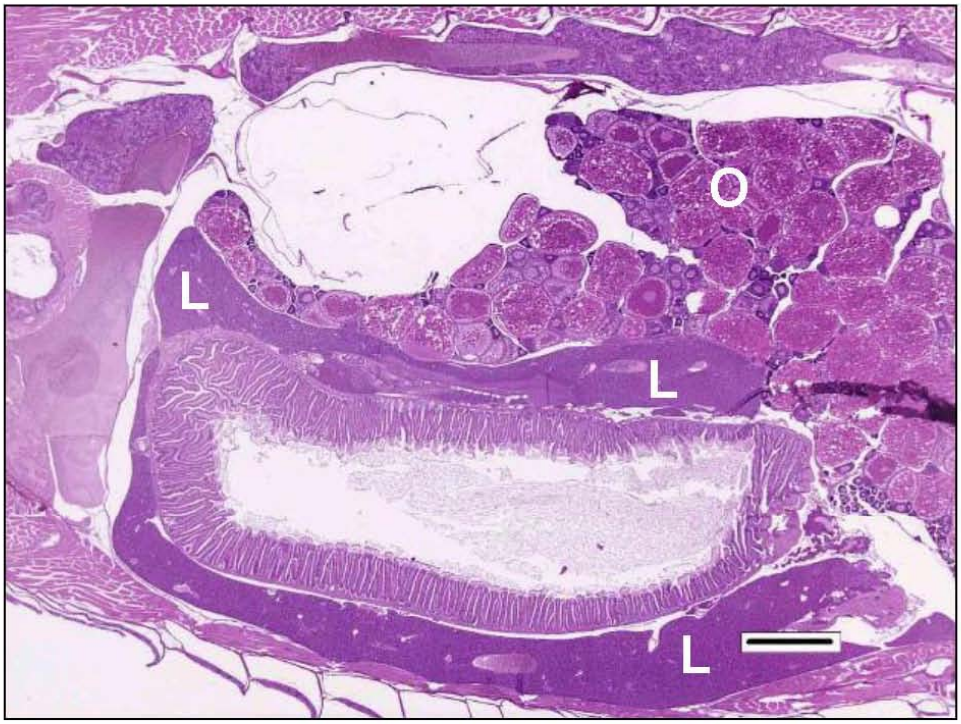


Figure 16. Zebrafish liver (L) and ovary (O), parasagittal section, Bar = 800 microns.

Figure 17. Zebrafish liver (L) and testis (T), frontal section, Bar = 800 microns

Figure 18. Medaka thyroid (arrow), liver (L), and testis (T), parasagittal section, Bar = 800 microns

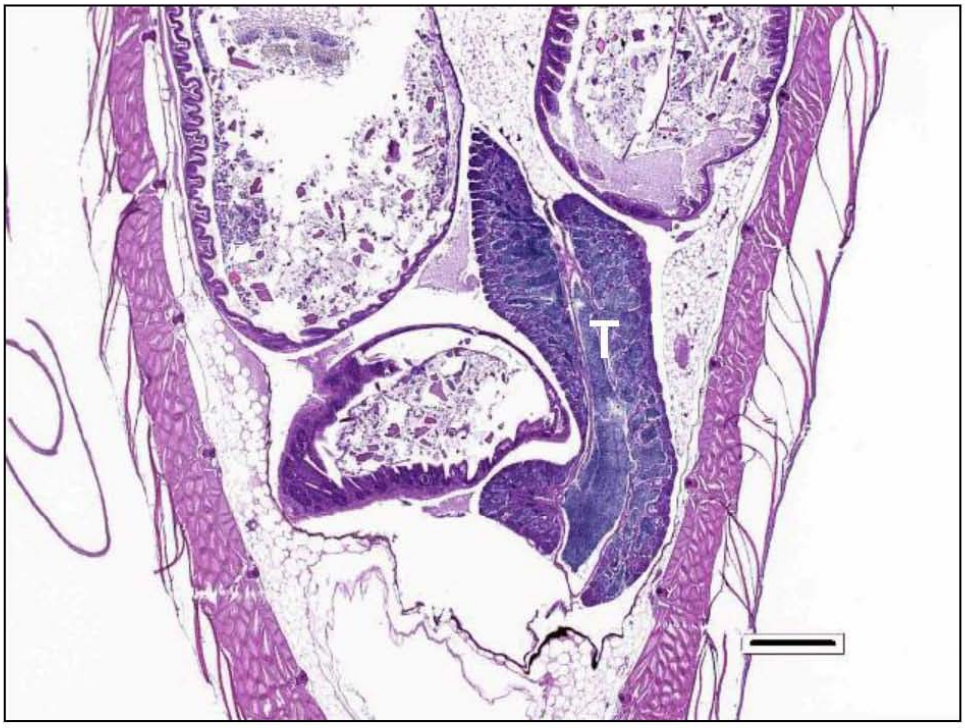


Figure 19. Medaka testis (T), frontal section, Bar = 500 microns

Figure 20. Medaka ovary (O), frontal section, Bar = 500 microns

Figure 21. Zebrafish testis (T), parasagittal section, Bar = 800 microns

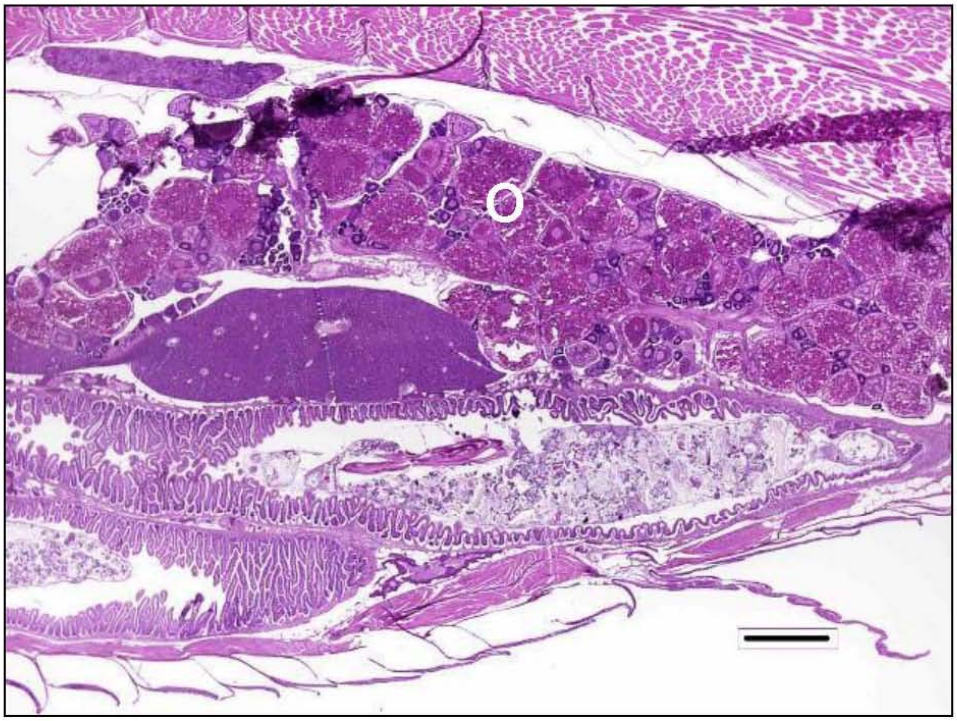


Figure 22. Zebrafish ovary (O), parasagittal section, Bar = 800 microns

APPENDIX B

EXCEL SPREADSHEET

Species	Sex	Section Plane	EPL No.	Fish No.	Total Number of Slides	Total Number of Sections	Thyroid + Sections	Thyroid ++ Sections	Thyroid +++ Sections	Total Thyroid Positive	% Total Thyroid Positive Sections	Mean Thyroid ++ and +++ Sections	Liver + Sections	Liver ++ Sections	Liver +++ Sections	Total Liver Positive	% Total Liver Positive Sections	Mean Liver ++ and +++ Sections	Gonad + Sections	Gonad ++ Sections	Gonad +++ Sections	Total Gonad Positive	% Total Gonad Positive Sections	Mean Gonad ++ and +++ Sections
medaka	m	parasagittal	E13554	1A	16	16	0	3	13	16	100%	16	0	0	16	16	100%	16	1	3	10	14	88%	13
medaka	m	parasagittal	E13554	2A	12	12	1	5	6	12	100%	11	0	1	11	12	100%	12	2	2	0	4	33%	2
											mean	**14**					mean	**14**					mean	**8**
medaka	f	parasagittal	E13555	1A	15	15	4	6	5	15	100%	11	0	0	15	15	100%	15	0	0	15	15	100%	15
medaka	f	parasagittal	E13555	2A	15	15	4	4	7	15	100%	11	0	0	15	15	100%	15	0	0	15	15	100%	15
											mean	**11**					mean	**15**					mean	**15**
zebrafish	m	parasagittal	E13552	1A	17	17	6	1	4	11	65%	5	0	7	10	17	100%	17	1	2	5	8	47%	7
zebrafish	m	parasagittal	E13552	2A	15	15	8	4	1	13	87%	5	2	12	1	15	100%	13	6	1	1	8	53%	2
zebrafish	m	parasagittal	E13553	2A	14	14	6	3	0	9	64%	3	2	7	5	14	100%	12	1	0	1	2	14%	1
											mean	**4**					mean	**14**					mean	**3**
zebrafish	f	parasagittal	E13553	1A	16	16	5	3	1	9	56%	4	0	0	16	16	100%	16	1	2	13	16	100%	15
											mean	**4**					mean	**16**					mean	**15**
medaka	m	frontal	E13554	1B	18	18	0	0	18	18	100%	18	0	3	15	18	100%	18	1	3	5	9	50%	8
medaka	m	frontal	E13554	2B	16	16	0	2	14	16	100%	16	1	4	11	16	100%	15	0	5	11	16	100%	16
											mean	**17**					mean	**17**					mean	**12**
medaka	f	frontal	E13555	1B	17	17	0	7	10	17	100%	17	0	1	16	17	100%	17	2	4	9	15	88%	13
medaka	f	frontal	E13555	2B	11	11	4	7	0	11	100%	7	1	1	6	8	73%	7	0	5	6	11	100%	11
											mean	**12**					mean	**12**					mean	**12**
zebrafish	m	frontal	E13552	1B	15	15	6	3	1	10	67%	4	0	7	8	15	100%	15	1	6	8	15	100%	14
zebrafish	m	frontal	E13552	2B	10	10	2	2	3	7	70%	5	1	3	6	10	100%	9	1	1	8	10	100%	9
zebrafish	m	frontal	E13553	1B	12	12	3	2	0	5	42%	2	0	5	7	12	100%	12	2	2	0	4	33%	2
zebrafish	m	frontal	E13553	2B	13	13	6	2	1	9	69%	3	0	5	8	13	100%	13	0	5	8	13	100%	13
											mean	**4**					mean	**12**					mean	**10**
medaka	u	transverse	E13554	1C	7	21	0	0	0	0	0%	0	1	4	16	21	100%	20	0	0	0	0	0%	0
medaka	u	transverse	E13554	2C	7	21	0	0	0	0	0%	0	0	7	14	21	100%	21	0	0	0	0	0%	0
medaka	u	transverse	E13554	3C	7	21	0	0	0	0	0%	0	0	0	21	21	100%	21	0	0	0	0	0%	0
medaka	u	transverse	E13554	4C	7	21	0	0	0	0	0%	0	0	2	19	21	100%	21	0	0	0	0	0%	0
											mean	**0**					mean	**21**					mean	**0**
zebrafish	u	transverse	E13552	1C	7	21	17	0	0	17	81%	0	0	0	0	0	0%	0	0	0	0	0	0%	0
zebrafish	u	transverse	E13552	2C	6	18	2	0	0	2	11%	0	1	1	3	5	28%	4	0	0	0	0	0%	0
zebrafish	u	transverse	E13553	1C	7	21	3	0	0	3	14%	0	0	0	3	3	14%	3	0	0	0	0	0%	0
zebrafish	u	transverse	E13553	2C	7	21	5	0	0	5	24%	0	0	0	0	0	0%	0	0	0	0	0	0%	0
											mean	**0**					mean	**2**					mean	**0**

BATT01-00404 FEL
Protocol Amendment 09

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka	
Amendment Number: 09	**Document ID Number:** BATT01-10 (00404)
Submitted By: Douglas J. Fort	**Date:** 5/1/2017

Amendment Relating To: BATT01-00404

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

[ ] Other (describe):

Original Specifications:

1. Protocol Amendment 7 and Protocol Amendment 3.

Changed To:

1. Eliminate Amendment 7, but add facility iodide analyses (attached) to Amendment 3.

Reason for Change:

1. Protocol Amendment 7 is an inadvertent duplicate of Amendment 3. Adds facility iodide analyses to Amendment 3.

Approval:

Study Director: _____ Date: 5/5/2017

Sponsor Representative: Vincent J. Brown Date: 5 MAY 2017

Page 1 of 2

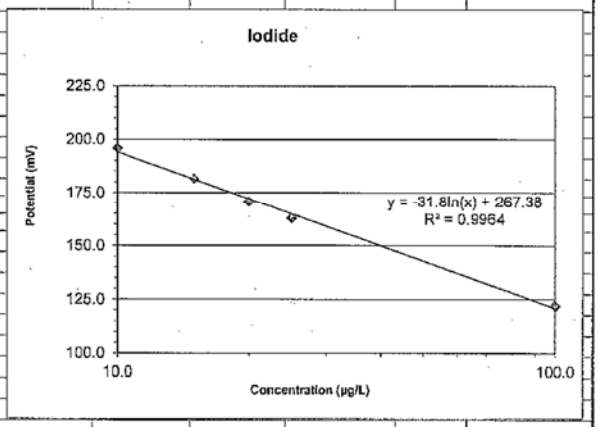
Attachment: Results of Dilution / Laboratory Control Water Iodide Analyses

FEL

FEL

FEL01-00000							
Iodide							
Test Article/Protocol:	ISE						
Kit:	ISE Probe						
Date:	1/16/2017						
Standard:	100 µg/L						
Sample Type:	Water						

Concentration (µg/L)	Standards mV Reading
0.0	0.0
10.0	196.0
15.0	181.7
20.0	170.8
25.0	162.9
100.0	122.1



FEL01-00000		Date:	1/16/2017		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	121.1	99.628		
	2		1	120.6	101.144		
	3		1	120.8	100.510		
	4		1	120.3	102.103		100.8
						SEM	0.104
Culture Room	1		1	195.8	9.497		
	2		1	195.9	9.468		
	3		1	197.1	9.117		
	4		1	196.2	9.379		9.4
						SEM	0.057
Exposure Room	1		1	196.4	9.320		
	2		1	197.2	9.088		
	3		1	196.9	9.174		
	4		1	195.8	9.497		9.3
						SEM	0.059
DI	1		1	855.0	0.000		

Analyst / Date: 1/16/2017

BATT01-00404
Protocol Amendment 10

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka	
Amendment Number: 10	**Document ID Number:** BATT01-10 (00404)
Submitted By: Douglas J. Fort	**Date:** 8/9/2017

Amendment Relating To: BATT01-00404

[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.

BATT01-00404 FEL

Protocol Amendment 10

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

BATT01-00404
Protocol Amendment 11

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

Amendment Number: 11 | **Document ID Number**: BATT01-10 (00404)

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

Amendment Relating To: BATT01-00404

[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 10, Original Specifications: Page 1, Title Page

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

3. Amendment 10, Original Specifications: 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.

4. Amendment 10, 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

BATT01-00404 FEL

Protocol Amendment 11

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

2. Amendment 10, Original Specifications: Page 1, Title Page

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

3. Amendment 10, Original Specifications: 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 leakt@eag.com.

4. Amendment 10, 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.

Reason for Change:

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

Approval:

Study Director: _____ Date: 9/28/2017

Sponsor Representative: _____ Date: 28 SEP 2017

BATT01-00404
Protocol Amendment 12

FEL

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka	
Amendment Number: 12	**Document ID Number:** BATT01-10 (00404)
Submitted By: Douglas J. Fort	**Date:** 2/12/2018

Amendment Relating To: BATT01-00404

[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: _____ Date: 13 FEB 2018

Page 1 of 1

BATT01-00404
Protocol Deviation 01

FEL

DOCUMENT DEVIATION FORM
Fort Environmental Laboratories

Document or Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka	
Deviation Number: 01	**Document ID Number:** BATT01-10 (00404)
Submitted By: Douglas J. Fort	**Date:** 9/18/2017
Deviation Relating To: BATT01-00404 **[X] Protocol [ ] Study Plan [ ] QAPP [ ] QMP [ ] SOP** **[ ] Other (describe):**	

Original Specifications:

1. Page 10, Section 10.6, Equipment

- "Analytical balance capable of measuring to 3 decimal places (mg);"

2. Page 14, Section 10.10.1.2. Endpoints Measured in All Study Fish

- **Body weights and length–** Whole body weight will be recorded at test termination and measured to the nearest 10 mg.

Deviation:

1. An analytical balance (Ohaus E10640) with 0.1 mg sensitivity was used to weigh fish and gonads.
2. Body weight was recorded to the nearest 0.1 mg rather than 10 mg.

Reason/Impact:

Since medaka are general smaller fish than fathead minnows, greater sensitivity in measurement was desired.

Schedule for Completion of Corrective Action: Immediate.

Approval:

Study Director: _____ Date: 9/20/2017

Sponsor: Vincent J. Brown Date: 20 SEP 2017

Page 1 of 1

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. Analytical Laboratory Data Report

ANALYTICAL DATA REPORT

Study Title

Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate
with Japanese Medaka

EAG Study Number: 84658
FEL Protocol Number: BATT01-10
FEL Study Number: BATT01-00404

Regulations

USEPA, FIFRA
Good Laboratory Practice (GLP) Standards, Final Rule (40 CFR Part 160, 1989),
US EPA OPPTS 890.1350, Fish Short-Term Reproduction Assay, modified,
and OECD, Test No. 229: Fish Short Term Reproduction Assay, modified

In-Life Testing Facility

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

Analytical Testing Facility

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

STATEMENT OF GLP COMPLIANCE

Compound: 2-Ethylhexyl 4-Hydroxybenzoate

Study Title: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

The study described in this report, with the following exception, 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.

This was the only exception 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 Laboratories along with facility records and a copy of the final report and the study plan.

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

John Aufderheide Date
Senior Director; Aquatic, Plant and Invertebrate Toxicology
Analytical Bio-Chemistry Laboratories, Inc.

QUALITY ASSURANCE STATEMENT

EAG Laboratories' Quality Assurance Unit (QAU) reviewed Study No. 84658 entitled, "Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka", 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
14 February 2017	Procedure: Sample Fortification and Dilution	15 February 2017 / 23 February 2017	16 February 2017 / 23 February 2017
02-04 May 2017	Raw Data and Draft Report	23 May 2017 / 25 October 2017	25 October 2017 / 25 October 2017
29 March 2018	Final Analytical Phase Report	29 March 2018 / 02 April 2018	02 April 2018 / 02 April 2018

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

Jeri Hofen 17 May 2018

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

Date

FEL Protocol Number: BATT01-10 FEL Study Number: BATT01-00404

APPROVAL

The following is to be signed by appropriate personnel:

Principal Investigator:

Name (signed): _____ Date: 17May15

Name (typed): Tom Leak, Ph.D.
Principal Scientist
Analytical Bio-Chemistry Laboratories, Inc.

Management:

Name (signed): _____ Date: 17May15

Name (typed): John Aufderheide
Senior Director; Aquatic, Plant and
Invertebrate Toxicology
Analytical Bio-Chemistry Laboratories, Inc.

STUDY PERSONNEL

Tom Leak, Ph.D.	Principal Scientist/ Principal Investigator
Wesley Fain	Senior Scientist
Danah O'Connor	Principal Technician
Ashley Seifert	Associate Scientist I

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1.0 INTRODUCTION

All medaka short-term reproduction test diluter and stock solution samples obtained from Fort Environmental Labs, Stillwater, Oklahoma (FEL) during this study (FEL Protocol No. BATT01-10, study number BATT01-00404) 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 0.106 to 26.6 µg/L.

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, was received from FEL on 26 October 2016 and given Reference No. MM-16870-00001. 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
CAS No.:	5153-25-3

2.2 Analytical Method

Samples were diluted with 50:50 methanol:water at FEL and 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).

2.3 Study Samples

2.3.1 Sample Sources, Storage, and Receipt Dates

A total of 134 study samples were received from Fort Environmental Labs from 17 November 2016 to 28 February 2017, stored at room temperature and typically analyzed within 24 hours of processing. 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.

The following instrument and instrument parameters were used in the analyses of the medaka extended one-generation reproductive test water samples:

Instrument:	ABSciex API-6500 Q-Trap
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:	5 µL
Column Temp:	40 °C
Mass Spec. Scan Type:	MRM
Polarity:	Negative

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

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. A linear standard curve was derived by plotting concentration vs. response for calibration standards using least squares regression with 1/x weighting. 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):

$$\frac{\left(\begin{array}{c}\text{µg/L from}\\ \text{standard curve}\end{array}\right)\left(\begin{array}{c}\text{analysis volume}\\ \text{in mL}\end{array}\right)}{\text{sample volume in mL}} = \text{µg/L}$$

Example calculation for low spike at initiation (EAG ID 84658-SPK19):

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

where:

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

Standard Curve: y = 40,791.24x + 3,450.511
Sample Peak Area: = 44,567

Substituting the Sample CPS in the following equation and solving for x gave the concentration of 2-ethylhexyl 4-hydroxybenzoate:

x = (44,567 – 3,450.511) / (40,791.24)
x = 1.00798 µg/L

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 2.02 µg/L.

Recovery of low spike sample at initiation:

$$\frac{2.02\ \mu g/L}{2.04\ \mu g/L} \times 100 = 99\%$$

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

Lowest standard concentration: 0.106 µg/L
Volume for analysis: 10 mL
Volume of sample: 5 mL

therefore:

$$MQL = \frac{(0.106\ \mu g/L) \times (10\ mL)}{5\ mL} = 0.212\ \mu g/L$$

3.0 RESULTS AND DISCUSSIONS

Analytical sets were typically run with six 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 medaka extended one-generation reproductive test 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 November 2016. The concentration range for 2-ethylhexyl 4-hydroxybenzoate was 0.106 to 26.6 µg/L in methanol.

A representative calibration curve is provided in Figure 1.

3.2 Method Validation

The method of analysis for 2-ethylhexyl 4-hydroxybenzoate was validated in EAG Study #83231.

3.3 Accuracy and Precision

The QC samples were prepared with each set from a 2-ethylhexyl 4-hydroxybenzoate spiking solution prepared on 17 November 2016. The QC sample concentrations ranged from 2.04 to 110 µg/L.

The results from the analysis of the QC fortifications are presented in Table 2.

3.4 Medaka Short-Term Reproduction Test Water Sample Concentrations

The calculated concentrations of 2-ethylhexyl 4-hydroxybenzoate in medaka short-term reproduction test water samples are provided in Table 3 and Table 4.

4.0 CONCLUSIONS

The percent of nominal concentration recovered from the 2.04 to 110 µg/L 2-ethylhexyl 4-hydroxybenzoate QC spikes in reagent water ranged from 92 to 108%, respectively.

These results indicate the method is acceptable for the recovery of 2-ethylhexyl 4-hydroxybenzoate in medaka short-term reproduction test 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 Medaka Fish Short-Term Reproduction Test 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
CI5038 84658 11222016A	2-EHHB Stock (001)	15 November 2016	21 November 2016	22 November 2016	NA	Accepted
CI5038 84658 12012016A	2-EHHB Stock (002)	27 November 2016	01 December 2016	01 December 2016	NA	Accepted
	2-EHHB Stock (003)	30 November 2016				
X 84658 01172017C	Blank (004)	11 January 2017	17 January 2017	17 January 2017	NA	Accepted
	0.0 µg/L (005)					
	3.6 µg/L (006)					
	10.9 µg/L (007)					
	33 µg/L (008)					
	100 µg/L (009)					
	Stock (010)					
X 84658 01182017G	Blank (011)	17 January 2017	18 January 2017	19 January 2017	NA	Accepted
	0.0 µg/L (012)					
	3.6 µg/L (013)					
	10.9 µg/L (014)					
	33 µg/L (015)					
	100 µg/L (016)					
	2-EHHB Stock (017)					

Table 1 Data Summary of Medaka Fish Short-Term Reproduction Test 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
X 84658 01242017C	Blank (018) 0.0 µg/L (019) 3.6 µg/L (020) 10.9 µg/L (021) 33 µg/L (022) 100 µg/L (023) 2-EHHB Stock (024)	23 January 2017	24 January 2017	24 January 2017	NA	Accepted
CI5038 84658 01272017A	Blank (025) 0.0 µg/L (026) 3.6 µg/L (027) 10.9 µg/L (028) 33 µg/L (029) 100 µg/L (030) 2-EHHB Stock (031)	25 January 2017	27 January 2017	27 January 2017	NA	Accepted
CI5038 84658 02012017A	Blank (032) 2-EHHB Stock (033)	30 January 2017	31 January 2017	01 February 2017	NA	Accepted
CI5038 84658 02022017A	Blank (034) 0.0 µg/L (035) 3.6 µg/L (036) 10.9 µg/L (037) 33 µg/L (038) 100 µg/L (039) 2-EHHB Stock (040)	01 February 2017	02 February 2017	02 February 2017	NA	Accepted

Table 1 Data Summary of Medaka Fish Short-Term Reproduction Test 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
CI5038 84658 02072017B	Blank (041)	06 February 2017	07 February 2017	07 February 2017	NA	Accepted
	0.0 µg/L A (042)					
	0.0 µg/L B (043)					
	0.0 µg/L C (044)					
	0.0 µg/L D (045)					
	3.6 µg/L A (046)					
	3.6 µg/L B (047)					
	3.6 µg/L C (048)					
	3.6 µg/L D (049)					
	10.9 µg/L A (050)					
	10.9 µg/L B (051)					
	10.9 µg/L C (052)					
	10.9 µg/L D (053)					
	33 µg/L A (054)					
	33 µg/L B (055)					
	33 µg/L C (056)					
	33 µg/L D (057)					
	100 µg/L A (058)					
	100 µg/L B (059)					
	100 µg/L C (060)					
	100 µg/L D (061)					
	2-EHHB Stock (062)					

Table 1 Data Summary of Medaka Fish Short-Term Reproduction Test 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
CI5038 84658 02142017A	Blank (065)	13 February 2017	14 February 2017	15 February 2017	NA	Accepted
	0.0 µg/L A (066)					
	0.0 µg/L B (067)					
	0.0 µg/L C (068)					
	0.0 µg/L D (069)					
	3.6 µg/L A (070)					
	3.6 µg/L B (071)					
	3.6 µg/L C (072)					
	3.6 µg/L D (073)					
	10.9 µg/L A (074)					
	10.9 µg/L B (075)					
	10.9 µg/L C (076)					
	10.9 µg/L D (077)					
	33 µg/L A (078)					
	33 µg/L B (079)					
	33 µg/L C (080)					
	33 µg/L D (081)					
	100 µg/L A (082)					
	100 µg/L B (083)					
	100 µg/L C (084)					
	100 µg/L D (085)					
	2-EHHB Stock (086)					

Table 1 Data Summary of Medaka Fish Short-Term Reproduction Test 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
CI5038 84658 02232017A	Blank (089)	20 February 2017	22 February 2017	23 February 2017	NA	Accepted
	0.0 µg/L A (090)					
	0.0 µg/L B (091)					
	0.0 µg/L C (092)					
	0.0 µg/L D (093)					
	3.6 µg/L A (094)					
	3.6 µg/L B (095)					
	3.6 µg/L C (096)					
	3.6 µg/L D (097)					
	10.9 µg/L A (098)					
	10.9 µg/L B (099)					
	10.9 µg/L C (100)					
	10.9 µg/L D (101)					
	33 µg/L A (102)					
	33 µg/L B (103)					
	33 µg/L C (104)					
	33 µg/L D (105)					
	100 µg/L A (106)					
	100 µg/L B (107)					
	100 µg/L C (108)					
	100 µg/L D (109)					
CI5038 84658 02272017A	2-EHHB Stock (110)			27 February 2017		

Table 1 Data Summary of Medaka Fish Short-Term Reproduction Test 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
X 84658 03012017A	Blank (113)	27 February 2017	28 February 2017	01 March 2017	NA	Accepted
	0.0 µg/L A (114)					
	0.0 µg/L B (115)					
	0.0 µg/L C (116)					
	0.0 µg/L D (117)					
	3.6 µg/L A (118)					
	3.6 µg/L B (119)					
	3.6 µg/L C (120)					
	3.6 µg/L D (121)					
	10.9 µg/L A (122)					
	10.9 µg/L B (123)					
	10.9 µg/L C (124)					
	10.9 µg/L D (125)					
	33 µg/L A (126)					
	33 µg/L B (127)					
	33 µg/L C (128)					
	33 µg/L D (129)					
	100 µg/L A (130)					
	100 µg/L B (131)					
	100 µg/L C (132)					
	100 µg/L D (133)					
	2-EHHB Stock (134)					

Table 2. QC Data of 2-Ethylhexyl 4-hydroxybenzoate Measured in Medaka Short-Term Reproduction Test Water Samples

	Nominal Concentration					
	Low Spike		High Spike		High Spike	
	2.04 µg/L		102 µg/L		110 µg/L	
LC-MS/MS Filename:	Measured Concentration (µg/L)	% Recovery	Measured Concentration (µg/L)	% Recovery	Measured Concentration (µg/L)	% Recovery
	2.05	100	--	--	116	105
CI5038 84658 11222016A	--	--	--	--	110	100
	--	--	--	--	110	100
CI5038 84658 12012016A	2.02	99	104	102	--	--
X 84658 01172017C	1.88	92	99.5	98	--	--
X 84658 01182017G	1.95	96	103	101	--	--
X 84658 01242017C	1.92	94	98.2	96	--	--
CI5038 84658 01272017A	2.04	100	104	102	--	--
CI5038 84658 02012017A	1.89	93	103	101	--	--
CI5038 84658 02022017A	1.97	97	110	108	--	--
CI5038 84658 02072017B	2.02	99	110	108	--	--
CI5038 84658 02142017A	1.93	95	98.6	97	--	--
CI5038 84658 02232017A	2.00	98	110	108	--	--
X 84658 03012017A	2.03	100	106	104	--	--

Table 3 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate in Medaka Short-Term Reproduction Test Pre-Exposure Samples

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
001	2-EHHB Stock	6,000	26,700
002	2-EHHB Stock	6,000	4,730
003	2-EHHB Stock	6,000	6,470
004	Blank	0.00	<MQL [b]
005	0.0 µg/L	0.00	<MQL [b]
006	3.6 µg/L	3.60	18.4
007	10.9 µg/L	10.9	54.4
008	33 µg/L	33.0	91.3
009	100 µg/L	100	266
010	Stock	6,000	15,600
011	Blank	0.00	<MQL [b]
012	0.0 µg/L	0.00	<MQL [b]
013	3.6 µg/L	3.60	16.2
014	10.9 µg/L	10.9	35.6
015	33 µg/L	33.0	71.5
016	100 µg/L	100	220
017	Stock	6,000	18,000
018	Blank	0.00	<MQL [b]
019	0.0 µg/L	0.00	1.45
020	3.6 µg/L	3.60	6.25
021	10.9 µg/L	10.9	17.1
022	33 µg/L	33.0	34.9
023	100 µg/L	100	102
024	Stock	6,000	8,990
025	Blank	0.00	<MQL [b]
026	0.0 µg/L	0.00	<MQL [b]
027	3.6 µg/L	3.60	4.34
028	10.9 µg/L	10.9	11.4
029	33 µg/L	33.0	24.5
030	100 µg/L	100	64.9
031	Stock	6,000	7,940

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

[b] MQL = 0.212 µg/L

NA = Not applicable

Table 3 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate in Medaka Short-Term Reproduction Test Pre-Exposure Samples (continued)

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
032	Blank	0.00	<MQL [b]
033	Stock	6,000	7,050
034	Blank	0.00	<MQL [b]
035	0.0 µg/L	0.00	<MQL [b]
036	3.6 µg/L	3.60	1.87
037	10.9 µg/L	10.9	6.63
038	33 µg/L	33.0	19.6
039	100 µg/L	100	84.4
040	Stock	6,000	3,850

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (µg/L) × Analysis Volume (mL) / Sample Volume (mL)
[b] MQL = 0.212 µg/L
NA = Not applicable

Table 4 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate in Medaka Short-Term Reproduction Test Exposure Samples

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
Study Day 0 (Initiation)			
041	Blank	0.00	<MQL [b]
042	0.0 µg/L A	0.00	<MQL [b]
043	0.0 µg/L B	0.00	<MQL [b]
044	0.0 µg/L C	0.00	<MQL [b]
045	0.0 µg/L D	0.00	<MQL [b]
046	3.6 µg/L A	3.60	4.38
047	3.6 µg/L B	3.60	4.05
048	3.6 µg/L C	3.60	3.47
049	3.6 µg/L D	3.60	3.44
050	10.9 µg/L A	10.9	12.3
051	10.9 µg/L B	10.9	11.4
052	10.9 µg/L C	10.9	10.9
053	10.9 µg/L D	10.9	14.4
054	33 µg/L A	33.0	42.8
055	33 µg/L B	33.0	38.8
056	33 µg/L C	33.0	34.2
057	33 µg/L D	33.0	39.7
058	100 µg/L A	100	130
059	100 µg/L B	100	114
060	100 µg/L C	100	128
061	100 µg/L D	100	120
062	Stock	6,000	9,700

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (µg/L) × Analysis Volume (mL) / Sample Volume (mL)
[b] MQL = 0.212 µg/L
NA = Not applicable

Table 4 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate in Medaka Short-Term Reproduction Test Exposure Samples (continued)

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
Study Day 7			
065	Blank	0.00	<MQL [b]
066	0.0 µg/L A	0.00	<MQL [b]
067	0.0 µg/L B	0.00	<MQL [b]
068	0.0 µg/L C	0.00	<MQL [b]
069	0.0 µg/L D	0.00	<MQL [b]
070	3.6 µg/L A	3.60	2.66
071	3.6 µg/L B	3.60	2.41
072	3.6 µg/L C	3.60	2.26
073	3.6 µg/L D	3.60	2.30
074	10.9 µg/L A	10.9	10.0
075	10.9 µg/L B	10.9	9.32
076	10.9 µg/L C	10.9	9.26
077	10.9 µg/L D	10.9	9.09
078	33 µg/L A	33.0	29.5
079	33 µg/L B	33.0	25.6
080	33 µg/L C	33.0	26.2
081	33 µg/L D	33.0	25.9
082	100 µg/L A	100	99.1
083	100 µg/L B	100	92.7
084	100 µg/L C	100	92.4
085	100 µg/L D	100	87.9
086	Stock	6,000	7,340

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (µg/L) × Analysis Volume (mL) / Sample Volume (mL)
[b] MQL = 0.212 µg/L
NA = Not applicable

Table 4 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate in Medaka Short-Term Reproduction Test Exposure Samples (continued)

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
Study Day 14			
089	Blank	0.00	<MQL [b]
090	0.0 µg/L A	0.00	<MQL [b]
091	0.0 µg/L B	0.00	<MQL [b]
092	0.0 µg/L C	0.00	<MQL [b]
093	0.0 µg/L D	0.00	<MQL [b]
094	3.6 µg/L A	3.60	3.36
095	3.6 µg/L B	3.60	3.15
096	3.6 µg/L C	3.60	2.88
097	3.6 µg/L D	3.60	2.95
098	10.9 µg/L A	10.9	13.1
099	10.9 µg/L B	10.9	11.6
100	10.9 µg/L C	10.9	11.4
101	10.9 µg/L D	10.9	12.2
102	33 µg/L A	33.0	38.5
103	33 µg/L B	33.0	33.0
104	33 µg/L C	33.0	33.5
105	33 µg/L D	33.0	32.6
106	100 µg/L A	100	106
107	100 µg/L B	100	95.6
108	100 µg/L C	100	93.1
109	100 µg/L D	100	91.4
110	Stock	6,000	28,900 [c]

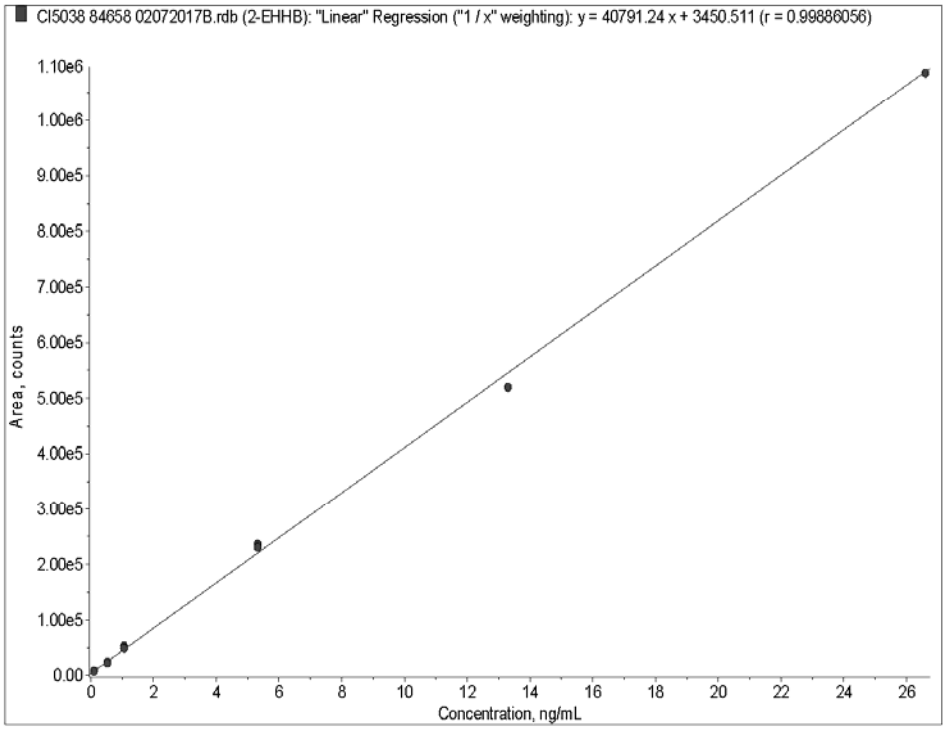
[a] Measured Conc. (µg/L) = Measured Conc. from Curve (µg/L) × Analysis Volume (mL) / Sample Volume (mL)
[b] MQL = 0.212 µg/L
[c] Original result is DNU due to being outside of the standard curve. Original final dilution was further diluted and analyzed.
NA = Not applicable

Table 4 Concentrations of 2-Ethylhexyl 4-Hydroxybenzoate in Medaka Short-Term Reproduction Test Exposure Samples (continued)

Sample Number	Sample ID	Nominal Conc. (µg/L)	Measured Conc. (µg/L) [a]
Study Day 21			
113	Blank	0.00	<MQL [b]
114	0.0 µg/L A	0.00	<MQL [b]
115	0.0 µg/L B	0.00	<MQL [b]
116	0.0 µg/L C	0.00	<MQL [b]
117	0.0 µg/L D	0.00	<MQL [b]
118	3.6 µg/L A	3.60	2.87
119	3.6 µg/L B	3.60	2.55
120	3.6 µg/L C	3.60	2.49
121	3.6 µg/L D	3.60	2.46
122	10.9 µg/L A	10.9	9.70
123	10.9 µg/L B	10.9	8.17
124	10.9 µg/L C	10.9	8.61
125	10.9 µg/L D	10.9	8.61
126	33 µg/L A	33.0	43.9
127	33 µg/L B	33.0	32.5
128	33 µg/L C	33.0	34.2
129	33 µg/L D	33.0	35.4
130	100 µg/L A	100	104
131	100 µg/L B	100	93.9
132	100 µg/L C	100	115
133	100 µg/L D	100	110
134	Stock	6,000	6,030

[a] Measured Conc. (µg/L) = Measured Conc. from Curve (µg/L) × Analysis Volume (mL) / Sample Volume (mL)
[b] MQL = 0.212 µg/L
NA = Not applicable

Figure 1 Typical Calibration Curve for 2-Ethylhexyl 4-Hydroxybenzoate from the Medaka Short-Term Reproduction Test Water Sample Analysis



APPENDIX 1 CERTIFICATE OF ANALYSIS

TCI

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 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.

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Stock
Data file name: CI5038 84658 11222016A

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-001	2-EHHB Stock	6000	33.40956	5	4000	26700	445%
84658-SPK1	Low Spike	2.04	1.02713	5	10	2.05	100%
84658-SPK2	High Spike A	110	5.81554	5	100	116	105%
84658-SPK3	High Spike B	110	5.48097	5	100	110	100%
84658-SPK4	High Spike C	110	5.47574	5	100	110	100%
					mean =	112	102%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106 \text{ ng/mL} \times 10 \text{ mL}}{5 \text{ mL}} = 0.212 \text{ ng/mL}$$

① Calculated Conc. (µg/L) = Concentration From Curve (µg/L) x Analysis Volume (mL) / Sample Volume (mL)

Prepared by: Sarah R. O'Connor Date: 30May17

Reviewed by: [illegible] Date: 31May17

Study Director: [illegible] Date: 31May17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_EQ Results.xls]Stock_17Nov16

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Stock
Data file name: CI5030 84658 12012016A

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-002	2-EHHB Stock	6000	5.90659	5	4000	4730	79%
84658-003	2-EHHB Stock	6000	8.08244	5	4000	6470	108%
84658-SPK5	Low Spike	2.04	1.00948	5	10	2.02	99%
84658-SPK6	High Spike	102	5.19494	5	100	104	102%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106 \text{ ng/mL} \times 10 \text{ mL}}{5 \text{ mL}} = 0.212 \text{ ng/mL}$$

① Calculated Conc. (µg/L) = Concentration From Curve (µg/L) x Analysis Volume (mL) / Sample Volume (mL)

Prepared by: Dawn R O'Connor Date: 04 Jan 17
Reviewed by: XyA Date: 22 Mar 17
Study Director: AJZ Date: 31 Mar 17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_EQ Results.xls]Stock_01Dec16

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Diluter EQ
Data file name: X 84658 01172017C

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-004	Blank	0.00	No Peak	5	10	< MQL	NA
84658-005	0.0 µg/L	0.00	< low std	5	10	< MQL	NA
84658-006	3.6 µg/L	3.60	9.18358	5	10	18.4	511%
84658-007	10.9 µg/L	10.9	13.59447	5	20	54.4	499%
84658-008	33 µg/L	33.0	9.12639	5	50	91.3	277%
84658-009	100 µg/L	100	13.28841	5	100	266	266%
84658-010	Stock	6000	19.52104	5	4000	15600	260%
84658-SPK7	Low Spike	2.04	0.93843	5	10	1.88	92%
84658-SPK8	High Spike	102	4.97660	5	100	99.5	98%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106\ \mu g/L \times 10\ mL}{5\ mL} = 0.212\ (\mu g/L)$$

① Calculated Conc. (µg/L) = Concentration From Curve (µg/L) x Analysis Volume (mL) / Sample Volume (mL)

Prepared by: Danah R. O'Connor Date: 24Mar17
Reviewed by: [illegible] Date: 27Mar17
Study Director: ATZ Date: 31 Mar 17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_EQ Results.xls]Diluter EQ_12Jan17

FEL Protocol Number: BATT01-10 FEL Study Number: BATT01-00404

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Diluter EQ
Data file name: X 84658 01182017G

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-011	Blank	0.00	No Peak	5	10	< MQL	NA
84658-012	0.0 µg/L	0.00	< low std	5	10	< MQL	NA
84658-013	3.6 µg/L	3.60	8.11977	5	10	16.2	450%
84658-014	10.9 µg/L	10.9	8.89400	5	20	35.6	327%
84658-015	33 µg/L	33.0	7.15468	5	50	71.5	217%
84658-016	100 µg/L	100	11.00426	5	100	220	220%
84658-017	Stock	6000	22.47566	5	4000	18000	300%
84658-SPK9	Low Spike	2.04	0.97570	5	10	1.95	96%
84658-SPK10	High Spike	102	5.15485	5	100	103	101%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106\ \mu g/L \times 10\ mL}{5\ mL} = 0.212\ (\mu g/L)$$

① Calculated Conc. (µg/L) = Concentration From Curve (µg/L) x Analysis Volume (mL) / Sample Volume (mL)

Prepared by: Derrick R. O'Connor Date: 24 May 17
Reviewed by: Date: 22 May 17
Study Director: Date: 31 May 17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_EQ Results.xls]Diluter EQ_18Jan17

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Diluter EQ
Data file name: X 84658 01242017C

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-018	Blank	0.00	No Peak	5	10	< MQL	NA
84658-019	0.0 µg/L	0.00	0.72380	5	10	1.45	NA
84658-020	3.6 µg/L	3.60	3.12507	5	10	6.25	174%
84658-021	10.9 µg/L	10.9	4.28474	5	20	17.1	157%
84658-022	33 µg/L	33.0	3.49387	5	50	34.9	106%
84658-023	100 µg/L	100	5.10135	5	100	102	102%
84658-024	Stock	6000	11.23245	5	4000	8990	150%
84658-SPK11	Low Spike	2.04	0.95816	5	10	1.92	94%
84658-SPK12	High Spike	102	4.90853	5	100	98.2	96%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106\ \mu g/L \times 10\ mL}{5\ mL} = 0.212\ (\mu g/L)$$

① Calculated Conc. (µg/L) = Concentration From Curve (µg/L) x Analysis Volume (mL) / Sample Volume (mL)

Prepared by: Dinah R O'Connor Date: 04 Feb 17
Reviewed by: [illegible] Date: 22 Mar 17
Study Director: AJZ Date: 31 March 17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_EQ Results.xls]Diluter EQ_24Jan17

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Diluter EQ
Data file name: CI5038 84658 01272017A

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-025	Blank	0.00	No Peak	5	10	< MQL	NA
84658-026	0.0 µg/L	0.00	< low std	5	10	< MQL	NA
84658-027	3.6 µg/L	3.60	2.17046	5	10	4.34	121%
84658-028	10.9 µg/L	10.9	2.85794	5	20	11.4	105%
84658-029	33 µg/L	33.0	2.44792	5	50	24.5	74%
84658-030	100 µg/L	100	3.24738	5	100	64.9	65%
84658-031	Stock	6000	9.93040	5	4000	7940	132%
84658-SPK13	Low Spike	2.04	1.02113	5	10	2.04	100%
84658-SPK14	High Spike	102	5.21499	5	100	104	102%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106\ \mu g/L \times 10\ mL}{5\ mL} = 0.212\ (\mu g/L)$$

① Calculated Conc. (µg/L) = Concentration From Curve (µg/L) x Analysis Volume (mL) / Sample Volume (mL)

Prepared by: _____ Date: 04Mar17

Reviewed by: _____ Date: 22Mar17

Study Director: _____ Date: 31Mar17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_EQ Results.xls]Diluter EQ_27Jan17

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Stock
Data file name: CI5038 84658 02012017A

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-032	Blank	0.00	No Peak	5	10	< MQL	NA
84658-033	Stock	6000	8.81461	5	4000	7050	118%
84658-SPK15	Low Spike	2.04	0.94399	5	10	1.89	93%
84658-SPK16	High Spike	102	5.13486	5	100	103	101%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106\ \mu g/L \times 10\ mL}{5\ mL} = 0.212\ (\mu g/L)$$

① Calculated Conc. (µg/L) = Concentration From Curve (µg/L) x Analysis Volume (mL) / Sample Volume (mL)

Prepared by: _____ Date: 14 Mar 17
Reviewed by: _____ Date: 22 Mar 17
Study Director: _____ Date: 31 Mar 17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_EQ Results.xls]Stock_31Jan17

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Diluter EQ
Data file name: CI5038 84658 02022017A

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-034	Blank	0.00	No Peak	5	10	< MQL	NA
84658-035	0.0 µg/L	0.00	No Peak	5	10	< MQL	NA
84658-036	3.6 µg/L	3.60	0.93588	5	10	1.87	52%
84658-037	10.9 µg/L	10.9	1.65670	5	20	6.63	61%
84658-038	33 µg/L	33.0	1.95776	5	50	19.6	59%
84658-039	100 µg/L	100	4.22096	5	100	84.4	84%
84658-040	Stock	6000	4.81358	5	4000	3850	64%
84658-SPK17	Low Spike	2.04	0.98434	5	10	1.97	97%
84658-SPK18	High Spike	102	5.49410	5	100	110	108%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106\ \mu g/L \times 10\ mL}{5\ mL} = 0.212\ (\mu g/L)$$

① Calculated Conc. (µg/L) = Concentration From Curve (µg/L) x Analysis Volume (mL) / Sample Volume (mL)

Prepared by: Date: 2/16/17
Reviewed by: Date: 2/16/17
Study Director: Date: 3/1/17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_EQ Results.xls]Diluter EQ_02Feb17

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Initiation
Data file name: CI5038 84658 02072017b

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-041	Blank	0.00	No Peak	5	10	< MQL	NA
84658-042	0.0 µg/L A	0.00	< low std	5	10	< MQL	NA
84658-043	0.0 µg/L B	0.00	No Peak	5	10	< MQL	NA
84658-044	0.0 µg/L C	0.00	No Peak	5	10	< MQL	NA
84658-045	0.0 µg/L D	0.00	< low std	5	10	< MQL	NA
84658-046	3.6 µg/L A	3.60	2.18864	5	10	4.38	122%
84658-047	3.6 µg/L B	3.60	2.02262	5	10	4.05	113%
84658-048	3.6 µg/L C	3.60	1.73500	5	10	3.47	96%
84658-049	3.6 µg/L D	3.60	1.72042	5	10	3.44	96%
84658-050	10.9 µg/L A	10.9	3.07001	5	20	12.3	113%
84658-051	10.9 µg/L B	10.9	2.83983	5	20	11.4	105%
84658-052	10.9 µg/L C	10.9	2.72686	5	20	10.9	100%
84658-053	10.9 µg/L D	10.9	3.60046	5	20	14.4	132%
84658-054	33 µg/L A	33.0	4.27607	5	50	42.8	130%
84658-055	33 µg/L B	33.0	3.88304	5	50	38.8	118%
84658-056	33 µg/L C	33.0	3.42123	5	50	34.2	104%
84658-057	33 µg/L D	33.0	3.97031	5	50	39.7	120%
84658-058	100 µg/L A	100	6.51914	5	100	130	130%
84658-059	100 µg/L B	100	5.68691	5	100	114	114%
84658-060	100 µg/L C	100	6.39805	5	100	128	128%
84658-061	100 µg/L D	100	6.01644	5	100	120	120%
84658-062	Stock	6000	12.11905	5	4000	9700	162%
84658-SPK19	Low Spike	2.04	1.00798	5	10	2.02	99%
84658-SPK20	High Spike	102	5.51620	5	100	110	108%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106\ \mu g/L \times 10\ mL}{5\ mL} = 0.212\ (\mu g/L)$$

① Calculated Conc. (µg/L) = Concentration from curve (µg/L) X Analysis Volume (mL) / Sample Volume (mL)

Prepared by: Sarah P. O'Connor Date: 06Mar17
Reviewed by: [illegible] Date: 22Mar17
Study Director: [illegible] Date: 31Mar17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_Results.xls]Initiation_07Feb17

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Week 1
Data file name: CI5038 84658 02142017A

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-065	Blank	0.00	No Peak	5	10	< MQL	NA
84658-066	0.0 µg/L A	0.00	< low std	5	10	< MQL	NA
84658-067	0.0 µg/L B	0.00	< low std	5	10	< MQL	NA
84658-068	0.0 µg/L C	0.00	< low std	5	10	< MQL	NA
84658-069	0.0 µg/L D	0.00	< low std	5	10	< MQL	NA
84658-070	3.6 µg/L A	3.60	1.32900	5	10	2.66	74%
84658-071	3.6 µg/L B	3.60	1.20371	5	10	2.41	67%
84658-072	3.6 µg/L C	3.60	1.12766	5	10	2.26	63%
84658-073	3.6 µg/L D	3.60	1.15136	5	10	2.30	64%
84658-074	10.9 µg/L A	10.9	2.50021	5	20	10.0	92%
84658-075	10.9 µg/L B	10.9	2.33095	5	20	9.32	86%
84658-076	10.9 µg/L C	10.9	2.31443	5	20	9.26	85%
84658-077	10.9 µg/L D	10.9	2.27187	5	20	9.09	83%
84658-078	33 µg/L A	33.0	2.95391	5	50	29.5	89%
84658-079	33 µg/L B	33.0	2.56411	5	50	25.6	78%
84658-080	33 µg/L C	33.0	2.62314	5	50	26.2	79%
84658-081	33 µg/L D	33.0	2.59111	5	50	25.9	78%
84658-082	100 µg/L A	100	4.95372	5	100	99.1	99%
84658-083	100 µg/L B	100	4.63407	5	100	92.7	93%
84658-084	100 µg/L C	100	4.62141	5	100	92.4	92%
84658-085	100 µg/L D	100	4.39670	5	100	87.9	88%
84658-086	Stock	6000	9.17788	5	4000	7340	122%
84658-SPK21	Low Spike	2.04	0.96675	5	10	1.93	95%
84658-SPK22	High Spike	102	4.92757	5	100	98.6	97%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106\ \mu g/L \times 10\ mL}{5\ mL} = 0.212\ (\mu g/L)$$

① Calculated Conc. (µg/L) = Concentration from curve (µg/L) X Analysis Volume (mL) / Sample Volume (mL)

Prepared by: Date: 24Mar17
Reviewed by: Date: 27Mar17
Study Director: Date: 31Mar17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_Results.xls]Week 1_14Feb17

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Week 2
Data file name: X 84658 02232017A and X 84658 02272017A

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal	
84658-089	Blank	0.00	No Peak	5	10	< MQL	NA	
84658-090	0.0 µg/L A	0.00	< low std	5	10	< MQL	NA	
84658-091	0.0 µg/L B	0.00	< low std	5	10	< MQL	NA	
84658-092	0.0 µg/L C	0.00	< low std	5	10	< MQL	NA	
84658-093	0.0 µg/L D	0.00	< low std	5	10	< MQL	NA	
84658-094	3.6 µg/L A	3.60	1.68165	5	10	3.36	93%	
84658-095	3.6 µg/L B	3.60	1.57607	5	10	3.15	88%	
84658-096	3.6 µg/L C	3.60	1.43923	5	10	2.88	80%	
84658-097	3.6 µg/L D	3.60	1.47698	5	10	2.95	82%	
84658-098	10.9 µg/L A	10.9	3.27458	5	20	13.1	120%	
84658-099	10.9 µg/L B	10.9	2.88751	5	20	11.6	106%	
84658-100	10.9 µg/L C	10.9	2.84167	5	20	11.4	105%	
84658-101	10.9 µg/L D	10.9	3.05743	5	20	12.2	112%	
84658-102	33 µg/L A	33.0	3.84723	5	50	38.5	117%	
84658-103	33 µg/L B	33.0	3.30492	5	50	33.0	100%	
84658-104	33 µg/L C	33.0	3.34613	5	50	33.5	102%	
84658-105	33 µg/L D	33.0	3.25895	5	50	32.6	99%	
84658-106	100 µg/L A	100	5.30478	5	100	106	106%	
84658-107	100 µg/L B	100	4.77755	5	100	95.6	96%	
84658-108	100 µg/L C	100	4.65576	5	100	93.1	93%	
84658-109	100 µg/L D	100	4.56875	5	100	91.4	91%	
84658-110	Stock	6000	3.60789	5	40,000	28900	482%	②
84658-SPK23	Low Spike	2.04	1.00249	5	10	2.00	98%	
84658-SPK24	High Spike	102	5.48902	5	100	110	108%	

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106\ \mu g/L \times 10\ mL}{5\ mL} = 0.212\ (\mu g/L)$$

① Calculated Conc. (µg/L) = Concentration from curve (µg/L) X Analysis Volume (mL) / Sample Volume (mL)
② Original result is DNU due to being outside standard curve. Original final dilution was further diluted and analyzed. Further dilution results from data set filename X 84658 02272017A are reported.

Prepared by: Sarah L. O'Connor Date: 04 Mar 17
Reviewed by: XJM Date: 31 Mar 17
Study Director: AMZ Date: 31 Mar 17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_Results.xls]Week 2_22Feb17

EAG Study Number: 84658
Test Substance: 2-ethylhexyl 4-hydroxybenzoate (2-EHHB)
Sample Point: Week 3
Data file name: X 84658 03012017A

Sample Number	Sample Identification	Nominal Concentration (µg/L)	Concentration from Curve (µg/L)	Sample Volume (mL)	Anaylsis Volume (mL)	Calculated Concentration (µg/L) ①	% of Nominal
84658-113	Blank	0.00	No Peak	5	10	< MQL	NA
84658-114	0.0 µg/L A	0.00	< low std	5	10	< MQL	NA
84658-115	0.0 µg/L B	0.00	< low std	5	10	< MQL	NA
84658-116	0.0 µg/L C	0.00	< low std	5	10	< MQL	NA
84658-117	0.0 µg/L D	0.00	< low std	5	10	< MQL	NA
84658-118	3.6 µg/L A	3.60	1.43656	5	10	2.87	80%
84658-119	3.6 µg/L B	3.60	1.27257	5	10	2.55	71%
84658-120	3.6 µg/L C	3.60	1.24319	5	10	2.49	69%
84658-121	3.6 µg/L D	3.60	1.22874	5	10	2.46	68%
84658-122	10.9 µg/L A	10.9	2.42516	5	20	9.70	89%
84658-123	10.9 µg/L B	10.9	2.04323	5	20	8.17	75%
84658-124	10.9 µg/L C	10.9	2.15330	5	20	8.61	79%
84658-125	10.9 µg/L D	10.9	2.15356	5	20	8.61	79%
84658-126	33 µg/L A	33.0	4.38838	5	50	43.9	133%
84658-127	33 µg/L B	33.0	3.25108	5	50	32.5	98%
84658-128	33 µg/L C	33.0	3.42333	5	50	34.2	104%
84658-129	33 µg/L D	33.0	3.53752	5	50	35.4	107%
84658-130	100 µg/L A	100	5.21980	5	100	104	104%
84658-131	100 µg/L B	100	4.69739	5	100	93.9	94%
84658-132	100 µg/L C	100	5.74718	5	100	115	115%
84658-133	100 µg/L D	100	5.51802	5	100	110	110%
84658-134	Stock	6000	7.53956	5	4,000	6030	101%
84658-SPK25	Low Spike	2.04	1.01715	5	10	2.03	100%
84658-SPK26	High Spike	102	5.29431	5	100	106	104%

NOTE: MQL = low std conc. x analysis volume / sample volume

$$= \frac{0.106\ \mu g/L \times 10\ mL}{5\ mL} = 0.212\ (\mu g/L)$$

① Calculated Conc. (µg/L) = Concentration from curve (µg/L) X Analysis Volume (mL) / Sample Volume (mL)

Prepared by: Daniel R. O'Connor Date: 01 Mar 17

Reviewed by: [signature] Date: 22 Mar 17

Study Director: [signature] Date: 31 Mar 17

File Location: X:\Shared\Client\Battelle\Study Folders\84658_Fresh Water_2-EHHB\Data\Analytical\[84658_Results.xls]Week 3_28Feb17

Appendix D. Histopathology Data Report

Experimental Pathology Laboratories, Inc.

FORT ENVIRONMENTAL LABORATORIES, INC.
STUDY NUMBER BATT01-00404
EPL PROJECT NUMBER 237-086

FISH SHORT-TERM REPRODUCTION ASSAY OF 2-ETHYLHEXYL 4-HYDROXYBENZOATE WITH JAPANESE MEDAKA

PATHOLOGY REPORT

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March 19, 2018

FINAL REPORT

Experimental Pathology Laboratories, Inc.

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PATHOLOGY NARRATIVE

EPL® Experimental Pathology Laboratories, Inc.

FORT ENVIRONMENTAL LABORATORIES, INC.
STUDY NUMBER BATT01-00404
EPL PROJECT NUMBER 237-086

FISH SHORT-TERM REPRODUCTION ASSAY OF 2-ETHYLHEXYL 4-HYDROXYBENZOATE WITH JAPANESE MEDAKA

PATHOLOGY NARRATIVE

INTRODUCTION

The primary objective of the study was to evaluate the potential reproductive toxicity of 2-ethylhexyl 4-hydroxybenzoate (2-EHHB) to Japanese medaka in accordance with the guidelines established in OPPTS 890.1350 Fish Short-Term Reproduction Assay (FSTRA) and OECD Guideline 229 with modification to use medaka *in lieu* of sexually mature male and female fathead minnows. The Fish Short-Term Reproduction Assay measures a suite of histological and biochemical endpoints that reflect effects associated with disturbance to the hypothalamus-pituitary-gonadal (HPG) endocrine axis. During a 21-day in-life exposure phase, survival, reproductive behavior, secondary sexual characteristics, fecundity, and fertility were monitored. At the conclusion of the in-life exposure phase, measurements were made that were indicative of the status of the reproductive endocrine system, including secondary sex characteristics; genetic sex, gonado-somatic index (GSI), liver vitellogenin (VTG) mRNA, and gonad, liver, and thyroid histopathology. These endpoints were generally inclusive of the traditional FSTRA endpoints, but also contain additional endpoints relative to medaka.

The experimental design is presented in Table 1.

Table 1. Study Design

2-EHHB Treatment Group (µg/L)	2-EHHB Measured Concentration (µg/L)	Number of Males per Treatment Group	Number of Females per Treatment Group
0 (control)	<MQL	12	12
3.6	2.98	12	12
10.9	10.6	12	12
33.0	34.1	12	12
100.0	105	12	12
TOTALS		60	60

MQL = Minimum Quantitation Level = 0.212 µg/L

METHODS

Whole body specimens from 120 Japanese medaka were received by Experimental Pathology Laboratories, Inc. (EPL®), Sterling, Virginia. Briefly, each whole fish specimen, minus the tail, was processed in an automated tissue processor and infiltrated with paraffin according to routine methods. Chemical decalcification was required. Each fish was embedded left side down in the paraffin block. Approximately 20 parasagittal sections were microtomed from each fish, three to four serial sections at each of five different levels, using prescribed anatomical landmarks to identify each level. The serial sections (each section 4-5 microns thick) obtained at each level were placed on a single glass slide, stained with hematoxylin and eosin, and mounted beneath a glass cover slip as per routine.

The focus of this investigation was the protocol-required target tissues: thyroid, liver, and gonad. The pathologist evaluated the slides by brightfield microscopy, beginning with all male and female medaka of the high dose (100.0 μg/L) and control groups. Upon Study Director approval, potential treatment-related effects in the high dose animals triggered evaluation of the corresponding tissue/sex combination in the mid-high concentration group (33.0 μg/L) and the mid-low dose group (10.9 μg/L).

Severity grading of findings was performed according to the following scale: X = not remarkable, Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, and Grade 4 = severe. Gonad phenotype scores were recorded as follows: Grade 1 = testicular tissue entirely, Grade 2 = testicular tissue predominates, Grade 3 = comparable amounts of testicular and ovarian tissue, Grade 4 = ovarian tissue predominates, and Grade 5 = ovarian tissue entirely. Findings were recorded into EPL's electronic Pathology Data Reporting System, and tabulated in the accompanying Histopathology Incidence Tables (HIT) and summarized in the Summary Incidence Tables (SIT).

RESULTS

Concerning the **thyroids** (Table 2), although there was a slight increase in the severity of follicular cell hypertrophy (minimal to mild) in the 100.0 μg/L dose group

males (4/11 had mild hypertrophy) relative to controls (2/12 had mild hypertrophy), this difference was potentially attributable to inter-animal variation. Similarly, modest decreases in the severity of follicular cell hyperplasia in 10.9 and 33.0 μg/L dose group males (7/12 and 8/12 had mild hyperplasia, respectively) as compared to controls (10/12 had mild hyperplasia) may also have been due to inter-animal variation, as there was no apparent dose-related pattern associated with this diagnosis (i.e., the severity of hyperplasia in the 100.0 μg/L dose group males did not differ substantially from that of controls). Because the amount and appearance of thyroid tissue in male medaka can vary appreciably depending on the precise location of the examined sections, slight grading differences should be interpreted cautiously. Hyaline degeneration of follicular cells was decreased statistically in females of the 33.0 and 100.0 μg/L dose groups relative to controls; however, a statistical decrease in that particular finding, as is true of most degenerative-type changes, is not considered to be toxicologically relevant.

Table 2. Selected Thyroid Findings

	Males					Females				
2-EHHB Treatment Group (μg/L)	0.0	3.6	10.9	33.0	100.0	0.0	3.6	10.9	33.0	100.0
Number Examined	12	12	12	12	12	12	12	12	12	12
Follicular Cell Hyperplasia	12	ne	12	12	12	0	ne	ne	ne	1
minimal	2	ne	5	4	2	-	ne	ne	ne	1
mild	10	ne	7	8	10	-	ne	ne	ne	-
Follicular Cell Hypertrophy	12	ne	12	11	11	0	ne	ne	ne	0
minimal	10	ne	11	10	7	-	ne	ne	ne	-
mild	2	ne	1	1	4	-	ne	ne	ne	-
Hyaline Degeneration	9	ne	8	5	6	2	ne	ne	ne	0
minimal	7	ne	6	2	6	1	ne	ne	ne	-
mild	2	ne	2	3	-	1	ne	ne	ne	-

ne = not evaluated

A treatment-related finding in the **livers** (Table 3; Figs. 1-2) of female medaka was a general decrease in hepatic basophilia (minimal to moderate) in fish of the 33.0 and

100.0 μg/L dose groups as compared to controls; the slight decrease in basophilia in fish of the 10.9 μg/L dose group relative to controls was insubstantial and not statistically significant. This occurred as decrease in the severity of this finding in the 100.0 μg/L dose group relative to controls, and a decrease of both the severity and prevalence of basophilia in the 33.0 μg/L dose group. On an individual fish basis, the presence of zero, minimal, or mild basophilia in females was often (but not always) associated with a low (i.e., Grade 1 or 2) ovarian stage score and/or oocyte atresia.

Foci of cellular alteration (minimal to moderate) were observed in the livers of three females, two of the 100.0 μg/L dose groups and one of the 33.0 μg/L dose groups, and were statistically different from controls in the 100.0 μg/L dose group. Because of the low prevalence of this finding, its relationship to treatment is uncertain.

Vacuolation was increased statistically in females of the 33.0 and 100.0 μg/L dose groups relative to controls at the mild and moderate severity levels.

Pigmented macrophage aggregates were decreased statistically in females of the 100.0 μg/L dose group relative to controls at the mild severity level; however, a statistical decrease in that particular finding is not likely to be toxicologically relevant.

Other statistically significant differences that were not considered to be toxicologically relevant included decreased cystic degeneration and decreased eosinophilic cytoplasmic inclusions in the livers of some male groups relative to controls, and increased granulomatous inflammation of the liver in 100.0 μg/L dose group males relative to controls.

Table 3. Selected Liver Findings

	Males					Females				
2-EHHB Treatment Group (μg/L)	0.0	3.6	10.9	33.0	100.0	0.0	3.6	10.9	33.0	100.0
Number Examined	12	12	12	12	12	12	12	12	12	12
Basophilia	0	ne	ne	ne	1	12	ne	12	6	11
minimal	-	ne	ne	ne	1	-	ne	1	2*	3*
mild	-	ne	ne	ne	-	2	ne	3	1*	-*
moderate	-	ne	ne	ne	-	10	ne	8	3*	8*
Focus of Cellular Alteration	0	ne	ne	ne	0	0	ne	0	1	2*
minimal	-	ne	ne	ne	-	-	ne	-	1	-*
moderate	-	ne	ne	ne	-	-	ne	-	-	2*
Vacuolation	8	ne	ne	ne	7	10	ne	11	11	11
minimal	8	ne	ne	ne	6	9	ne	9	3	3
mild	-	ne	ne	ne	1	-	ne	2	2*	4*
moderate	-	ne	ne	ne	-	1	ne	-	6*	4*

ne = not evaluated; *Significantly different from control at the 0.05 level

There were no treatment-related findings in the **testes** of male medaka.

A treatment-related finding in the **ovaries** (Table 4; Figs. 3-4) of female medaka was an increase in the prevalence and severity of oocyte atresia (minimal to severe) in fish of the 33.0 and 100.0 μg/L dose groups as compared to controls. The severity of oocyte atresia was also increased slightly, but not substantially, in females of the 10.9 μg/L dose group relative to controls.

Mean ovarian stage scores were slightly greater than controls in females of the 100.0 μg/L dose group, but were substantially lower than controls in 10.9 and 33.0 μg/L group females. Especially in the 33.0 μg/L dose group, the high prevalence of oocyte atresia appeared to contribute to the low stage scores, as atresia often affected the more mature vitellogenic follicles preferentially.

Egg debris in the oviduct was decreased statistically in females of the 33.0 and 100.0 μg/L dose groups relative to controls at the minimal and mild severity levels; however, statistical decreases in that particular finding are not likely to be toxicologically relevant.

Table 4. Selected Ovary Findings					
2-EHHB Treatment Group (μg/L)	0.0	3.6	10.9	33.0	100.0
Number Examined	12	12	12	12	12
Oocyte Atresia	5	ne	5	9	8
minimal	5	ne	3	7	3
mild	-	ne	2	-	4
moderate	-	ne	-	1	1
severe	-	ne	-	1	-
Ovary Stage					
Stage 1	1	ne	4	5	3
Stage 2	4	ne	2	3	6
Stage 3	5	ne	6	1	9
Stage 4	2	ne	-	1	12
Mean Stage Score	2.7	ne	2.2	1.8	2.5
Unable to Stage	-	ne	-	2	-

ne = not evaluated

There were six gonadal germ cell neoplasms observed in this study (Figs. 5-14, Table 5), none of which were considered to be treatment related. Benign seminomas occurred in the testes of one control male (Animal No. 143-B1) and two 100.0 μg/L group males (Animal Nos. 235-A3 and 247-C3). Malignant seminomas, with intra-coelomic metastasis (to the anterior coelomic wall in one case, and ventral liver capsule in another), were evident in the ovaries of one control female (Animal No. 140-A4) and one 100.0 μg/L dose group female (Animal No. 254-D4). Interestingly, while the malignant seminomas of the ovaries were of the spermatocytic type (which is characteristic of most teleosts), the testicular seminomas were of the less common spermatogonial type. A benign dysgerminoma occurred in the testis of a single 100.0 μg/L dose group male (Animal No. 233-A1). The labelling of benign versus malignant for these tumors was based solely on the presence or absence of metastasis, as the cytologic features are not a consistently useful indicator of malignant potential. The relatively high prevalence (for medaka) of these germ cell tumors and their presence in controls suggests that this is likely a strain-related background issue.

Table 5. Prevalence of Gonadal Germ Cell Tumors										
Sex	Males					Females				
2-EHHB Treatment Group (µg/L)	0.0	3.6	10.9	33.0	100.0	0.0	3.6	10.9	33.0	100.0
Number Examined	12	12	12	12	12	12	12	12	12	12
Dysgerminoma, Benign	0	ne	ne	ne	1	0	ne	0	0	0
Seminoma, Benign	1	ne	ne	ne	2	0	ne	0	0	0
Seminoma, Malignant	0	ne	ne	ne	0	1	ne	0	0	1

ne = not evaluated

Other diagnoses recorded in this study tended to be background types of findings observed typically in adult medaka, and were present in comparable numbers of control and 2-EHHB-treated fish generally at low prevalence and severity; thus, none were considered to be treatment-related.

DISCUSSION

Vitellogenin is a phospholipoglycoprotein essential for egg yolk formation in fish, and it has been demonstrated that the relative degree of liver basophilia in histologic sections is a sensitive marker of the level of hepatic vitellogenin synthesis (van der Ven et al., 2003). Because hepatic vitellogenin synthesis is estrogen driven, at one or more levels, the treatment-related decrease in liver basophilia in this study is suggestive of interference with the production or hepatic utilization of estrogen. The concomitant increase in liver vacuolation, as observed in this study, was a sequela of decreased basophilia, as energy not required for vitellogenin synthesis and egg production may then be stored in the liver in the form of lipid and/or glycogen. Since the production of hepatic vitellogenin is to some degree dependent on the estrogen (17β-estradiol) generated by ovarian follicles in response to pituitary secretion of follicle stimulating hormone (FSH) (Hara et al., 2016), disruption of follicular integrity (i.e., oocyte atresia) or suppression of ovarian cycling (as suggested by the presence of Stage 1 ovaries) could conceivably result in diminished estrogen production. Conversely, although oocyte atresia can result from exposure to excess estrogen (Dietrich and Krieger, 2009), it is

also possible that the failure to maintain the integrity of vitellogenic oocytes may stem from diminished estrogen availability for hepatic vitellogenin production.

In the current study, gonadal germ cell tumors were observed in four male and two female medaka. The occurrence of these neoplasms was not considered treatment-related, based on the comparable presence of such tumors in control fish and those exposed to 2-EHHB. However, the recording of gonadal tumors in six of 72 examined medaka (8%) is considered unusual, based on the experience of the pathologist and the results of at least one published report (Hawkins et al., 1996), in which germ cell neoplasms were detected in only 26 of approximately 10,000 medaka (0.3%). In that study, the authors reported that the tumors were associated with age rather than chemical exposure, and they suggested that the development of the neoplasms was most likely attributable to "culture practices, genetic factors, or a virus", based on the tendency for multiple occurrences to cluster in the fish of the same aquarium or cohort. In the current study, the occurrence of the six germ cell neoplasms was distributed among control and high dose groups from both sexes, and thus their presence did not affect the experimental outcome.

CONCLUSIONS

Treatment-related findings associated with 2-EHHB exposure in the current study included decreased hepatic basophilia, increased hepatocellular vacuolation, and increased ovarian oocyte atresia in 33.0 and 100.0 μg/L dose group females compared to controls. This combination of findings is suggestive of decreased estrogen availability, the mechanism of which cannot be determined based on the histopathology results alone.

A slight increase in the incidence of hepatic foci of cellular alteration occurred in 100.0 μg/L dose group females relative to controls; however, due to the low overall incidence of that finding (3 of 72 examined fish), its relationship to treatment is uncertain.

There were no treatment-related effects in the thyroid glands of male or female medaka, or in the testes of male medaka.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Memorial Institute Study Number BATT01-00404

Benign and malignant germ cell neoplasms were observed in six of 72 examined male and female medaka. Although the frequency of these tumors was higher than would typically be expected, their comparable prevalence in control and treated fish indicates that their development was not related to 2-EHHB exposure. Additionally, because these tumors were distributed among male and female fish from control and high dose groups, their presence did not impact the study outcome.

19 March 2018

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

JCW/skh

REFERENCES

Dietrich DR, Krieger HO. (2009). Germ Cell Atresia. *Histological Analysis of Endocrine Disruptive Effects in Small Laboratory Fish*. **(6.2.2.5):**224.

Hara A, Hiramatsu N, Fujita T. (2016). Vitellogenesis and choriogenesis in fishes. *Fisheries Science*. **82:**187-202.

Hawkins WE, Fournie JW, Ishikawa T, and Walker WW. (1996). Germ Cell Neoplasms in Japanese Medaka. *Journal of Aquatic Animal Health*. **8:**120-123.

OECD. (2012). Fish Short Term Reproduction Assay. OECD Guidelines for the Testing of Chemicals: Test No. 229. Adopted 2 October 2012.

US EPA. (2009). Endocrine Disruptor Screening Program Test Guidelines: OPPTS 890.1350 Fish Short-Term Reproduction Assay.

van der Ven LT, Wester PW, Vos JG. (2003). Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (*Danio rerio*). *Environmental Toxicology Chemistry*. **22(4):**908-913.

EPL® Experimental Pathology Laboratories, Inc.

COMPLIANCE STATEMENT

Test Facility	Fort Environmental Laboratories, Inc.	EPL Principal Investigator	Dr. Jeffrey C. Wolf
Study No.	BATT01-00404	EPL Pathologist	Dr. Jeffrey C. Wolf
Species	*Oryzias latipes* (Medaka)	EPL Project Number	237-086
Study Title	Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka		
Test Article	2-Ethylhexyl 4-Hydroxybenzoate		

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: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

Client Study: BATT01-00404 EPL Principal Investigator: Dr. Jeffrey C. Wolf

EPL Project Number: 237-086 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	5/3,4/17	5/4/17
Project Setup	5/5,8/17	5/5,8/17
In-Process - Checkout	6/14/17	6/14/17
Data Review	6/13,15/17; 7/13/17	6/13,15/17; 7/13/17
Draft Pathology Report	7/12-14/17; 3/13-15/18	7/14/17; 3/15/18
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 Harwick 3/19/18

EPL Quality Assurance Unit Date

SUMMARY INCIDENCE TABLES

SUMMARY INCIDENCE TABLE

BATT01-00404
Terminal Sacrifice
Male
Oryzias latipes (Medaka)

	GROUP 0.0µg/L	GROUP 10.9µg/L	GROUP 33.0µg/L	GROUP 100.0µg/L		
LIVER (NO. EXAMINED)	(12)			(12)		
Basophilia				1		
Bile Pigment	1					
Cystic Degeneration	7			9		
Cytoplasmic Inclusions, Eosinophilic	4			1		
Focus of Cellular Alteration						
Inflammation, Granulomatous	1			4		
Lymphoma/Leukemia						
Pigmented Macrophage Aggregates	3			5		
Single Cell Necrosis						
Vacuolation	8			7		
TESTIS (NO. EXAMINED)	(12)			(12)		
Dysgerminoma, Benign				1		
Seminoma, Benign	1			2		
Increased Spermatagonia	2			1		
Interstitial Cells, Increased	1			3		
Phenotype 1	12			12		
Stage 1	9			11		
Stage 2	3			1		
Testicular Degeneration	11			11		
THYROID (NO. EXAMINED)	(12)	(12)	(12)	(12)		
Colloid, Inspissated		3	2	1		
Follicle Size, Decreased	1	1	1	1		
Follicular Cell Hyperplasia	12	12	12	12		
Follicular Cell Hypertrophy	12	12	11	11		
Follicular Cells, Hyaline Degeneration	9	8	5	6		

SUMMARY INCIDENCE TABLE

BATT01-00404
Terminal Sacrifice
Female
Oryzias latipes (Medaka)

	GROUP 0.0µg/L	GROUP 10.9µg/L	GROUP 33.0µg/L	GROUP 100.0µg/L		
LIVER (NO. EXAMINED)	(12)	(12)	(12)	(12)		
Basophilia	12	12	6	11		
Bile Pigment						
Cystic Degeneration	12	7	9	8		
Cytoplasmic Inclusions,						
Eosinophilic	4	4	1	3		
Focus of Cellular Alteration			1	2		
Inflammation, Granulomatous	1	2				
Lymphoma/Leukemia			1			
Pigmented Macrophage						
Aggregates	3	3	3	3		
Single Cell Necrosis	2	1	1			
Vacuolation	10	11	11	11		
OVARY (NO. EXAMINED)	(12)	(12)	(12)	(12)		
Seminoma, Malignant	1			1		
Egg Debrin Oviduct	2					
Inflammation, Granulomatous	2	4	2	1		
Lymphoma/Leukemia			1			
Oocyte Atresia	5	5	9	8		
Oviduct, Fibrovascular						
Proliferation			2			
Perifollicular Cell						
Hyperplasia-Hypertrophy			1			
Phenotype 5	12	12	12	12		
Post-Ovulatory Follicles	10	9	5	9		
Post-Ovulatory Follicles,						
Vacuolation			1	1		
Stage 1	1	4	5	3		
Stage 2	4	2	3	3		
Stage 3	5	6	1	3		
Stage 4	2		1	3		
Unable to Stage			2			
THYROID (NO. EXAMINED)	(12)			(12)		
Colloid, Inspissated						
Follicle Size, Decreased						
Follicular Cell Hyperplasia				1		
Follicular Cell Hypertrophy						
Follicular Cells, Hyaline						
Degeneration	2					

HISTOPATHOLOGY INCIDENCE TABLES

HISTOPATHOLOGY INCIDENCE TABLE

BATT01-00404 Terminal Sacrifice Male *Oryzias latipes* (Medaka)	GROUP 0.0µg/L ANIMAL																				
	137-A1	138-A2	139-A3	143-B1	144-B2	145-B3	149-C1	150-C2	151-C3	155-D1	156-D2	157-D3									
LIVER									X												
Basophilia																					
Bile Pigment				1																	
Cystic Degeneration	1	2	1	2		2		1		1											
Cytoplasmic Inclusions,																					
Eosinophilic				1			2				1	1									
Focus of Cellular Alteration																					
Inflammation, Granulomatous		1																			
Lymphoma/Leukemia																					
Pigmented Macrophage																					
Aggregates						1				1	1										
Single Cell Necrosis																					
Vacuolation	1	1	1	1	1	1				1	1										
TESTIS																					
Dysgerminoma, Benign																					
Seminoma, Benign				P																	
Increased Spermatagonia		1	1																		
Interstitial Cells, Increased							1														
Phenotype 1	P	P	P	P	P	P	P	P	P	P	P	P									
Stage 1	P	P	P	P	P	P	P	P				P									
Stage 2									P	P	P										
Testicular Degeneration	2	1	2	2	2	2	1		2	2	1	1									
THYROID																					
Colloid, Inspissated																					
Follicle Size, Decreased					2																
Follicular Cell Hyperplasia	2	2	1	2	2	2	2	2	2	2	2	1									
Follicular Cell Hypertrophy	1	1	1	1	1	2	1	1	1	1	2	1									
Follicular Cells, Hyaline																					
Degeneration		2	1	1		2	1		1	1	1	1									

EPL Experimental Pathology Laboratories, Inc.

II-1

Key: X=Not Remarkable N=No Section
1=minimal 2=mild 3=moderate 4=severe
P=Present

HISTOPATHOLOGY INCIDENCE TABLE

GROUP 10.9µg/L

BATT01-00404
Terminal Sacrifice
Male
Oryzias latipes (Medaka)

ANIMAL	185-A1	186-A2	187-A3	191-B1	192-B2	193-B3	197-C1	198-C2	199-C3	203-D1	204-D2	205-D3
THYROID												
Colloid, Inspissated					2					1		2
Follicle Size, Decreased	1											
Follicular Cell Hyperplasia	2	2	1	2	1	1	2	1	1	2	2	2
Follicular Cell Hypertrophy	1	1	1	1	1	2	1	1	1	1	1	1
Follicular Cells, Hyaline												
Degeneration		2		1	1	1	2	1	1	1		

EPL Experimental Pathology Laboratories, Inc.

II-2

Key: X=Not Remarkable N=No Section
1=minimal 2=mild 3=moderate 4=severe
P=Present

HISTOPATHOLOGY INCIDENCE TABLE

GROUP
33.0µg/L

BATT01-00404
Terminal Sacrifice
Male
Oryzias latipes (Medaka)

ANIMAL	209-A1	210-A2	211-A3	215-B1	216-B2	217-B3	221-C1	222-C2	223-C3	227-D1	228-D2	229-D3
THYROID												
Colloid, Inspissated									1	1		
Follicle Size, Decreased									2			
Follicular Cell Hyperplasia	2	1	1	2	2	1	2	2	2	2	2	1
Follicular Cell Hypertrophy	1	1		2	1	1	1	1	1	1	1	1
Follicular Cells, Hyaline												
Degeneration	2			1			2		1	2		

EPL
Experimental Pathology Laboratories, Inc.

II-3

Key: X=Not Remarkable N=No Section
1=minimal 2=mild 3=moderate 4=severe
P=Present

HISTOPATHOLOGY INCIDENCE TABLE

GROUP
100.0µg/L

BATT01-00404
Terminal Sacrifice
Male
Oryzias latipes (Medaka)

ANIMAL	233-A1	234-A2	235-A3	239-B1	240-B2	241-B3	245-C1	246-C2	247-C3	251-D1	252-D2	253-D3
LIVER						X						
Basophilia											1	
Bile Pigment												
Cystic Degeneration	1	1	2	2			2	2	1	2	2	
Cytoplasmic Inclusions, Eosinophilic		1										
Focus of Cellular Alteration												
Inflammation, Granulomatous	2		3	2			3					
Lymphoma/Leukemia												
Pigmented Macrophage Aggregates		1	1	1			1	1				
Single Cell Necrosis												
Vacuolation		1	1		1			1	1	1		2
TESTIS												
Dysgerminoma, Benign	P											
Seminoma, Benign			P						P			
Increased Spermatagonia			1									
Interstitial Cells, Increased	1		1							1		
Phenotype 1	P	P	P	P	P	P	P	P	P	P	P	P
Stage 1	P	P	P	P	P	P	P	P	P	P	P	
Stage 2												P
Testicular Degeneration	1	1	2	1	2	2	1	2	1		2	1
THYROID												
Colloid, Inspissated		1										
Follicle Size, Decreased				1								
Follicular Cell Hyperplasia	2	2	1	2	2	2	2	2	2	2	2	1
Follicular Cell Hypertrophy	1	1	1	2	1	2	1	2	1	2	1	
Follicular Cells, Hyaline Degeneration	1	1	1				1		1	1		

EPL
Experimental Pathology Laboratories, Inc.

II-4

Key: X=Not Remarkable N=No Section
1=minimal 2=mild 3=moderate 4=severe
P=Present

HISTOPATHOLOGY INCIDENCE TABLE

GROUP 0.0µg/L

BATT01-00404
Terminal Sacrifice
Female
Oryzias latipes (Medaka)

ANIMAL	140-A4	141-A5	142-A6	146-B4	147-B5	148-B6	152-C4	153-C5	154-C6	158-D4	159-D5	160-D6
LIVER												
Basophilia	3	2	3	3	3	3	2	3	3	3	3	3
Bile Pigment												
Cystic Degeneration	2	2	1	1	1	1	2	2	2	1	3	2
Cytoplasmic Inclusions, Eosinophilic	1		1	1						1		
Focus of Cellular Alteration												
Inflammation, Granulomatous									1			
Lymphoma/Leukemia												
Pigmented Macrophage Aggregates	1			2			3					
Single Cell Necrosis							1					1
Vacuolation	1	1	1		1	1	3	1	1	1	1	
OVARY												
Seminoma, Malignant	P											
Egg Debrin Oviduct				3			2					
Inflammation, Granulomatous				3			1					
Lymphoma/Leukemia												
Oocyte Atresia				1			1	1		1		1
Oviduct, Fibrovascular Proliferation												
Perifollicular Cell Hyperplasia-Hypertrophy												
Phenotype 5	P	P	P	P	P	P	P	P	P	P	P	P
Post-Ovulatory Follicles	1	1	1		1		1	1	1	2	1	1
Post-Ovulatory Follicles, Vacuolation												
Stage 1				P								
Stage 2						P		P		P		P
Stage 3		P	P		P				P		P	
Stage 4	P						P					
Unable to Stage												
THYROID	X	X		X	X	X		X	X	X	X	X
Colloid, Inspissated												
Follicle Size, Decreased												
Follicular Cell Hyperplasia												
Follicular Cell Hypertrophy												

Key: X=Not Remarkable N=No Section
1=minimal 2=mild 3=moderate 4=severe
P=Present

HISTOPATHOLOGY INCIDENCE TABLE

GROUP
0.0µg/L

BATT01-00404
Terminal Sacrifice
Female
Oryzias latipes (Medaka)

ANIMAL

	140-A4	141-A5	142-A6	146-B4	147-B5	148-B6	152-C4	153-C5	154-C6	158-D4	159-D5	160-D6
THYROID (CONTINUED)												
Follicular Cells, Hyaline												
Degeneration			1				2					

EPL Experimental Pathology Laboratories, Inc.

II-6

Key: X=Not Remarkable N=No Section
1=minimal 2=mild 3=moderate 4=severe
P=Present

HISTOPATHOLOGY INCIDENCE TABLE

GROUP 10.9µg/L

BATT01-00404
Terminal Sacrifice
Female
Oryzias latipes (Medaka)

ANIMAL	188-A4	189-A5	190-A6	194-B4	195-B5	196-B6	200-C4	201-C5	202-C6	206-D4	207-D5	208-D6
LIVER												
Basophilia	2	3	2	3	1	2	3	3	3	3	3	3
Bile Pigment												
Cystic Degeneration	1	3	1	2			2		2			1
Cytoplasmic Inclusions, Eosinophilic				1			1	1	1			
Focus of Cellular Alteration												
Inflammation, Granulomatous	1									2		
Lymphoma/Leukemia												
Pigmented Macrophage Aggregates				2			1			1		
Single Cell Necrosis				1								
Vacuolation	2	1	1	1	1	1	1	1	1	2		1
OVARY												
Seminoma, Malignant												
Egg Debrin Oviduct												
Inflammation, Granulomatous	2			1	1					2		
Lymphoma/Leukemia												
Oocyte Atresia	2		1		2	1		1				
Oviduct, Fibrovascular Proliferation												
Perifollicular Cell Hyperplasia-Hypertrophy												
Phenotype 5	P	P	P	P	P	P	P	P	P	P	P	P
Post-Ovulatory Follicles		1	1	2			1	2	1	1	1	1
Post-Ovulatory Follicles, Vacuolation												
Stage 1	P		P		P	P						
Stage 2		P						P				
Stage 3				P			P		P	P	P	P
Stage 4												
Unable to Stage												

Key: X=Not Remarkable N=No Section
1=minimal 2=mild 3=moderate 4=severe
P=Present

HISTOPATHOLOGY INCIDENCE TABLE

GROUP
33.0µg/L

BATT01-00404
Terminal Sacrifice
Female
Oryzias latipes (Medaka)

ANIMAL	212-A4	213-A5	214-A6	218-B4	219-B5	220-B6	224-C4	225-C5	226-C6	230-D4	231-D5	232-D6
LIVER												
Basophilia			3	1				1	3	2		3
Bile Pigment												
Cystic Degeneration		1		2	1	1	2	1	1		2	2
Cytoplasmic Inclusions, Eosinophilic											1	
Focus of Cellular Alteration		1										
Inflammation, Granulomatous												
Lymphoma/Leukemia					P							
Pigmented Macrophage Aggregates			1							1		1
Single Cell Necrosis												1
Vacuolation	3	3	2	3	3	3	3	2	1		1	1
OVARY												
Seminoma, Malignant												
Egg Debrin Oviduct												
Inflammation, Granulomatous										2	1	
Lymphoma/Leukemia					P							
Oocyte Atresia		1	1	1	1	1	1			3	4	1
Oviduct, Fibrovascular Proliferation										3	2	
Perifollicular Cell Hyperplasia-Hypertrophy											1	
Phenotype 5	P	P	P	P	P	P	P	P	P	P	P	P
Post-Ovulatory Follicles			1	2				2	1			1
Post-Ovulatory Follicles, Vacuolation			1									
Stage 1	P	P			P	P	P					
Stage 2				P					P			P
Stage 3			P									
Stage 4								P				
Unable to Stage										P	P	

EPL Experimental Pathology Laboratories, Inc.

II-8

Key: X=Not Remarkable N=No Section
1=minimal 2=mild 3=moderate 4=severe
P=Present

HISTOPATHOLOGY INCIDENCE TABLE

GROUP 100.0µg/L

BATT01-00404
Terminal Sacrifice
Female
Oryzias latipes (Medaka)

ANIMAL	236-A4	237-A5	238-A6	242-B4	243-B5	244-B6	248-C4	249-C5	250-C6	254-D4	255-D5	256-D6
LIVER												
Basophilia	1	3	3	3	3	3	3	3	3		1	1
Bile Pigment												
Cystic Degeneration	1	2	1	1	1	2	1			1		
Cytoplasmic Inclusions, Eosinophilic			1			1		1				
Focus of Cellular Alteration			3					3				
Inflammation, Granulomatous												
Lymphoma/Leukemia												
Pigmented Macrophage Aggregates		1				1			1			
Single Cell Necrosis												
Vacuolation	3	2	1	1		1	2	2	2	3	3	3
OVARY												
Seminoma, Malignant										P		
Egg Debrin Oviduct												
Inflammation, Granulomatous					1							
Lymphoma/Leukemia												
Oocyte Atresia	2			2	1	2	1		1	2	3	
Oviduct, Fibrovascular Proliferation												
Perifollicular Cell Hyperplasia-Hypertrophy												
Phenotype 5	P	P	P	P	P	P	P	P	P	P	P	P
Post-Ovulatory Follicles	1	1	1	1	1		2	1	1			1
Post-Ovulatory Follicles, Vacuolation	1											
Stage 1	P									P	P	
Stage 2					P	P	P					
Stage 3		P							P			P
Stage 4			P	P				P				
Unable to Stage												
THYROID		X	X	X	X	X	X	X	X	X	X	X
Colloid, Inspissated												
Follicle Size, Decreased												
Follicular Cell Hyperplasia	1											
Follicular Cell Hypertrophy												

Key: X=Not Remarkable N=No Section
1=minimal 2=mild 3=moderate 4=severe
P=Present

HISTOPATHOLOGY INCIDENCE TABLE

GROUP
100.0µg/L

BATT01-00404
Terminal Sacrifice
Female
Oryzias latipes (Medaka)

ANIMAL	236-A4	237-A5	238-A6	242-B4	243-B5	244-B6	248-C4	249-C5	250-C6	254-D4	255-D5	256-D6								
THYROID (CONTINUED)																				
Follicular Cells, Hyaline																				
Degeneration																				

Key: X=Not Remarkable N=No Section
1=minimal 2=mild 3=moderate 4=severe
P=Present

APPENDIX A

FIGURES AND LEGENDS

FIGURES

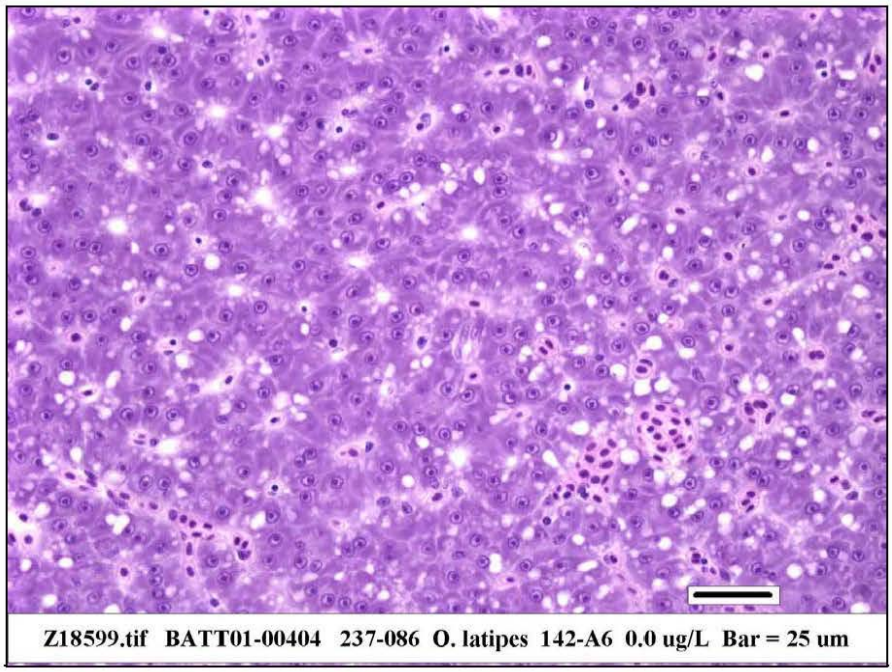

Figure 1 (Z18599). Liver from a control female, Animal No. 142-A6. Grade 3 basophilia provided for comparison. H&E.

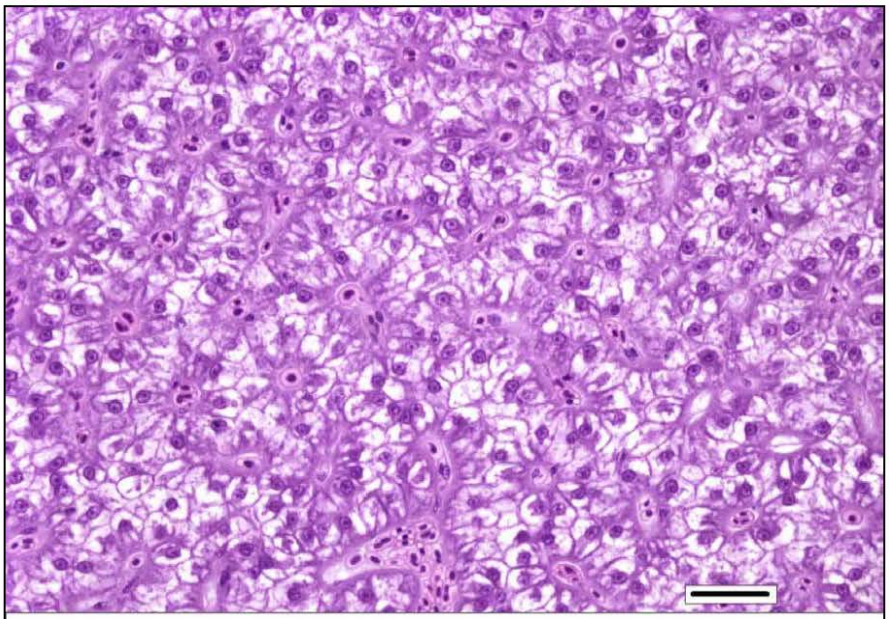

Figure 2 (Z18601). Liver from a female exposed to 100.0 mg/L 2-EHHB, Animal No. 254-D4. In addition to increased vacuolation, hepatocytes display a lack of basophilia. H&E.

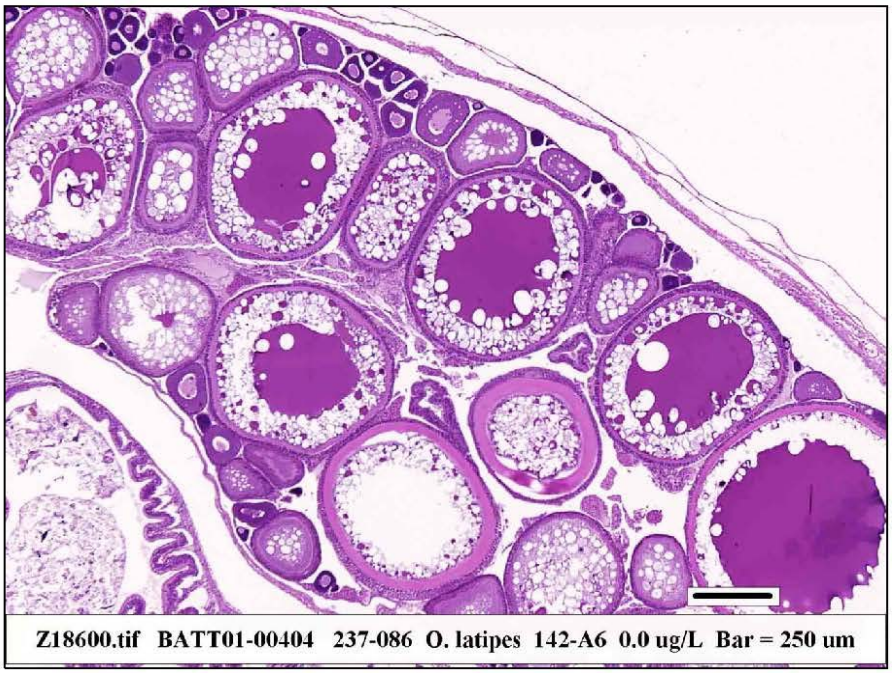

Figure 3 (Z18600). Ovary from a control female, Animal No. 142-A6. Stage 3 ovary without oocyte atresia provided for comparison. H&E.

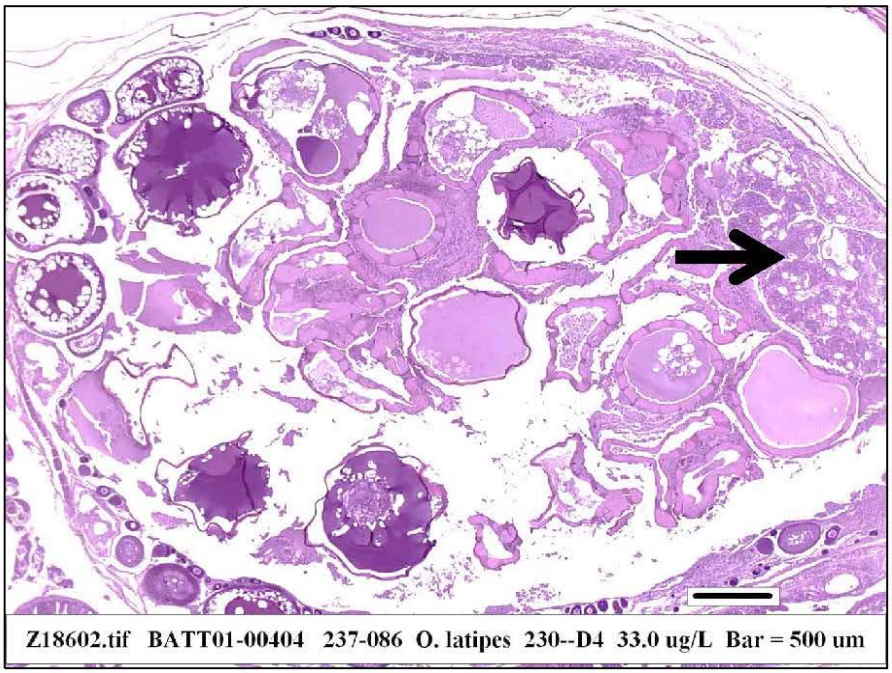

Figure 4 (Z18602). Ovary from a female exposed to 33.0 mg/L 2-EHHB, Animal No. 230-D4. Grade 3 oocyte atresia. Proliferation of fibrovascular tissue in the wall of the oviduct (arrow) is likely a reaction to the massive atresia. H&E.

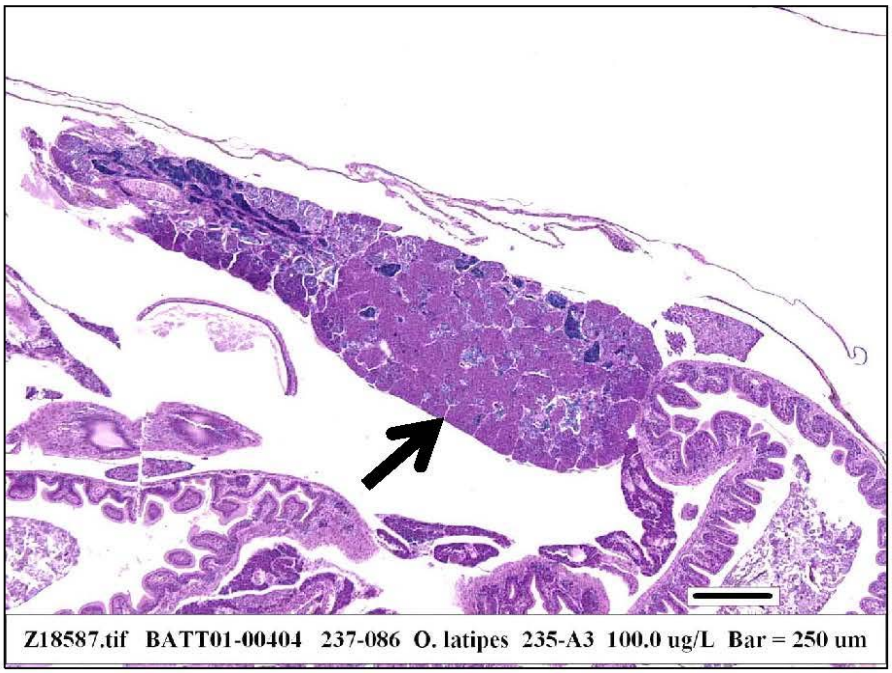

Figure 5 (Z18587). Testis from a male exposed to 100.0 mg/L 2-EHHB, Animal No. 235-A3. The ovoid mass in the posterior testis (arrow) is a benign seminoma. H&E.

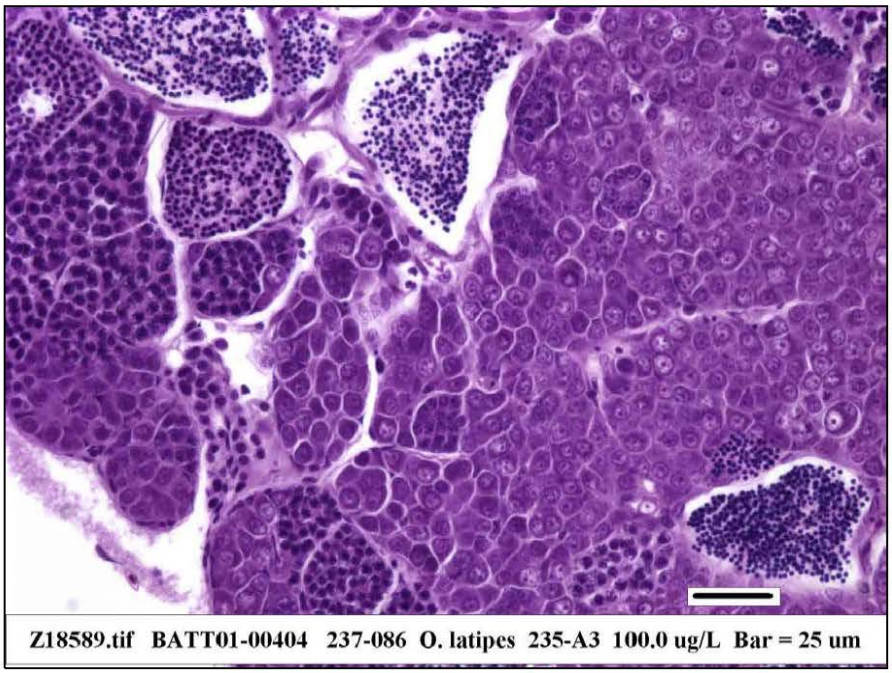

Figure 6 (Z18589). Higher magnification of testis from previous image. The tumor (right side of image) is comprised primarily of spermatogonia-like cells that surround entrapped spermatocysts of non-neoplastic testis. H&E.

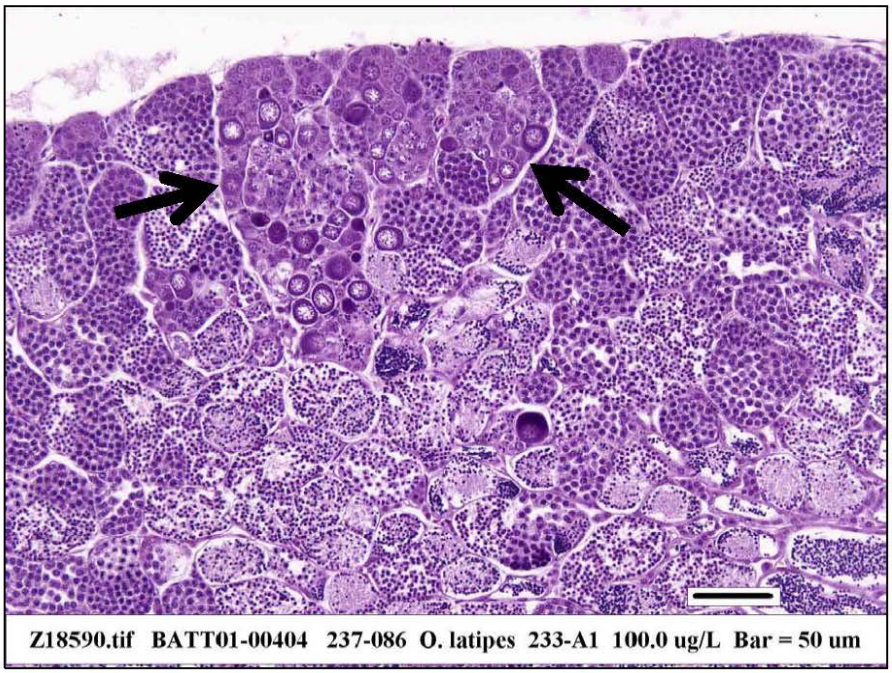

Figure 7 (Z18590). Testis from another male exposed to 100.0 mg/L 2-EHHB, Animal No. 233-A1. A benign dysgerminoma of the testis (arrows) presents as a patchy area of oogenic tissue. H&E.

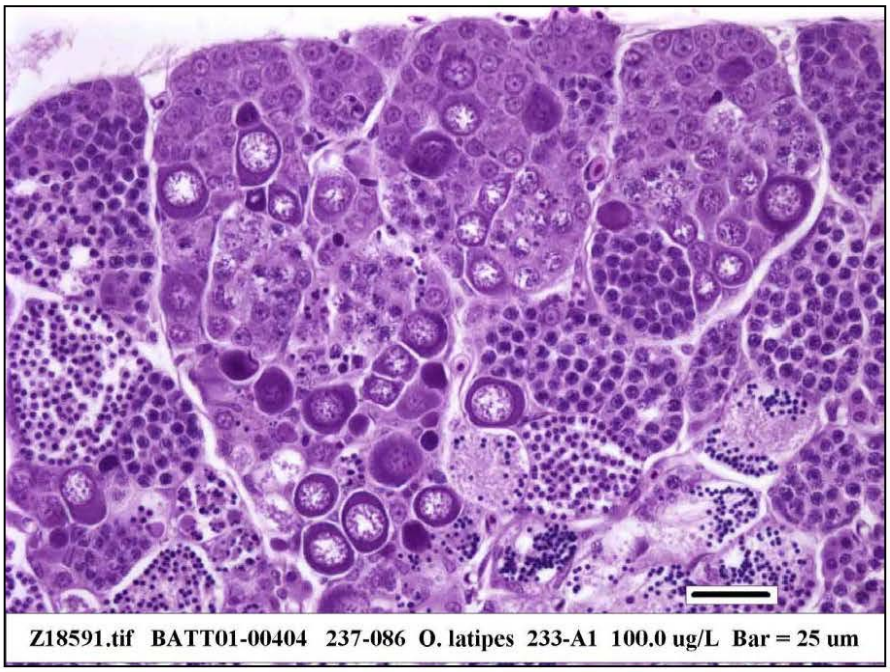

Figure 8 (Z18591). Higher magnification of testis with benign dysgerminoma from preceding figure. H&E.

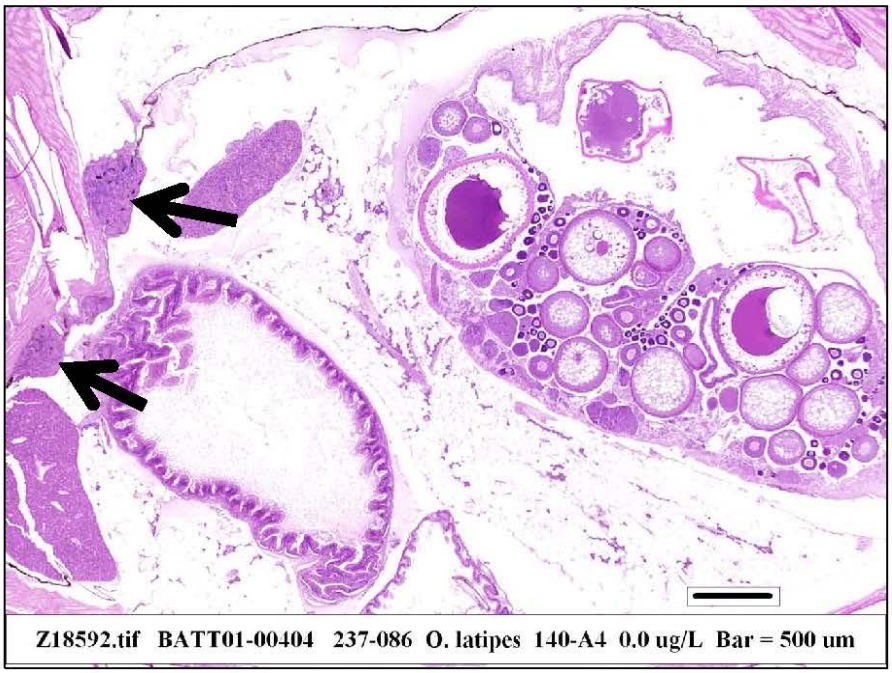

Figure 9 (Z18592). Ovary and anterior coelomic cavity from a control female, Animal No. 140-A4. Note nodular masses attached to the anterior coelomic wall (arrows). H&E.

Figure 10 (Z18595). Higher magnification of one of the anterior coelomic masses from the prior figure. The mass consists of neoplastic spermatogenic tissue (metastatic seminoma). H&E.

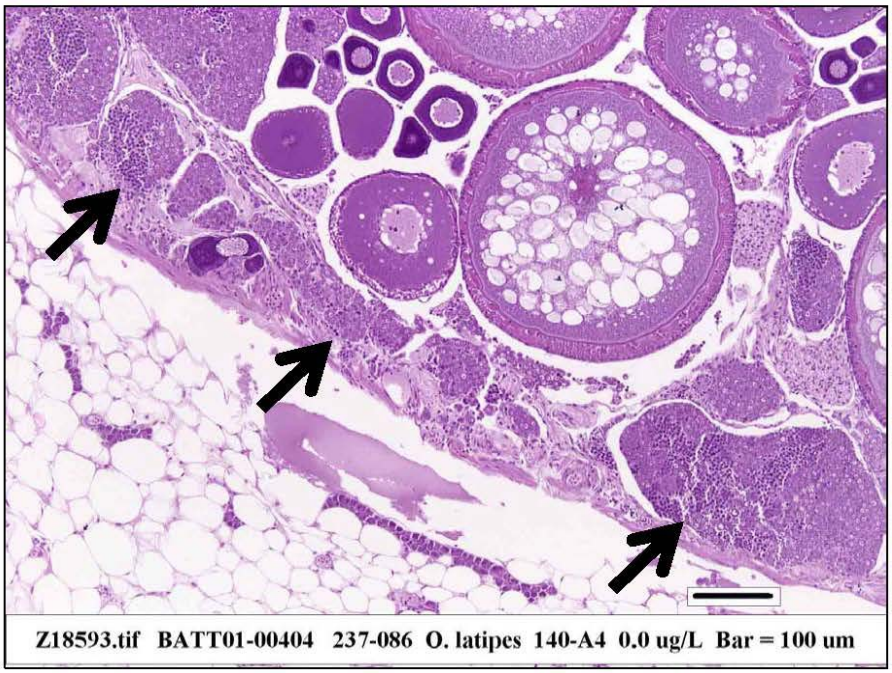

Figure 11 (Z18593). Higher magnification of ovary from Fig. 9. There are multiple variably-sized nodular areas of neoplastic spermatogenic tissue (arrows) in the ventral ovary. This is the primary site of the malignant seminoma. H&E.

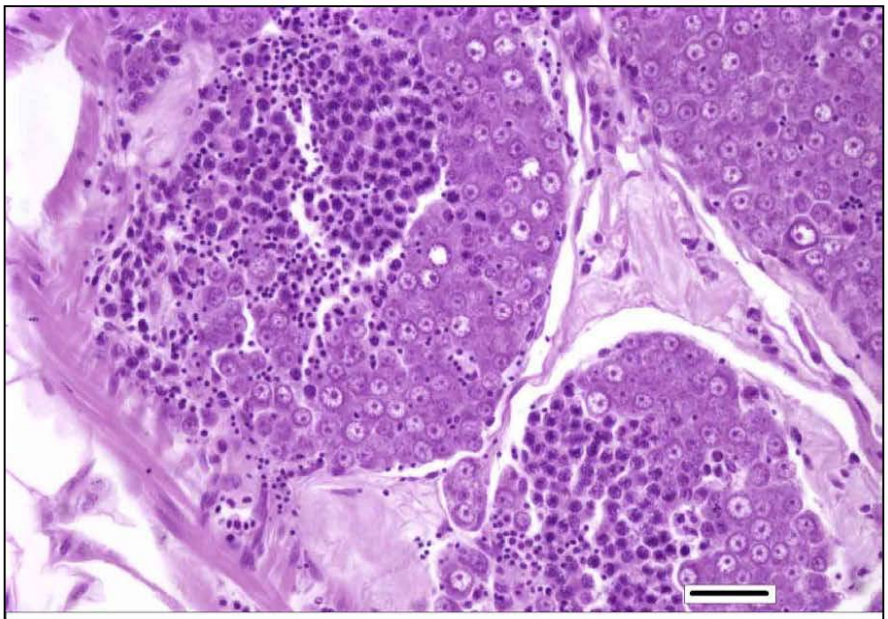

Figure 12 (Z18594). Higher magnification of ovarian seminoma from preceding figure. This is a spermatocytic-type seminoma. H&E.

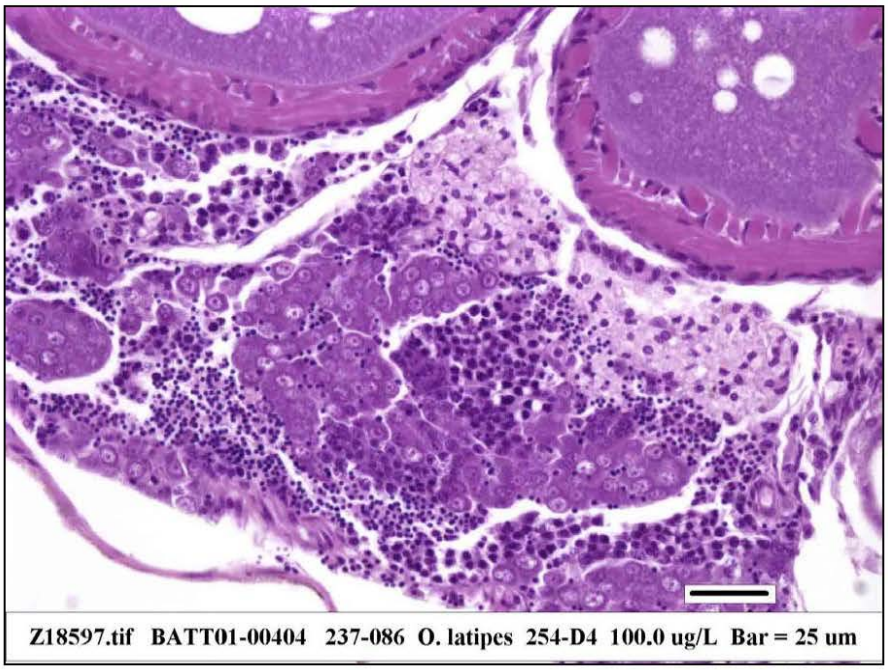

Figure 13 (Z18597). Ovary from another female exposed to 100.0 mg/L 2-EHHB, Animal No. 254-D4. Another spermatocytic seminoma. H&E.

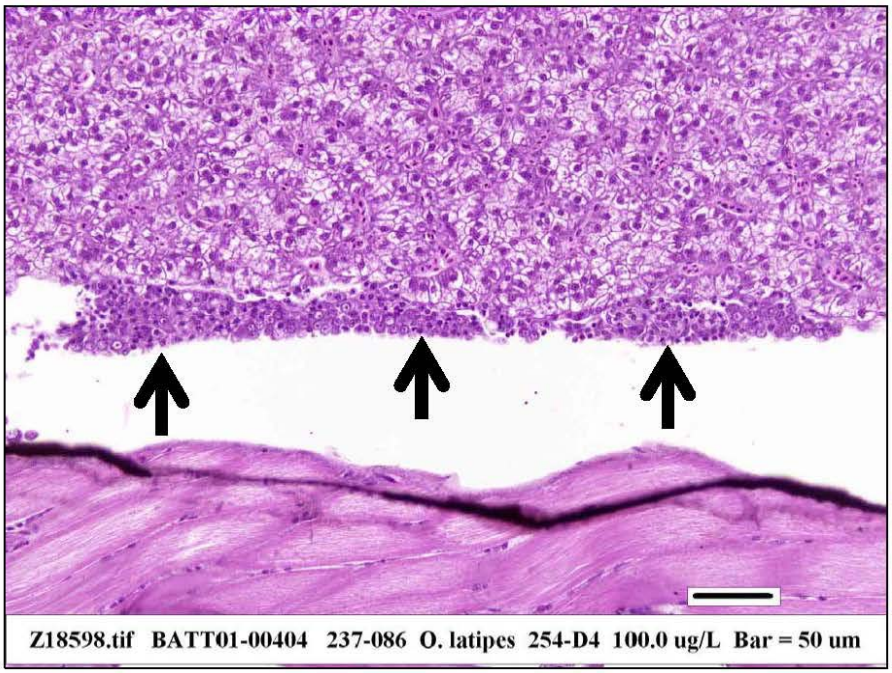

Figure 14 (Z18598). Liver from the female illustrated in the previous figure. Metastatic seminoma tissue (arrows) is adherent to the ventral liver capsule. H&E.

Appendix E. Liver Vitellogenin mRNA Expression Report

FISH SHORT-TERM REPRODUCTION ASSAY OF 2-ETHYLHEXYL 4-HYDROXYBENZOATE WITH JAPANESE MEDAKA: ANALYSIS OF HEPATIC VITELLOGENIN

FINAL REPORT

EAG LABORATORIES – EASTON PROJECT NUMBER: 607A-110
FEL STUDY NUMBER: BATT01-00404

U.S. EPA OPPTS NUMBER 890.1350
OECD GUIDELINE 229

AUTHORS:

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VTG ANALYSIS EXPERIMENTAL INITIATION DATE: July 6, 2017
VTG ANALYSIS EXPERIMENTAL TERMINATION DATE: July 10, 2017
STUDY COMPLETION DATE: May 7, 2018

SUBMITTED TO:

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EAG LABORATORIES

PROJECT NO.: 607A-110

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: U.S. Environmental Protection Agency

TITLE: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka: Analysis of Hepatic Vitellogenin

EAG LABORATORIES – EASTON PROJECT NUMBER: 607A-110

FEL STUDY NUMBER: BATT01-00404

STUDY COMPLETION DATE: May 7, 2018

The analysis performed at EAG Laboratories - Easton was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Part 160), USEPA Federal Insecticide, Fungicide and Rodenticide Act (FIFRA).

PRINCIPAL INVESTIGATOR:

Amanda L. Milligan, B.S.
Senior Biologist
EAG Laboratories-Easton

May 7, 2018
Date

EAG LABORATORIES

PROJECT NO.: 607A-110

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Part 160), USEPA Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO: PRINCIPLE INVESTIGATOR	MANAGEMENT:	DATE REPORTED TO: STUDY DIRECTOR:	MANAGEMENT:
VTG Standards Preparation*	June 8, 2017	July 17, 2017	July 17, 2017	July 20, 2017	July 20, 2017
Vitellogenin Analysis Data and Draft Report	August 15 to 17, 2017	August 17, 2017	August 25, 2017	August 25, 2017	August 25, 2017
Final Report	May 7, 2018	May 7, 2018	May 7, 2018	May 7, 2018	May 7, 2018

All inspections were study-based unless otherwise noted.
*Inspection was facility-based.

Allison M. Jacobs
Quality Assurance Representative III
EAG Laboratories-Easton

May 7, 2018
Date

EAG LABORATORIES

PROJECT NO.: 607A-110

REPORT APPROVAL

SPONSOR: U.S. Environmental Protection Agency

TITLE: Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka: Analysis of Hepatic Vitellogenin

EAG LABORATORIES PROJECT NUMBER: 607A-110

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PRINCIPAL INVESTIGATOR:

Amanda L. Milligan, B.S.
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EAG Laboratories - Easton

7 May 2018
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7 May 2018
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INTRODUCTION

The analysis of vitellogenin 1 (*Vtg* 1) mRNA in Japanese medaka liver tissue treated with 2-EHHB (FEL study 404) was conducted by EAG Laboratories for the U.S. Environmental Protection Agency at the EAG Laboratories aquatic toxicology facility in Easton, Maryland. The analyses were conducted from July 6, 2017 to July 10, 2017. The original raw data and the original report will be sent to Battelle Memorial Institute for archiving.

Liver tissues preserved in RNA*later* Solution were received at the EAG Laboratories, Easton, Maryland facility on April 19, 2017 from Fort Environmental Laboratories, Inc. Upon receipt, samples were visually inspected to confirm presence of tissue and to note condition of the sample, assigned unique EAG Laboratories accession numbers, logged into the in-house sample tracking system, and stored at -20˚C. The measurement of copies *Vtg* 1 mRNA/ ng total RNA was analyzed in livers from 2 male and 2 female fish per replicate and performed using real-time quantitative PCR (RT-qPCR) methodology (1).

MATERIALS AND METHODS

Preparation of RNA Standards

Extraction of RNA

To prepare the RNA standards, medaka livers were aseptically removed from surplus female medaka housed in the culture unit of EAG Laboratories, Easton, Maryland facility. Livers were immediately placed in micro-tubes containing of RNA*later*, incubated at 4˚C overnight to allow perfusion of the tissue with RNA*later*, and then transferred for long-term storage in a freezer at -20˚C. Extraction of the RNA from the livers was done as described in Appendix 1.1. A method outline for the preparation of standards is included in Appendix 1.2.

Dilution and Reverse Transcription

After extraction of the RNA, a 2 µL volume of each sample was analyzed on the NanoDrop 2000 (Thermo Scientific) to determine RNA concentration. Each standard was analyzed in duplicate and the values averaged to determine mean RNA concentration. Next, RNASTD1, RNASTD2, RNASTD3, RNASTD4, RNASTD5 and RNASTD6 were diluted with Nuclease free water to 200 ng total RNA/µL. Diluted samples were used with the SuperScript™ II Reverse Transcription kit (Life Technologies, Frederick, MD) to produce cDNA. Component volumes used and incubation time and temperatures are presented Appendix 1.3.

Amplification of cDNA and Transcription to RNA

cDNA generated from the SuperScript™ II (Life Technologies) reaction was then selectively amplified using AmpliTaq Gold® DNA Polymerase kit via PCR with primers specific to a region surrounding the vitellogenin 1 gene. The nucleotide sequence positions of the forward and reverse primers used in mRNA standard preparation are presented in Appendix 1.4. The *Vtg* 1 standard forward and reverse primer sequences are detailed in Appendix 1.5. Typical component volumes for the PCR reaction and cycling parameters are listed in Appendix 1.6 and 1.7, respectively. After amplification of the cDNA, a transcription step was initiated to produce RNA strands similar to naturally occurring vitellogenin 1 mRNA using MEGAscript® kit. A typical reaction mix is shown in Appendix 1.8.

DNase Treatment and Sample Clean-up

Upon completion of the second reverse transcription, standard samples were treated with DNase I (Life Technologies, Frederick, MD). A 1 µL aliquot of DNase I was added directly to each reverse transcription tube, mixed by pipetting and incubated at 37˚C for 10 minutes. At the end of the incubation period, samples were spun in a centrifuge to collect the mixture in the bottom of the tube and were immediately processed through the MEGAClear™ kit (Life Technologies, Frederick, Maryland) as outlined in Appendix 1.9.

Adjustment of Standard Concentration

In order to calculate the starting concentration of the prepared standards, a 2.0 µL aliquot of each sample was analyzed in duplicate on the NanoDrop 2000 (Thermo Scientific) (2). Values for each standard were averaged to determine the mean concentration for use in further calculations. The RNA sample with the highest purity and concentration was chosen as the stock solution for the high calibration standard, from which all subsequent standard concentrations were prepared. Starting concentrations of copy number per µL were determined by the equation given by the U.S. EPA (Appendix 1.10).

Once the number of *Vtg* 1 copies per µL of RNA was determined, the high calibration standard was diluted to a concentration of 2.5×10^9 copies *Vtg* 1 mRNA/µL with nuclease-free water. The high calibration standard was then portioned into 20 µL aliquots in individual tubes for storage at -80˚C.

Dilution of Standards

Prior to the master mix preparation for each assay, a serial dilution of the RNA standard was made from a single 20 µL aliquot. The aliquot was removed from frozen storage, placed on an ice tray to gradually thaw and a serial dilution was prepared. Dilutions were made as shown in Appendix 1.11.

Sample Analysis of Vitellogenin mRNA

Extraction of RNA

Prior to being processed for RNA extraction, samples were removed from frozen storage and placed on an ice plate and allowed to gradually equilibrate to room temperature. Extraction of total RNA from liver tissues was performed using the RNeasy® Protect Mini Kit, product #74126 (Qiagen, Louisville, KY and Germantown, Maryland) with methodology presented by the U.S. EPA (1). Liver tissue was aseptically removed from the RNA*later* with sterile forceps and placed in a 1.5 mL micro-centrifuge tube containing 600 µL of Buffer RLT and a small amount of glass beads. The tubes were then placed on a pulse vortexer and allowed to vortex for ≥ 5 minutes to disrupt the tissue sample. Samples were then processed further as outlined in Appendix 1.1.

RNA Quantitation of Samples

Once the samples were extracted a small volume (1.5 µL) of each sample was used to determine the RNA concentration using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, NanoDrop Products, Wilmington, DE) (2). Prior to and periodically during analysis of samples, RNase-free water supplied with the RNeasy Protect Mini Kit (Qiagen) was used as a blank on the NanoDrop 2000. Each sample was analyzed in duplicate for total RNA concentration (ng/µL), and the results were averaged to determine mean total RNA concentration per sample. Total RNA in each study sample was then normalized to a concentration of approximately 40 ng/µL by adding 15 µL of RNA template to a calculated amount of nuclease-free water. If less than 5 µL of nuclease-free water was required for the dilution, or if the concentration of total RNA in the study sample was less than 40 ng/µL, the normalization step was not performed and the total RNA concentration as determined by the NanoDrop 2000 Spectrophotometer was used in the final calculation of copies of *Vtg* 1 mRNA/ng total RNA. Although normalizing the concentration of total RNA is not specified in the guideline (1), it was discovered during the non-GLP optimization process that normalizing RNA/cDNA to 40 ng/µL produced uniform and consistent conditions across the tested samples during qPCR analysis.

Primer/Probe Design

New primers and probe were designed by EAG Laboratories – Easton, since the forward primer sequence listed in the guideline (1) was not successful in amplifying *Vtg* 1 gene during the non-GLP method optimization. To verify the accuracy of EAG Laboratories designed primers and probe (sequences shown in Appendix 2.2), which are used to measure the absolute quantity of *Vtg* 1 mRNA in medaka study samples, these sequences were entered in the NCBI nucleotide BLAST program and aligned to GenBank accession number XM_020702291.1 (*Oryzias latipes* vitellogenin-1-like (LOC101173135)), mRNA (3, 4). In order to validate EAG forward primer, reverse primer and probe sequences, the whole XM_020702291.1 FASTA sequence of mRNA Japanese medaka (*Oryzias latipes*) was selected from GenBank and entered into the PrimerQuest tool of Integrated DNA Technologies (5). The custom design section was chosen and the EAG forward primer, reverse primer and probe sequences were entered into partial design input of the custom target region section under the custom design parameters within the IDT platform. The nucleotide position information including start and stop positions, amplicon length (106 bp), melting temperature (Tm), GC content of primer (designed by PrimerQuest) are presented in Appendix 2.1. Primers and probe sequences are provided in Appendix 2.2. The BLAST analysis of EAG primers and probe sequences showed 100% genetic similarities with mRNA Japanese medaka under the accession number of XM_020702291.1 (6).

Measurement of Vitellogenin mRNA

Analysis for vitellogenin 1 mRNA was conducted on the Rotor-Gene Q platform (Qiagen) using the 72-place locking ring. Cycling parameters for the assays are outlined in Appendix 2.3. The quantitative reverse transcription PCR (RT-qPCR) master mix used for the assays was made by keeping all components on ice blocks, adding each component individually to a clean 2.0 mL micro-tube and vortexing the mixture. Component concentrations for a typical run are presented in Appendix 2.4. After vortexing, 20.5 µL aliquots of the Master Mix were added to individual PCR clean strip tubes and 4.5 µL of extracted RNA was added to its designated tube.

For each sample set analyzed, one or two No-Template Control (NTC) samples and two Positive Control samples were analyzed. The NTC samples consisted of 20.5 µL of Master Mix and 4.5 µL of RNase/DNase free water and the Positive Control samples consisted of 20.5 µL Master Mix and 4.5 µL of a known concentration of *Vtg* 1 standard (Appendix 1.11). The blank was an empty tube. The RNA

standards, analyzed in triplicate, consisted of 4.5 µL of each RNA standard and 20.5 µL of the Master Mix (Appendix 2.4). A sample layout for a typical 72-place locking ring is included in Appendix 2.5.

After a RT-qPCR run was finished, the Rotor-Gene Q software (7) was used to calculate the standard curve and vitellogenin 1 mRNA concentrations in test samples. An example of a typical standard curve is presented in Appendix 2.6. Calculated values of replicate samples were determined by the software through comparison to the standard curve. The concentrations generated by the Rotor-Gene Q software were then used to calculate a final concentration of Copies *Vtg* 1 mRNA/ng Total RNA using the equation listed in Appendix 2.7. Measured concentrations of vitellogenin mRNA in male and female liver samples are presented in Appendices 2.8 and 2.9, respectively. The results of the NTC and Positive Control samples are presented in Appendix 2.10.

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Appendix 1

Preparation of mRNA Standards

Appendix 1.1

Method Outline for Extraction of Total RNA from Liver Tissue

Transfer liver tissue (typically ≤ 30 mg) into a micro-centrifuge tube containing 600 µL of Buffer RLT and a small amount of glass beads

↓

Vortex sample in tube for ≥5 minutes

↓

Centrifuge sample at 17,000 x *g* for 3 minutes and transfer 550 µL of supernatant to a new, clean 1.5 mL micro-centrifuge tube

↓

Add 550 µL of 70% ethanol and mix

↓

Transfer 700 µL to a RNeasy® Mini spin column, fitted in a 2 mL collection tube and centrifuge at 8,000 x *g* for 1 to 2 minutes

↓

Discard flow-through; add 700 µL of Buffer RW1 to spin column and centrifuge at 8,000 x *g* for 30 seconds to 1 minute

↓

Discard flow-through; add 500 µL of Buffer RPE to spin column and centrifuge at 8,000 x *g* for 30 seconds to 1 minute

↓

Discard flow-through, add 500 µL volume of Buffer RPE to spin column and centrifuge at 8,000 x *g* for 1 to 2 minutes

↓

Discard flow-through and collection tube. Fit spin column into new 2.0 mL collection tube and centrifuge at 17,000 x *g* for 1 minute

↓

Discard flow-through and collection tube, place spin column in new, clean, labeled micro-centrifuge tube, add 50 µL of RNase-free water to center of spin column membrane, incubate at room temperature for 1 minute and centrifuge at 8,000 x *g* for 1 minute

↓

Discard spin column and analyze immediately by NanoDrop or store eluate at -80˚C for later analysis

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Appendix 1.2

Method Outline for Preparation of mRNA Standards

Extract total RNA from the livers of female medaka

↓

Adjust concentration of extracted RNA to 200 ng Total RNA/µL using Nuclease-free water

↓

Create cDNA from extracted RNA via SuperScript™ II Reverse Transcription kit

↓

Selectively amplify vitellogenin cDNA via PCR using AmpliTaq Gold® DNA Polymerase Kit

↓

Convert amplified vitellogenin cDNA into RNA via MEGAScript® kit

↓

Treat newly transcribed vitellogenin RNA with DNase I

↓

Clean vitellogenin RNA samples with MEGAClear™ kit

↓

Determine the number of vitellogenin copies per µL in standards using concentration given by the NanoDrop

↓

Dilute the standard with the highest purity and concentration to $2.5x10^9$ copies/µL

Appendix 1.3

SuperScript™ II Reverse Transcription Preparation

Component	Volume (µL)/Sample[1]	Process
RNase/DNase-Free Water[2]	5.89	Combine these components into one clean microfuge tube. Briefly vortex to mix. Pipette 8.0 µL of this mixture into six clean microfuge tubes.
dNTP mix (10mM)[3]	1.06	
Oligo d(T) primer[4]	1.06	
Template RNA sample @ 200 ng/µL	4.0	Add 4.0 µL of RNA template sample to each microfuge tube. Incubate microfuge tubes at 65˚C for 5 minutes and immediately chill on ice.
SuperScript™ II 0.1M DTT[5]	2	Combine these components into one clean microfuge tube. Briefly vortex to mix. Pipette 7.0 µL of this mixture to each sample tube. Pipette to mix. Incubate microfuge tubes at 42˚C for 2 minutes.
SuperScript™ II 5X Buffer[5]	4	
RNasin[6]	1	
SuperScript™ II RT[5]	1.0	Add 1.0 µL to each microfuge tube. Pipette to mix. Incubate microfuge tubes at 42˚C for 50 minutes.
Inactivate reaction by heating at 70˚C for 15 minutes		

1 Component volumes were scaled up to accommodate 6 samples, plus additional samples to account for any losses during processing.
2 Supplied by Life Technologies, Carlsbad, CA
3 Supplied by Promega, Madison, WI
4 Supplied by Promega, Madison, WI
5 Supplied by Life Technologies, Frederick, MD
6 Supplied by Promega, Madison, WI

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Appendix 1.4

Nucleotide Sequence Positions of Forward and Reverse Primer for mRNA Standards

Sequence NM_001104677.1 Vitellogenin1, mRNA downloaded from National Center for Biotechnology Information (NCBI).
Forward and reverse primer sequences for mRNA standard preparation provided by the U.S. EPA OCSPP 890.2200 guideline (1).

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Appendix 1.5

Primers Used for Amplification of Standards

Primer	Sequence (5' to 3')
Vtg 1 Standard Forward	TAA TAC GAC TCA CTA TAG GGA GAT CCC TCG GAT ACG GCA CAA T
Vtg 1 Standard Reverse	TAG ACA GCT TTG CTG TAA CGT AAG C

Note: Underlined nucleotides are the T7 promoter sequence.

Appendix 1.6

Master Mix Preparation for Amplification of cDNA per Sample

Master Mix Component[1,2]	**Volume Required Per Sample (µL)**
RNase/DNase free water[3]	15.4
AmpliTaq® Gold Buffer[4]	2.5
AmpliTaq® Gold $MgCl_2$[4]	1.5
dNTP mix (10 mM)[5]	0.5
Vtg 1 Standard Forward Primer @ 10 µM[6]	0.5
Vtg 1 Standard Reverse Primer @ 10 µM[6]	0.5
AmpliTaq Gold® DNA Polymerase[4]	0.16
Template cDNA	4.0

1 Component volumes were scaled up to accommodate samples, plus additional samples to account for any losses during processing. Components were mixed and 21 µL was aliquoted to individual microfuge tubes followed by the addition of 4 µL of template cDNA.
2 Primer sequences given in EPA methodology (1) are listed in Appendix 2.2.
3 Supplied by Life Technologies, Carlsbad, CA
4 Supplied by Life Technologies, Frederick, MD
5 Supplied by Promega, Madison, WI
6 Supplied by Integrated DNA Technologies, Coralville, IA

Appendix 1.7

Cycling Parameters for Amplification of cDNA

Step	Temperature (˚C)	Time (s)	Number of Cycles
1 (Initial Denaturation)	95	600	1
2 (Denaturation)	94	30	35
3 (Anneal)	58	60	
4 (Extension)	72	60	

Appendix 1.8

Reaction Preparation for Transcription of Amplified cDNA

Component [1]	**Required Volume (µL)**
RNase/DNase-free water[2]	4.0
MEGAScript® 10mM ATP[3]	2.0
MEGAScript® 10mM CTP[3]	2.0
MEGAScript® 10mM GTP[3]	2.0
MEGAScript® 10mM UTP[3]	2.0
MEGAScript® 10X Buffer[3]	2.0
cDNA template	4.0
MEGAScript® RNA Polymerase[3]	2.0

[1] Components were added individually to tubes in the order listed above and mixed by pipetting. Once all tubes contained all components the tubes were incubated at 37°C overnight.
[2] Supplied by Life Technologies, Carlsbad, CA
[3] Supplied by Life Technologies, Frederick, MD.

Appendix 1.9

Method Outline for Clean-up of DNase I Treated Standards

Add 80 µL of Elution Solution to sample and mix gently by pipetting in a 200 µL tube

↓

Transfer up to 100 µL of the mixture to a new, clean 1.5 mL micro-centrifuge tube and add 350 µL Binding Solution and 250 µL 100% Ethanol, mixing thoroughly by pipetting after each addition

↓

Pipette up to 700 µL of the mixture onto a filter cartridge fitted in a collection tube and centrifuge at 10,000 x *g* for 1 minute

↓

Discard flow-through, add 500 µL Wash Solution to the filter cartridge and centrifuge at 10,000 x *g* for 1 minute

↓

Discard flow-through; add a second 500 µL volume of Wash Solution to the filter cartridge and centrifuge at 10,000 x *g* for 1 minute

↓

Discard flow-through; centrifuge at 10,000 x *g* for 1 minute to dry the filter and place filter cartridge into a new micro-centrifuge tube

↓

Add 50 µL of Elution Solution to the center of the membrane, incubate at 70˚C for 10 minutes and centrifuge at 10,000 x *g* for 1 minute

↓

Add a second 50 µL volume of Elution Solution to the center of the membrane; incubate at ~ 70˚C for 10 minutes and centrifuge at 10,000 x *g* for 1 minute

↓

Discard filter cartridge, analyze on NanoDrop and store samples at -80˚C

Appendix 1.10

Example Calculation of Copy Number per µL in Standards

$$\frac{\frac{6x10^{23}copies}{mol}\ x\ Concentration\ (\frac{g}{\mu L})}{Molecular\ weight\left(\frac{g}{mol}\right)} = amount\frac{copies}{\mu L}$$

Molecular weight (g/mol) of ssRNA[1] = $640\frac{Daltons}{bp} \times \#\ of\ base\ pairs$

Vtg 1 standard RNA[1] = 612bp

Sample RNA has a concentration of $\frac{150ng}{\mu L}$

$$\frac{\frac{6x10^{23}copies}{mol}\ x\ 1.5x10^{-7}\ (\frac{g}{\mu L})}{3.92x10^{5}\left(\frac{g}{mol}\right)} = \frac{2.3x10^{11}\ copies}{\mu L}$$

[1] Values provided by the U.S. EPA (1)

Appendix 1.11

Dilution of Standards Used in RT-qPCR Assay

Initial Standard Concentration (copies/µL)	Standard Volume (µL)	RNase/DNase-free Water Volume (µL)	Final Volume (µL) [1]	Final Standard Concentration (copies/µL)	Final Standard Identification (607A-110-)
$2.5x10^9$	10	90	90	$2.5x10^8$	STD9
$2.5x10^8$	10	90	90	$2.5x10^7$	STD8
$2.5x10^7$	10	90	90	$2.5x10^6$	STD7
$2.5x10^6$	10	90	90	$2.5x10^5$	STD6 [2]
$2.5x10^5$	10	90	90	$2.5x10^4$	STD5
$2.5x10^4$	10	90	90	$2.5x10^3$	STD4
$2.5x10^3$	10	90	100	$2.5x10^2$	STD3

[1] Serial dilutions were performed from highest standard (STD10) resulting in a reduced final volume.
[2] Standard was used for the positive control sample during sample analysis.

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Appendix 2

The Analysis of Vitellogenin 1 (*Vtg* 1) by RT-qPCR Analysis

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Appendix 2.1

Nucleotide Sequence Positions of Primer and Probe for Study Samples

Parameter Set: RT-qPCR (Primers with Probe)
Sequence Name: EAG Primers/Probe Sequence
Amplicon Length: 106

		Start	Stop	Length	Tm	GC%
Forward	TCTAAGGGCGAAACAGAAGAC (Sense)	1460	1481	21	62	47.6
Probe	TTGGGAAATGCAGGACACCCTAGT (Sense)	1502	1526	24	68	50
Reverse	TGCCGTGAATGGGCATAA (AntiSense)	1548	1566	18	62	50

Base	Sequence
1	TAAAAGGCCAGCACAAATGTGAGCACAGCCCCATTTAACCATGAGAGCAGTTGTGCTCGCTCTGTCCTCTAGCCCTAGTGGCTGGACAAGCTTCCAACCAT
101	GGTGCAACTGTGTTTCCCTGAATTTTCTGCTGGCAAAACGTATGTGTATATGTATGAGATGTCCGTCATTAACGGTCTACCCGAGGAGGGTTTGGCAAGAG
201	CTAAGATGAACGCCAGCTGCAAATTTCTCATAAGTGCATTTGACAAAAACACATACATGTTAAAGGTTGAGGATGTGATGATGCAAGAGTTCACTGGCTT
301	TTGGCCAAAGGAACATGCAGAATCAATTGGGAATATTCCTAAAGCATTTACACCAGAGTTCAATATTCCCATCAAGTTTGAATACAGCAATGGTGTTGTA
401	GGAAAAGTTTATGCCCCCGAAGGAGTCTCAGATTTGGTCCTGAACTTCTACAGAGCCGTCCTTAACATCATTCAGCTTAACATCAAGAAGACACACAACG
501	TTTATGATCTCCAGGAGACTGGAATTCAAGGCGTGTGCAAGACCCTCTACTCTGTTAATGAAGATGTAAAGGCTGAACGCATTCACCTGACTAAAACCAG
601	GGACATGAACCACTGTCAGGAAAGGGTCTATAGAGACATGGGTTTGGCATACACAGAGAAATGCGACAAGTGCCAGCAGGAATCCAAGAATTTAAGAGGT
701	TCAACATCATACAGATACACCCTGAAACCAGTTCTTAGTGGTGTTATGATCCTGGAGGCAGATGTAAATGAGCTTATCCAGTTCTCACCGTTCTCTGAGA
801	GTTATGGGGCTGCTCAAACAGAGACCAAACAAACCTTCGTCTTCCAAGAGATTCAGGATTCCTCCATTGCACCAATTTCTGCTGAGTATCATCACCGTGG
901	ATCTCTCAAGTATGAGTTCACTAATGACTTTGAGTGGACACCACTTCAGCTTGTCAATGTCACTGATGAACAGGCACAGACAGAGGAGCTTCTGAATCAT
1001	CTAGTTACCCATAATGCAGAGAAAGTCACTGATCATGCTCCTCTGAAATATTTGGAATTGGTTGAGTTTTACGTCTTGCCCGCTATGAAGACCTGGAAG
1101	CATTCTGGAGCAAGTACAGAGATACGCCTTCTCACAGACTCTGGCTCTTGGAAGCTATTCCAGCCACAGGAACGTCTGCTGCTCTCAGATTCATCAAGGA
1201	GAAATTTACATCTGAAGACTTAAGTGTTACTGAGGCAGTTAGAACCTTGGTTGCAGCTGTTCACATGGTTACAGCAAACCCTGAATCCATCAAGCTGTTT
1301	GAGACCCTGACAGAAGACAGCAAACTCAACGCCAACCCAGTTCTACGTGAGATCGTCTTCCTCGGATACGGCACAATGATTCCAAATATTGTGCAGAGT
1401	CAGATGTCTGTCCTGTCGAATATATAAAACCAATTCAGAAGCGTCCTCCAGAGGCAGTTTCTAAGGGCGAAACAGAAGACATCATTTTGTATGTGAAGGT
1501	TTTGGGAAATGCAGGACACCCTAGTAGCCTCAAGTCAATCACAAAGATTATGCCCATTCACGGCACTGCTGCTGCTTCTCTGCCAATCACAGTCCATATT
1601	GAAGCCATCATGGCTCTGAGGAACATTGCAAAGAAAGAACCAAGAATGGTTCAGGAACTGGCTCTTCAGCTCTACATGGACAAGGCTCTTGACCCAGAGC
1701	TTCGTATGCTTTCATGCATTGTGCCTGTTTGAGACCAATCCTTCAATGGCGCTGATTTCCACCCTTGCCAATGCTGTTAAGTCAGAGGAGAATTTGCAGGT
1801	TGCCAGCTTCACATACTCTGACATGAAGTCCCTGAGCAGGAGCGCTTCCATGATTCATCCATCAGTAGCTGCAGCTTGTAATGTTGCCATGAAGATCCTG
1901	AGTCCAAAGCTGGACAGACTGAGCTTACGTTACAGCAAAGCTGTCTATGGGGAAGCCTATAGCAGCTCTTTTATGCTTGGTGCTGCTGCTACCGCTTTCT
2001	ACATCAACGATGCTGCTACATTCCTGCCAAGATCTGTTGTTGCCAAGACCAAAGCCTTCTTTGTTGGAGCTGCTGCTGATGTTCTGGAGATTGGAGTAAG
2101	AACTGATGGACTTCAAGAGACCCTTCTGAAAAACCCTTCAGTTCTAAAAAGTGGTGACAGGATCACCAAGATGAAACGTGTTATCAAGGCCTTTCTCAG
2201	TGGAGGTCCTTGCCCAACAACAAACCCTTAGCTTCTTCTATATCAAGCTTTTTGGACAAGAGATTGCCTTGCGAACATCGATAAACCCATGGTTGAGC
2301	AGGCAATTGCGCTAGCCAGTGGTCCCTCTGTTCAGGCTTTTGGTGTAAAAGCTCTGAAGACTCTTTTACTCTCTGGTGTCAGTTTCAACTATACAATGCC
2401	TGTGATGTATACTGAGATGCGACGCGCAATACCTACTGCTACTGGTATTTCAGTGCAGCTCAGTCTTTTTTCTGCTGCTGTGGCTGGAGCTGTCGTTAAA
2501	ATCGAACCAACTCCTGTTGCCACATCTGCCTGAAGACTTTCCTCTCTCTCGCCTTCTGGAGACAGACATCAAGCTTGAGACTGAAATCAGACCCAGTTTTG
2601	CTATGGACACCTATGCTGTGATGGGAATTAACAATCCTTTCTACCATGCAATCATTGTGTCAAGAGCTAAATTTAACTCAACTCTGCCCTCCAAAATCTC
2701	CGCAAGACTTGACATTAAAGAGGGTGACTTCAAGATAGAAGTAAGATGTCCCACTTAGATTTTATCAGAAATGACAGTTTATCAAATGACCAGCTCTTCA
2801	CTTCTTTTACTTTATATGCTCTTGCTCCGTCTTGCCTCTGCATTGCCACATCCTCAAAAGCACACTTTTCCCATTTTCTGTAACTCATTCCCTGTTCTT
2901	TTTTACTATATGATGGTGCTTGTTGTCCATTAGGTGATATGATGCCCTCAAATTTTCCATATTGTTCACAAAGTGATTAACATGGTCTTTATAAGATCTGAC
3001	ACCTTCTTTGCTTTGTCCTTTCCCATTTTCAGTTATTTATGATAACAATTTCAAATACTCTTGATTATGATTCAAACAACAGAAACGCCAGTCATTCG
3101	CTTCTCATTCAGACATTCACAGCGGTGGATTTCATTCACAACAATCCACCAGTCACACAACGCTTGCGCTTGTCCGGATAAAAATAAAAATAAAATGA
3201	AGGTCTTTAAGGAAACCAGTCAGGAAACCACATCTGACATGTGCGTATTGATCCTTAGGTCGCGAAGCACAACAATGCCAGAACACATCTGACAAATTTC
3301	CACAACCACCTAGAAAAATACCACGGTACCTGATAGAGTCCTAGAA

Appendix 2.2

Primers and Probe Used for Amplification of Study Samples

Primer/Probe	Sequence (5' to 3')
Vtg 1 Forward	TCT AAG GGC GAA ACA GAA GAC
Vtg 1 Reverse	TGC CGT GAA TGG GCA TAA
Vtg 1 Probe	/56-FAM/TTG GGA AAT/ZEN/GCA GGA CAC CCT AGT /3IABkFQ/

Appendix 2.3

Cycling Parameters for RT-qPCR Assay

Step	Temperature (˚C)	Time (s)	Number of Cycles
Hold 1 (Reverse Transcription)	50	600	1
Hold 2 (Initial Denaturation)	95	300	
Step 1 (Denaturation)	95	15	35
Step 2 (Anneal/Extension)	60	45	

Appendix 2.4

Target RT-qPCR Master Mix Preparation per Sample

Master Mix Component[1,2]	**Starting Concentration**	**Final Concentration**	**Volume Required (µL)**
Nuclease-Free Water			6.75
TaqMan® Fast Virus 1-step PCR Master Mix	4 X	1 X	6.25
Vtg 1 Forward Primer	10 µM	0.90 µM	2.25
Vtg 1 Reverse Primer	10 µM	0.50 µM	1.25
Vtg 1 Probe	1.25 µM	0.20 µM	4.00

[1] Volumes listed are for a single sample. Actual volumes used for assay were typically scaled up for 72 samples plus extra to account for any loss.

[2] Primer and Probe sequences are listed in Appendix 2.2.

Appendix 2.5

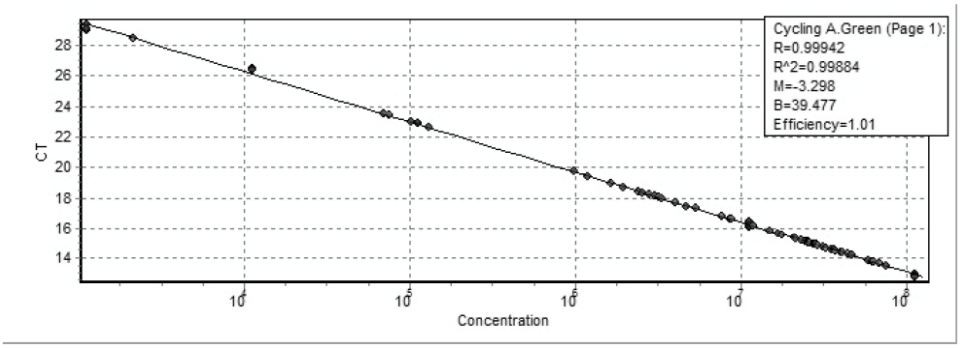
Typical Place Assignment of Standards, Samples and Controls

Tube #	Sample	Tube #	Sample	Tube #	Sample
1	STD8	**25**	Unknown5	**49**	Unknown17
2	STD8	**26**	Unknown5	**50**	Unknown17
3	STD8	**27**	Unknown6	**51**	Unknown18
4	STD7	**28**	Unknown6	**52**	Unknown18
5	STD7	**29**	Unknown7	**53**	Unknown19
6	STD7	**30**	Unknown7	**54**	Unknown19
7	STD5	**31**	Unknown8	**55**	Unknown20
8	STD5	**32**	Unknown8	**56**	Unknown20
9	STD5	**33**	Unknown9	**57**	Unknown21
10	STD4	**34**	Unknown9	**58**	Unknown21
11	STD4	**35**	Unknown10	**59**	Unknown22
12	STD4	**36**	Unknown10	**60**	Unknown22
13	STD3	**37**	Unknown11	**61**	Unknown23
14	STD3	**38**	Unknown11	**62**	Unknown23
15	STD3	**39**	Unknown12	**63**	Unknown24
16	Blank[1]	**40**	Unknown12	**64**	Unknown24
17	Unknown1	**41**	Unknown13	**65**	Unknown25
18	Unknown1	**42**	Unknown13	**66**	Unknown25
19	Unknown2	**43**	Unknown14	**67**	Unknown26
20	Unknown2	**44**	Unknown14	**68**	Unknown26
21	Unknown3	**45**	Unknown15	**69**	Positive Control[2]
22	Unknown3	**46**	Unknown15	**70**	Positive Control[2]
23	Unknown4	**47**	Unknown16	**71**	NTC[3]
24	Unknown4	**48**	Unknown16	**72**	NTC[3]

[1] Blank = empty tube.
[2] Positive control = Standard 6 (STD6).
[3] NTC = No-Template Control.

Appendix 2.6

Typical Standard Curve for Vitellogenin mRNA Analysis



Standard Curve from 607A-110 Assay 1 Females

Appendix 2.7

Calculation Used to Determine copies *Vtg* 1 mRNA/ng Total RNA in Samples

$$\frac{\left(\frac{(Sample\ value\ replicate\ 1 + Sample\ value\ replicate\ 2)}{2}\right)}{\left(\frac{ng\ Total\ RNA\ of\ Sample}{\mu L} \times 4.5\ \mu L\right)} = \frac{Copies\ Vtg1\ mRNA}{ng\ Total\ RNA}$$

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Appendix 2.8

Summary of *Vtg* 1 mRNA copies/ng Total RNA for Males

Sample ID (607A-110-RNA-)	Concentration (µg/L)	Phenotypic Sex	Normalized Concentration of Total RNA (ng/µL)[1]	Volume of Total RNA Sample Added per Reaction (µL)	ng Total RNA per Reaction[2]	Average Rotor-Gene Q Output[3,6]	VTG[4,5,6]	Log_{10} VTG[4,5,6]	%CV[6,8]
497	Control	Male	50	4.5	226	6.70E+03	2.97E+01	1.47	8.44
498	Control	Male	39	4.5	176	6.05E+03	3.43E+01	1.53	0.819
501	Control	Male	40	4.5	180	7.03E+03	3.90E+01	1.59	11.6
502	Control	Male	40	4.5	180	1.86E+04	1.03E+02	2.01	5.72
505	Control	Male	40	4.5	180	2.84E+04	1.58E+02	2.20	4.48
506	Control	Male	49[7]	4.5	221	5.16E+03	2.34E+01	1.37	2.33
509	Control	Male	40	4.5	180	1.44E+04	7.97E+01	1.90	6.41
510	Control	Male	40[6]	4.5	180	1.15E+04	6.39E+01	1.81	2.46
513	3.6	Male	40	4.5	180	4.96E+05	2.76E+03	3.44	3.14
514	3.6	Male	40	4.5	180	4.63E+04	2.57E+02	2.41	4.43
517	3.6	Male	40	4.5	180	1.80E+05	1.00E+03	3.00	3.93
518	3.6	Male	40	4.5	180	1.73E+05	9.61E+02	2.98	7.36
521	3.6	Male	40	4.5	180	8.33E+05	4.63E+03	3.67	4.50
522	3.6	Male	40	4.5	180	2.67E+06	1.48E+04	4.17	3.18
525	3.6	Male	40	4.5	180	1.68E+06	9.31E+03	3.97	8.87
526	3.6	Male	40	4.5	180	9.03E+05	5.02E+03	3.70	5.79

[1] If less than 5 µL of nuclease-free water was required for the dilution, or if the concentration of total RNA in the study sample was less than 40 ng/µL, the normalization step was not performed and the total RNA concentration as determined by the NanoDrop 2000 Spectrophotometer was used in the final calculation of copies of *Vtg* 1 mRNA/ng total RNA.

[2] ng total RNA per reaction = normalized concentration of total RNA (ng/µL) X volume of total RNA sample added to reaction (µL).

[3] Presented as (copies *Vtg* 1 mRNA per ng total RNA).

[4] Average Rotor-Gene Q Output presented as copies *Vtg* 1 mRNA/ reaction.

[5] VTG = Average (copies *Vtg* 1 mRNA per reaction) / ng total RNA per reaction.

[6] Results generated using Excel 2010 in Full Precision mode. Manual calculations may differ slightly.

[7] Upon receipt, sample was found to be very pale in color and fragmented.

[8] Coefficient of variation calculated based on Rotor-Gene Q results of duplicate sample analysis.

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Appendix 2.8 (Continued)

Summary of *Vtg* 1 mRNA copies/ng Total RNA for Males

Sample ID (607A-110-RNA-)	Concentration (µg/L)	Phenotypic Sex	Normalized Concentration of Total RNA (ng/µL)[1]	Volume of Total RNA Sample Added per Reaction (µL)	ng Total RNA per Reaction[2]	Average Rotor-Gene Q Output[3,6]	VTG[4,5,6]	Log_{10} VTG[4,5,6]	%CV[6,8]
529	10.9	Male	40	4.5	180	3.76E+06	2.09E+04	4.32	6.59
530	10.9	Male	40	4.5	180	1.27E+05	7.06E+02	2.85	3.34
533	10.9	Male	40	4.5	180	1.40E+06	7.75E+03	3.89	21.8
534	10.9	Male	40	4.5	180	2.17E+05	1.21E+03	3.08	5.21
537	10.9	Male	40	4.5	180	4.62E+04	2.57E+02	2.41	9.18
538	10.9	Male	40	4.5	180	5.36E+04	2.98E+02	2.47	5.15
541	10.9	Male	40	4.5	180	2.57E+05	1.43E+03	3.15	15.2
542	10.9	Male	40[7]	4.5	180	1.94E+05	1.08E+03	3.03	1.46
545	33	Male	47	4.5	213	7.29E+03	3.42E+01	1.53	14.2
546	33	Male	40	4.5	180	7.04E+03	3.91E+01	1.59	1.71
549	33	Male	40	4.5	180	1.44E+05	7.97E+02	2.90	4.43
550	33	Male	40	4.5	180	1.12E+04	6.19E+01	1.79	6.98
553	33	Male	40	4.5	180	1.20E+06	6.67E+03	3.82	1.18
554	33	Male	40	4.5	180	1.50E+05	8.33E+02	2.92	0.943
557	33	Male	40	4.5	180	1.33E+04	7.36E+01	1.87	8.00
558	33	Male	40	4.5	180	8.13E+03	4.51E+01	1.65	1.48

[1] If less than 5 µL of nuclease-free water was required for the dilution, or if the concentration of total RNA in the study sample was less than 40 ng/µL, the normalization step was not performed and the total RNA concentration as determined by the NanoDrop 2000 Spectrophotometer was used in the final calculation of copies of *Vtg* 1 mRNA/ng total RNA.

[2] ng total RNA per reaction = normalized concentration of total RNA (ng/µL) X volume of total RNA sample added to reaction (µL).

[3] Presented as (copies *Vtg* 1 mRNA per ng total RNA).

[4] Average Rotor-Gene Q Output presented as copies *Vtg* 1 mRNA/ reaction.

[5] VTG = Average (copies *Vtg* 1 mRNA per reaction) / ng total RNA per reaction.

[6] Results generated using Excel 2010 in Full Precision mode. Manual calculations may differ slightly.

[7] Sample provided appeared to have tissue present that was not liver tissue.

[8] Coefficient of variation calculated based on Rotor-Gene Q results of duplicate sample analysis.

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Appendix 2.8 (Continued)

Summary of *Vtg* 1 mRNA copies/ng Total RNA for Males

Sample ID (607A-110-RNA-)	Concentration (µg/L)	Phenotypic Sex	Normalized Concentration of Total RNA (ng/µL)	Volume of Total RNA Sample Added per Reaction (µL)	ng Total RNA per Reaction [1]	Average Rotor-Gene Q Output[2,5]	VTG[3,4,5]	Log_{10} VTG[3,4,5]	%CV[5,6]
561	100	Male	40	4.5	180	1.65E+04	9.14E+01	1.96	16.8
562	100	Male	40	4.5	180	5.70E+04	3.17E+02	2.50	2.98
565	100	Male	40	4.5	180	3.84E+03	2.13E+01	1.33	14.4
566	100	Male	40	4.5	180	3.24E+03	1.80E+01	1.25	14.2
569	100	Male	40	4.5	180	7.45E+04	4.14E+02	2.62	3.13
570	100	Male	40	4.5	180	8.36E+03	4.64E+01	1.67	6.77
573	100	Male	40	4.5	180	1.22E+05	6.75E+02	2.83	4.07
574	100	Male	40	4.5	180	5.75E+03	3.19E+01	1.50	6.64

[1] ng total RNA per reaction = normalized concentration of total RNA (ng/µL) X volume of total RNA sample added to reaction (µL).
[2] Presented as (copies *Vtg* 1 mRNA per ng total RNA).
[3] Average Rotor-Gene Q Output presented as copies *Vtg* 1 mRNA/ reaction.
[4] VTG = Average (copies *Vtg* 1 mRNA per reaction) / ng total RNA per reaction.
[5] Results generated using Excel 2010 in Full Precision mode. Manual calculations may differ slightly.
[6] Coefficient of variation calculated based on Rotor-Gene Q results of duplicate sample analysis.

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Appendix 2.9

Summary of *Vtg* 1 mRNA copies/ng Total RNA for Females

Sample ID (607A-110-RNA-)	Concentration (µg/L)	Phenotypic Sex	Normalized Concentration of Total RNA (ng/µL)	Volume of Total RNA Sample Added per Reaction (µL)	ng Total RNA per Reaction [1]	Average Rotor-Gene Q Output[2,5]	VTG[3,4,5]	Log_{10} VTG[3,4,5]	%CV[5,7]
499	Control	Female	40	4.5	180	3.35E+07	1.86E+05	5.27	17.1
500	Control	Female	40	4.5	180	7.27E+04	4.04E+02	2.61	5.45
503	Control	Female	40	4.5	180	2.59E+07	1.44E+05	5.16	0.274
504	Control	Female	40	4.5	180	1.16E+05	6.44E+02	2.81	17.1
507	Control	Female	40	4.5	180	2.83E+07	1.57E+05	5.20	3.25
508	Control	Female	40	4.5	180	4.02E+07	2.23E+05	5.35	25.0
511	Control	Female	40	4.5	180	1.49E+07	8.28E+04	4.92	27.5
512	Control	Female	40	4.5	180	8.69E+06	4.83E+04	4.68	0.896
515	3.6	Female	40[6]	4.5	180	2.68E+06	1.49E+04	4.17	6.33
516	3.6	Female	40	4.5	180	3.61E+07	2.00E+05	5.30	3.33
519	3.6	Female	40	4.5	180	1.83E+06	1.01E+04	4.01	12.0
520	3.6	Female	40	4.5	180	6.29E+07	3.49E+05	5.54	1.01
523	3.6	Female	40	4.5	180	3.63E+07	2.02E+05	5.30	17.5
524	3.6	Female	40	4.5	180	4.26E+07	2.37E+05	5.37	4.98
527	3.6	Female	40	4.5	180	6.37E+07	3.54E+05	5.55	10.2
528	3.6	Female	40	4.5	180	1.84E+07	1.02E+05	5.01	24.6

[1] ng total RNA per reaction = normalized concentration of total RNA (ng/µL) X volume of total RNA sample added to reaction (µL).
[2] Presented as (copies *Vtg* 1 mRNA per ng total RNA).
[3] Average Rotor-Gene Q Output presented as copies *Vtg* 1 mRNA/ reaction.
[4] VTG = Average (copies *Vtg* 1 mRNA per reaction) / ng total RNA per reaction.
[5] Results generated using Excel 2010 in Full Precision mode. Manual calculations may differ slightly.
[6] Sample provided appeared to have tissue present that was not liver tissue.
[7] Coefficient of variation calculated based on Rotor-Gene Q results of duplicate sample analysis.

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Appendix 2.9 (Continued)

Summary of *Vtg* 1 mRNA copies/ng Total RNA for Females

Sample ID (607A-110-RNA-)	Concentration (µg/L)	Phenotypic Sex	Normalized Concentration of Total RNA (ng/µL)[1]	Volume of Total RNA Sample Added per Reaction (µL)	ng Total RNA per Reaction[2]	Average Rotor-Gene Q Output[3,6]	VTG[4,5,6]	Log_{10} VTG[4,5,6]	%CV[6,8]
531	10.9	Female	40	4.5	180	3.18E+06	1.76E+04	4.25	7.35
532	10.9	Female	40	4.5	180	6.28E+07	3.49E+05	5.54	1.58
535	10.9	Female	40	4.5	180	2.07E+07	1.15E+05	5.06	27.1
536	10.9	Female	40[7]	4.5	180	8.23E+06	4.57E+04	4.66	9.28
539	10.9	Female	40	4.5	180	6.99E+07	3.88E+05	5.59	12.1
540	10.9	Female	40	4.5	180	2.68E+07	1.49E+05	5.17	6.08
543	10.9	Female	40	4.5	180	2.21E+07	1.23E+05	5.09	7.04
544	10.9	Female	40	4.5	180	5.06E+06	2.81E+04	4.45	8.53
547	33	Female	40	4.5	180	3.63E+06	2.01E+04	4.30	16.2
548	33	Female	40	4.5	180	2.62E+06	1.46E+04	4.16	9.72
551	33	Female	40	4.5	180	1.39E+07	7.72E+04	4.89	1.02
552	33	Female	40	4.5	180	4.79E+07	2.66E+05	5.43	6.20
555	33	Female	49	4.5	218	6.53E+06	2.99E+04	4.48	2.28
556	33	Female	40	4.5	180	2.35E+04	1.30E+02	2.11	3.32
559	33	Female	40	4.5	180	2.25E+06	1.25E+04	4.10	0.315
560	33	Female	40	4.5	180	1.12E+07	6.19E+04	4.79	6.98

1 If less than 5 µL of nuclease-free water was required for the dilution, or if the concentration of total RNA in the study sample was less than 40 ng/µL, the normalization step was not performed and the total RNA concentration as determined by the NanoDrop 2000 Spectrophotometer was used in the final calculation of copies of Vtg 1 mRNA/ng total RNA.

2 ng total RNA per reaction = normalized concentration of total RNA (ng/µL) X volume of total RNA sample added to reaction (µL).

3 Presented as (copies *Vtg* 1 mRNA per ng total RNA).

4 Average Rotor-Gene Q Output presented as copies *Vtg* 1 mRNA/ reaction.

5 VTG = Average (copies *Vtg* 1 mRNA per reaction) / ng total RNA per reaction.

6 Results generated using Excel 2010 in Full Precision mode. Manual calculations may differ slightly.

7 Sample starting to degrade in microfuge tube. As much sample as possible was transferred for RNA extraction.

8 Coefficient of variation calculated based on Rotor-Gene Q results of duplicate sample analysis.

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Appendix 2.9 (Continued)

Summary of *Vtg* 1 mRNA copies/ng Total RNA for Females

Sample ID (607A-110-RNA-)	Concentration (µg/L)	Phenotypic Sex	Normalized Concentration of Total RNA (ng/µL)	Volume of Total RNA Sample Added per Reaction (µL)	ng Total RNA per Reaction [1]	Average Rotor-Gene Q Output[2,5]	VTG[3,4,5]	Log_{10} VTG[3,4,5]	%CV[5,6]
563	100	Female	40	4.5	180	3.04E+06	1.69E+04	4.23	2.79
564	100	Female	40	4.5	180	1.11E+05	6.14E+02	2.79	7.04
567	100	Female	40	4.5	180	3.94E+06	2.19E+04	4.34	12.2
568	100	Female	40	4.5	180	9.49E+04	5.27E+02	2.72	12.1
571	100	Female	40	4.5	180	5.65E+07	3.14E+05	5.50	8.64
572	100	Female	40	4.5	180	3.86E+06	2.14E+04	4.33	7.33
575	100	Female	40	4.5	180	6.95E+07	3.86E+05	5.59	15.1
576	100	Female	40	4.5	180	1.65E+05	9.14E+02	2.96	8.17

[1] ng total RNA per reaction = normalized concentration of total RNA (ng/µL) X volume of total RNA sample added to reaction (µL).
[2] Presented as (copies *Vtg* 1 mRNA per ng total RNA).
[3] Average Rotor-Gene Q Output presented as copies *Vtg* 1 mRNA/ reaction.
[4] VTG = Average (copies *Vtg* 1 mRNA per reaction) / ng total RNA per reaction.
[5] Results generated using Excel 2010 in Full Precision mode. Manual calculations may differ slightly.
[6] Coefficient of variation calculated based on Rotor-Gene Q results of duplicate sample analysis.

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Appendix 2.10

Summary of *Vtg* 1 No-Template Control (NTC) and Positive Control Results

Sample Type	Assay No.	Rotor-Gene Q Output (copies *Vtg* 1 mRNA/ reaction)			
		Sample 1	Percent of Nominal[1]	Sample 2	Percent of Nominal[1]
Positive Control[1]	1	9.88×10^5	87.4%	1.20×10^6	106%
	2	1.24×10^6	110%	1.37×10^6	121%
	3	6.90×10^5	61.1%	9.15×10^5	81.0%
	4	1.05×10^6	92.9%	1.26×10^6	112%
No-Template Control[2,3]	1	2.15×10^3	NA	<Threshold	NA
	2	2.11×10^3	NA	<Threshold	NA
	3	1.56×10^3	NA	5.88×10^2 *	NA
	4	1.42×10^4	NA	6.62×10^2 *	NA

[1] Positive Control is STD 6 (nominal concentration of 1.13×10^6 copies *Vtg* 1 mRNA/ reaction).

[2] Although measured concentration in NTC for Assays 1 and 2 were slightly above the low standard, it was due to capping procedure, not considered to be a contamination and does not affect the interpretation of the study results. A second NTC sample was added to the sample runs of Assays 3 and 4.

[3] The threshold was manually set at 0.240.

* While there was a measured concentration of *Vtg* 1 mRNA in the NTC, it was below the concentration of the lowest standard (1.13×10^3 copies *Vtg* 1 mRNA/ reaction) and therefore, does not affect the interpretation of the study results.

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Appendix 3

Personnel Involved in the Study

The following key EAG Laboratories – Easton personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., VP, Aquatic, Plant and Insect Toxicology
2. Sean P. Gallagher, B.S., Scientific Advisor - Ecotoxicology
3. Suzanne Z. Schneider, Ph.D., Manager of Study Directors
4. Timothy L. Ross, B.S., Senior Scientist
5. Jeremy M. Claude, B.S., Senior Scientist
6. Amanda L. Milligan, B.S., Senior Biologist, Principle Investigator
7. Brittany R. Heffelfinger, B.S., Biologist
8. John A. Aufderheide, B.A., Senior Director

Appendix F. DEST

DEST BATT01-00404 Pre-test Body Weight

Sex (M or F)	Pre-Test Animal ID	Initial Body Weight (g)	Tank ID
M	1	0.3201	N/A
M	2	0.3746	N/A
M	3	0.3633	N/A
M	4	0.3948	N/A
M	5	0.3521	N/A
M	6	0.3308	N/A
M	7	0.3819	N/A
M	8	0.3728	N/A
M	9	0.4035	N/A
M	10	0.3964	N/A
M	11	0.3239	N/A
M	12	0.3012	N/A
M	13	0.3146	N/A
M	14	0.2989	N/A
M	15	0.3310	N/A
M	16	0.3727	N/A
M	17	0.3588	N/A
M	18	0.3670	N/A
M	19	0.3453	N/A
M	20	0.3819	N/A
M	21	0.3242	N/A
M	22	0.4113	N/A
M	23	0.3744	N/A
M	24	0.3286	N/A
M	25	0.3499	N/A
M	26	0.3672	N/A
M	27	0.4085	N/A
M	28	0.3336	N/A
M	29	0.3182	N/A
M	30	0.3473	N/A
M	31	0.3611	N/A
M	32	0.3954	N/A
M	33	0.3723	N/A
M	34	0.2992	N/A
M	35	0.4081	N/A
M	36	0.3680	N/A
M	37	0.3294	N/A
M	38	0.3155	N/A
M	39	0.3336	N/A
M	40	0.3758	N/A
M	41	0.3914	N/A
M	42	0.4007	N/A
M	43	0.3698	N/A
M	44	0.3715	N/A

Sex (M or F)	Mean Initial Body Weight (g)	Max Initial Body Weight (g)	Min Initial Body Weight (g)
M	0.3546	0.4119	0.2971
F	0.3521	0.4102	0.2889

DEST BATT01-00404 Pre-test Body Weight

Sex (M or F)	Pre-Test Animal ID	Initial Body Weight (g)	Tank ID
M	45	0.3146	N/A
M	46	0.3066	N/A
M	47	0.2972	N/A
M	48	0.2981	N/A
M	49	0.3582	N/A
M	50	0.3911	N/A
M	51	0.3572	N/A
M	52	0.3126	N/A
M	53	0.4027	N/A
M	54	0.3755	N/A
M	55	0.3261	N/A
M	56	0.3343	N/A
M	57	0.2971	N/A
M	58	0.3006	N/A
M	59	0.3772	N/A
M	60	0.3961	N/A
M	61	0.4103	N/A
M	62	0.3975	N/A
M	63	0.3612	N/A
M	64	0.3520	N/A
M	65	0.3771	N/A
M	66	0.3664	N/A
M	67	0.3992	N/A
M	68	0.4011	N/A
M	69	0.4103	N/A
M	70	0.3728	N/A
M	71	0.3915	N/A
M	72	0.3274	N/A
M	73	0.3683	N/A
M	74	0.3025	N/A
M	75	0.3169	N/A
M	76	0.3164	N/A
M	77	0.3372	N/A
M	78	0.3873	N/A
M	79	0.3944	N/A
M	80	0.3618	N/A
M	81	0.3748	N/A
M	82	0.3265	N/A
M	83	0.3118	N/A
M	84	0.3074	N/A
M	85	0.3283	N/A
M	86	0.3781	N/A
M	87	0.3956	N/A
M	88	0.3162	N/A
M	89	0.4056	N/A

DEST BATT01-00404 Pre-test Body Weight

Sex (M or F)	Pre-Test Animal ID	Initial Body Weight (g)	Tank ID
M	90	0.3511	N/A
M	91	0.3559	N/A
M	92	0.3033	N/A
M	93	0.4119	N/A
M	94	0.3751	N/A
M	95	0.3128	N/A
M	96	0.3462	N/A
M	97	0.2988	N/A
M	98	0.3462	N/A
M	99	0.3583	N/A
M	100	0.3148	N/A
F	1	0.3092	N/A
F	2	0.3658	N/A
F	3	0.4053	N/A
F	4	0.3969	N/A
F	5	0.3140	N/A
F	6	0.3273	N/A
F	7	0.2922	N/A
F	8	0.3180	N/A
F	9	0.3378	N/A
F	10	0.3865	N/A
F	11	0.3923	N/A
F	12	0.3358	N/A
F	13	0.3729	N/A
F	14	0.3816	N/A
F	15	0.3216	N/A
F	16	0.3184	N/A
F	17	0.3347	N/A
F	18	0.3918	N/A
F	19	0.3526	N/A
F	20	0.3170	N/A
F	21	0.3918	N/A
F	22	0.4062	N/A
F	23	0.3183	N/A
F	24	0.3516	N/A
F	25	0.3825	N/A
F	26	0.2889	N/A
F	27	0.2975	N/A
F	28	0.3217	N/A
F	29	0.3348	N/A
F	30	0.2952	N/A
F	31	0.3542	N/A
F	32	0.3939	N/A
F	33	0.4008	N/A
F	34	0.3756	N/A

DEST BATT01-00404 Pre-test Body Weight

Sex (M or F)	Pre-Test Animal ID	Initial Body Weight (g)	Tank ID
F	35	0.3818	N/A
F	36	0.3925	N/A
F	37	0.3568	N/A
F	38	0.3274	N/A
F	39	0.2926	N/A
F	40	0.3058	N/A
F	41	0.3146	N/A
F	42	0.3358	N/A
F	43	0.3928	N/A
F	44	0.3831	N/A
F	45	0.3656	N/A
F	46	0.3708	N/A
F	47	0.3526	N/A
F	48	0.3001	N/A
F	49	0.3248	N/A
F	50	0.3562	N/A
F	51	0.3508	N/A
F	52	0.3882	N/A
F	53	0.3651	N/A
F	54	0.4041	N/A
F	55	0.3926	N/A
F	56	0.2973	N/A
F	57	0.3158	N/A
F	58	0.3262	N/A
F	59	0.3580	N/A
F	60	0.3164	N/A
F	61	0.2889	N/A
F	62	0.3574	N/A
F	63	0.3862	N/A
F	64	0.3335	N/A
F	65	0.3629	N/A
F	66	0.3816	N/A
F	67	0.3158	N/A
F	68	0.3214	N/A
F	69	0.3742	N/A
F	70	0.3825	N/A
F	71	0.3275	N/A
F	72	0.3348	N/A
F	73	0.3628	N/A
F	74	0.3954	N/A
F	75	0.3016	N/A
F	76	0.4102	N/A
F	77	0.3183	N/A
F	78	0.3250	N/A
F	79	0.3571	N/A

DEST BATT01-00404 Pre-test Body Weight

Sex (M or F)	Pre-Test Animal ID	Initial Body Weight (g)	Tank ID
F	80	0.3684	N/A
F	81	0.399	N/A
F	82	0.3758	N/A
F	83	0.3089	N/A
F	84	0.3256	N/A
F	85	0.4085	N/A
F	86	0.4001	N/A
F	87	0.3875	N/A
F	88	0.3168	N/A
F	89	0.3337	N/A
F	90	0.3142	N/A
F	91	0.3826	N/A
F	92	0.3946	N/A
F	93	0.3651	N/A
F	94	0.4018	N/A
F	95	0.3746	N/A
F	96	0.3917	N/A
F	97	0.3258	N/A
F	98	0.3155	N/A
F	99	0.3482	N/A
F	100	0.3771	N/A

DEST BATT01-00404 Pre-exposure Observations

Tank ID	Replicate ID	Pre-Exposure Day (1 to 11)	Number of Surviving Males	Number of Surviving Females	Number of Spawns	Number of Eggs	Number of Fertilized Eggs	Comments
0.0µg/L	A	PE1	5	5	1	38	38	
0.0µg/L	B	PE1	5	5	1	42	42	
0.0µg/L	C	PE1	5	5	1	26	26	
0.0µg/L	D	PE1	5	5	1	41	41	
3.6µg/L	A	PE1	5	5	1	37	37	
3.6µg/L	B	PE1	5	5	1	46	46	
3.6µg/L	C	PE1	5	5	1	30	30	
3.6µg/L	D	PE1	5	5	1	18	18	
10.9µg/L	A	PE1	5	5	1	24	24	
10.9µg/L	B	PE1	5	5	1	17	17	
10.9µg/L	C	PE1	5	5	1	31	31	
10.9µg/L	D	PE1	5	5	1	19	19	
33µg/L	A	PE1	5	5	1	22	22	
33µg/L	B	PE1	5	5	1	26	26	
33µg/L	C	PE1	5	5	1	34	34	
33µg/L	D	PE1	5	5	1	14	14	
100µg/L	A	PE1	5	5	1	19	19	
100µg/L	B	PE1	5	5	1	17	17	
100µg/L	C	PE1	5	5	1	23	23	
100µg/L	D	PE1	5	5	1	40	40	
0.0µg/L	A	PE2	5	5	1	98	98	
0.0µg/L	B	PE2	5	5	1	88	88	
0.0µg/L	C	PE2	5	5	1	74	74	
0.0µg/L	D	PE2	5	5	1	96	96	
3.6µg/L	A	PE2	5	5	1	92	92	
3.6µg/L	B	PE2	5	5	1	113	113	
3.6µg/L	C	PE2	5	5	1	86	86	
3.6µg/L	D	PE2	5	5	1	117	117	
10.9µg/L	A	PE2	5	5	1	116	116	
10.9µg/L	B	PE2	5	5	1	142	141	
10.9µg/L	C	PE2	5	5	1	135	135	
10.9µg/L	D	PE2	5	5	1	146	145	
33µg/L	A	PE2	5	5	1	141	140	
33µg/L	B	PE2	5	5	1	139	137	
33µg/L	C	PE2	5	5	1	93	93	
33µg/L	D	PE2	5	5	1	135	134	
100µg/L	A	PE2	5	5	1	125	123	
100µg/L	B	PE2	5	5	1	142	140	
100µg/L	C	PE2	5	5	1	161	160	
100µg/L	D	PE2	5	5	1	118	118	
0.0µg/L	A	PE3	5	5	1	123	123	
0.0µg/L	B	PE3	5	5	1	97	97	
0.0µg/L	C	PE3	5	5	1	124	122	
0.0µg/L	D	PE3	5	5	1	117	117	

DEST BATT01-00404 Pre-exposure Observations

Tank ID	Replicate ID	Pre-Exposure Day (1 to 11)	Number of Surviving Males	Number of Surviving Females	Number of Spawns	Number of Eggs	Number of Fertilized Eggs	Comments
3.6µg/L	A	PE3	5	5	1	121	119	
3.6µg/L	B	PE3	5	5	1	103	103	
3.6µg/L	C	PE3	5	5	1	110	109	
3.6µg/L	D	PE3	5	5	1	139	137	
10.9µg/L	A	PE3	5	5	1	102	102	
10.9µg/L	B	PE3	5	5	1	118	118	
10.9µg/L	C	PE3	5	5	1	122	121	
10.9µg/L	D	PE3	5	5	1	131	130	
33µg/L	A	PE3	5	5	1	126	125	
33µg/L	B	PE3	5	5	1	126	125	
33µg/L	C	PE3	5	5	1	152	150	
33µg/L	D	PE3	5	5	1	121	120	
100µg/L	A	PE3	5	5	1	116	116	
100µg/L	B	PE3	5	5	1	128	127	
100µg/L	C	PE3	5	5	1	142	140	
100µg/L	D	PE3	5	5	1	137	136	
0.0µg/L	A	PE4	5	5	1	88	88	
0.0µg/L	B	PE4	5	5	1	106	106	
0.0µg/L	C	PE4	5	5	1	103	102	
0.0µg/L	D	PE4	5	5	1	85	85	
3.6µg/L	A	PE4	5	5	1	103	101	
3.6µg/L	B	PE4	5	5	1	110	110	
3.6µg/L	C	PE4	5	5	1	89	87	
3.6µg/L	D	PE4	5	5	1	76	75	
10.9µg/L	A	PE4	5	5	1	93	92	
10.9µg/L	B	PE4	5	5	1	84	84	
10.9µg/L	C	PE4	5	5	1	79	77	
10.9µg/L	D	PE4	5	5	1	85	84	
33µg/L	A	PE4	5	5	1	88	87	
33µg/L	B	PE4	5	5	1	92	90	
33µg/L	C	PE4	5	5	1	102	99	
33µg/L	D	PE4	5	5	1	83	81	
100µg/L	A	PE4	5	5	1	78	76	
100µg/L	B	PE4	5	5	1	96	95	
100µg/L	C	PE4	5	5	1	81	78	
100µg/L	D	PE4	5	5	1	81	80	
0.0µg/L	A	PE5	5	5	1	98	97	
0.0µg/L	B	PE5	5	5	1	116	114	
0.0µg/L	C	PE5	5	5	1	88	86	
0.0µg/L	D	PE5	5	5	1	121	119	
3.6µg/L	A	PE5	5	5	1	93	91	
3.6µg/L	B	PE5	5	5	1	87	86	
3.6µg/L	C	PE5	5	5	1	119	118	
3.6µg/L	D	PE5	5	5	1	116	114	

DEST BATT01-00404 Pre-exposure Observations

Tank ID	Replicate ID	Pre-Exposure Day (1 to 11)	Number of Surviving Males	Number of Surviving Females	Number of Spawns	Number of Eggs	Number of Fertilized Eggs	Comments
10.9µg/L	A	PE5	5	5	1	115	113	
10.9µg/L	B	PE5	5	5	1	126	125	
10.9µg/L	C	PE5	5	5	1	108	106	
10.9µg/L	D	PE5	5	5	1	120	120	
33µg/L	A	PE5	5	5	1	90	90	
33µg/L	B	PE5	5	5	1	84	82	
33µg/L	C	PE5	5	5	1	79	78	
33µg/L	D	PE5	5	5	1	96	95	
100µg/L	A	PE5	5	5	1	127	126	
100µg/L	B	PE5	5	5	1	118	118	
100µg/L	C	PE5	5	5	1	128	127	
100µg/L	D	PE5	5	5	1	123	122	
0.0µg/L	A	PE6	5	5	1	73	70	
0.0µg/L	B	PE6	5	5	1	54	51	
0.0µg/L	C	PE6	5	5	1	59	55	
0.0µg/L	D	PE6	5	5	1	54	51	
3.6µg/L	A	PE6	5	5	1	59	57	
3.6µg/L	B	PE6	5	5	1	66	64	
3.6µg/L	C	PE6	5	5	1	54	50	
3.6µg/L	D	PE6	5	5	1	59	56	
10.9µg/L	A	PE6	5	5	1	73	70	
10.9µg/L	B	PE6	5	5	1	58	55	
10.9µg/L	C	PE6	5	5	1	48	47	
10.9µg/L	D	PE6	5	5	1	70	66	
33µg/L	A	PE6	5	5	1	83	80	
33µg/L	B	PE6	5	5	1	74	70	
33µg/L	C	PE6	5	5	1	59	55	
33µg/L	D	PE6	5	5	1	80	76	
100µg/L	A	PE6	5	5	1	59	56	
100µg/L	B	PE6	5	5	1	72	68	
100µg/L	C	PE6	5	5	1	70	66	
100µg/L	D	PE6	5	5	1	66	64	
0.0µg/L	A	PE7	5	5	1	66	64	
0.0µg/L	B	PE7	5	5	1	65	61	
0.0µg/L	C	PE7	5	5	1	63	61	
0.0µg/L	D	PE7	5	5	1	78	76	
3.6µg/L	A	PE7	5	5	1	68	65	
3.6µg/L	B	PE7	5	5	1	79	76	
3.6µg/L	C	PE7	5	5	1	61	60	
3.6µg/L	D	PE7	5	5	1	66	64	
10.9µg/L	A	PE7	5	5	1	86	82	
10.9µg/L	B	PE7	5	5	1	67	65	
10.9µg/L	C	PE7	5	5	1	69	66	
10.9µg/L	D	PE7	5	5	1	80	77	

DEST BATT01-00404 Pre-exposure Observations

Tank ID	Replicate ID	Pre-Exposure Day (1 to 11)	Number of Surviving Males	Number of Surviving Females	Number of Spawns	Number of Eggs	Number of Fertilized Eggs	Comments
33µg/L	A	PE7	5	5	1	78	76	
33µg/L	B	PE7	5	5	1	79	76	
33µg/L	C	PE7	5	5	1	64	62	
33µg/L	D	PE7	5	5	1	72	68	
100µg/L	A	PE7	5	5	1	73	70	
100µg/L	B	PE7	5	5	1	56	55	
100µg/L	C	PE7	5	5	1	61	57	
100µg/L	D	PE7	5	5	1	69	66	
0.0µg/L	A	PE8	5	5	1	72	69	
0.0µg/L	B	PE8	5	5	1	74	72	
0.0µg/L	C	PE8	5	5	1	70	67	
0.0µg/L	D	PE8	5	5	1	70	69	
3.6µg/L	A	PE8	5	5	1	74	70	
3.6µg/L	B	PE8	5	5	1	80	77	
3.6µg/L	C	PE8	5	5	1	78	75	
3.6µg/L	D	PE8	5	5	1	72	71	
10.9µg/L	A	PE8	5	5	1	73	70	
10.9µg/L	B	PE8	5	5	1	74	71	
10.9µg/L	C	PE8	5	5	1	82	80	
10.9µg/L	D	PE8	5	5	1	65	62	
33µg/L	A	PE8	5	5	1	79	75	
33µg/L	B	PE8	5	5	1	80	77	
33µg/L	C	PE8	5	5	1	70	66	
33µg/L	D	PE8	5	5	1	65	63	
100µg/L	A	PE8	5	5	1	81	77	
100µg/L	B	PE8	5	5	1	80	77	
100µg/L	C	PE8	5	5	1	80	76	
100µg/L	D	PE8	5	5	1	70	70	
0.0µg/L	A	PE9	5	5	1	63	60	
0.0µg/L	B	PE9	5	5	1	78	75	
0.0µg/L	C	PE9	5	5	1	56	53	
0.0µg/L	D	PE9	5	5	1	66	63	
3.6µg/L	A	PE9	5	5	1	70	68	
3.6µg/L	B	PE9	5	5	1	69	66	
3.6µg/L	C	PE9	5	5	1	72	70	
3.6µg/L	D	PE9	5	5	1	75	70	
10.9µg/L	A	PE9	5	5	1	65	63	
10.9µg/L	B	PE9	5	5	1	79	75	
10.9µg/L	C	PE9	5	5	1	78	76	
10.9µg/L	D	PE9	5	5	1	69	66	
33µg/L	A	PE9	5	5	1	85	82	
33µg/L	B	PE9	5	5	1	63	60	
33µg/L	C	PE9	5	5	1	58	56	
33µg/L	D	PE9	5	5	1	72	67	

DEST BATT01-00404 Pre-exposure Observations

Tank ID	Replicate ID	Pre-Exposure Day (1 to 11)	Number of Surviving Males	Number of Surviving Females	Number of Spawns	Number of Eggs	Number of Fertilized Eggs	Comments
100µg/L	A	PE9	5	5	1	75	72	
100µg/L	B	PE9	5	5	1	79	76	
100µg/L	C	PE9	5	5	1	59	57	
100µg/L	D	PE9	5	5	1	60	56	
0.0µg/L	A	PE10	5	5	1	71	68	
0.0µg/L	B	PE10	5	5	1	59	57	
0.0µg/L	C	PE10	5	5	1	79	75	
0.0µg/L	D	PE10	5	5	1	80	78	
3.6µg/L	A	PE10	5	5	1	69	66	
3.6µg/L	B	PE10	5	5	1	58	57	
3.6µg/L	C	PE10	5	5	1	75	72	
3.6µg/L	D	PE10	5	5	1	88	85	
10.9µg/L	A	PE10	5	5	1	72	70	
10.9µg/L	B	PE10	5	5	1	81	77	
10.9µg/L	C	PE10	5	5	1	63	60	
10.9µg/L	D	PE10	5	5	1	70	67	
33µg/L	A	PE10	5	5	1	61	58	
33µg/L	B	PE10	5	5	1	65	62	
33µg/L	C	PE10	5	5	1	57	55	
33µg/L	D	PE10	5	5	1	71	66	
100µg/L	A	PE10	5	5	1	71	76	
100µg/L	B	PE10	5	5	1	59	56	
100µg/L	C	PE10	5	5	1	73	70	
100µg/L	D	PE10	5	5	1	68	66	
0.0µg/L	A	PE11	5	5	1	64	62	
0.0µg/L	B	PE11	5	5	1	69	66	
0.0µg/L	C	PE11	5	5	1	58	55	
0.0µg/L	D	PE11	5	5	1	71	68	
3.6µg/L	A	PE11	5	5	1	75	72	
3.6µg/L	B	PE11	5	5	1	68	66	
3.6µg/L	C	PE11	5	5	1	76	73	
3.6µg/L	D	PE11	5	5	1	70	67	
10.9µg/L	A	PE11	5	5	1	78	74	
10.9µg/L	B	PE11	5	5	1	79	76	
10.9µg/L	C	PE11	5	5	1	59	56	
10.9µg/L	D	PE11	5	5	1	78	75	
33µg/L	A	PE11	5	5	1	85	81	
33µg/L	B	PE11	5	5	1	76	73	
33µg/L	C	PE11	5	5	1	64	62	
33µg/L	D	PE11	5	5	1	68	64	
100µg/L	A	PE11	5	5	1	66	63	
100µg/L	B	PE11	5	5	1	71	67	
100µg/L	C	PE11	5	5	1	63	60	
100µg/L	D	PE11	5	5	1	74	71	

Analytical Results Analysis

BATT01-00404

Substance 2-EHHB
Study BATT01-00404

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 CVs (Within)	
0.0 µg/L A	<MQL	0.106	0.106	0.106	0.106	0.106				3
0.0 µg/L B	<MQL	0.106	0.106	0.106	0.106	0.106				
0.0 µg/L C	<MQL	0.106	0.106	0.106	0.106	0.106				
0.0 µg/L D	<MQL	0.106	0.106	0.106	0.106	0.106				
0.0 µg/L Mean		0.106	0.106	0.106	0.106	0.106				
Replicate CV		0.0	0.0	0.0	0.0	0.0				
3.6 µg/L A	3.60	4.38	2.66	3.36	2.87	3.32				83
3.6 µg/L B	3.60	4.05	2.41	3.15	2.55	3.04				
3.6 µg/L C	3.60	3.47	2.26	2.88	2.49	2.78				
3.6 µg/L D	3.60	3.44	2.30	2.95	2.46	2.79				
3.6 µg/L Mean		3.84	2.41	3.09	2.59	2.98	2.98	8.43	9%	
Replicate CV		12.0	7.5	7.0	7.3	8.6				
10.9 µg/L A	10.9	12.3	10.0	13.1	9.70	11.3				98
10.9 µg/L B	10.9	11.4	9.32	11.6	8.17	10.1				
10.9 µg/L C	10.9	10.9	9.26	11.4	8.61	10.0				
10.9 µg/L D	10.9	14.4	9.09	12.2	8.61	11.1				
10.9 µg/L Mean		12.3	9.42	12.1	8.77	10.6	10.6	7.66	6%	
Replicate CV		12.6	4.3	6.3	7.4	6.0				
33.0 µg/L A	33.0	42.8	29.5	38.5	43.9	38.7				103
33.0 µg/L B	33.0	38.8	25.6	33.0	32.5	32.5				
33.0 µg/L C	33.0	34.2	26.2	33.5	34.2	32.0				
33.0 µg/L D	33.0	39.7	25.9	32.6	35.4	33.4				
33 µg/L Mean		38.9	26.8	34.4	36.5	34.1	34.1	9.46	9%	
Replicate CV		9.1	6.8	8.0	13.9	9.0				
100 µg/L A	100	130	99.1	106	104	110				105
100 µg/L B	100	114	92.7	95.6	93.9	99.1				
100 µg/L C	100	128	92.4	93.1	115	107				
100 µg/L D	100	120	87.9	91.4	110	102				
100 µg/L Mean		123	93.0	96.5	106	105	104.6	6.58	5%	
Replicate CV		6.0	5.0	6.8	8.6	4.6				

NOTE: <MQL reported as 1/2 of MQL (0.212)

IQR Calculations

	0.0	3.6	10.9	33	100
1st Quartile	0.106	2.48	9.22	31.8	93.0
3rd Quartile	0.106	3.38	11.8	38.6	114
IQR	0.000	0.90	2.53	6.83	21.3
Upper	0.106	4.73	15.5	48.8	146
Lower	0.106	1.14	5.42	21.5	61.1

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
(Ctrl) 0.0µg/L	0.0	A	PE0		25.3	7.7	7.6	686	132	52					$DeCl_2$ Tap Water
(Ctrl) 0.0µg/L	0.0	B	PE0		25.4	7.7	7.6	653							
(Ctrl) 0.0µg/L	0.0	C	PE0		25.4	7.8	7.5	675							
(Ctrl) 0.0µg/L	0.0	D	PE0		25.4	7.8	7.3	701							
3.6µg/L	3.6	A	PE0		25.2	7.8	7.3	710							
3.6µg/L	3.6	B	PE0		25.3	7.8	7.4	723							
3.6µg/L	3.6	C	PE0		25.3	7.8	7.5	747							
3.6µg/L	3.6	D	PE0		25.2	7.8	7.5	736							
10.9µg/L	10.9	A	PE0		25.4	7.8	7.5	781							
10.9µg/L	10.9	B	PE0		25.4	7.8	7.3	779							
10.9µg/L	10.9	C	PE0		25.3	7.8	7.3	753							
10.9µg/L	10.9	D	PE0		25.4	7.8	7.3	768							
33µg/L	33	A	PE0		25.5	7.8	7.5	801							
33µg/L	33	B	PE0		25.4	7.8	7.5	822							
33µg/L	33	C	PE0		25.4	7.8	7.5	813							
33µg/L	33	D	PE0		25.4	7.8	7.4	816							
100µg/L	100	A	PE0		25.3	7.8	7.4	798							
100µg/L	100	B	PE0		25.4	7.8	7.3	763							
100µg/L	100	C	PE0		25.4	7.8	7.4	757							
100µg/L	100	D	PE0		25.3	7.8	7.5	799							
(Ctrl) 0.0µg/L	0.0	A	PE1		25.5										
(Ctrl) 0.0µg/L	0.0	B	PE1		25.4										
(Ctrl) 0.0µg/L	0.0	C	PE1		25.4										
(Ctrl) 0.0µg/L	0.0	D	PE1		25.5										
3.6µg/L	3.6	A	PE1		25.4										
3.6µg/L	3.6	B	PE1		25.3										
3.6µg/L	3.6	C	PE1		25.4										
3.6µg/L	3.6	D	PE1		25.4										
10.9µg/L	10.9	A	PE1		25.4										
10.9µg/L	10.9	B	PE1		25.3										
10.9µg/L	10.9	C	PE1		25.4										
10.9µg/L	10.9	D	PE1		25.4										
33µg/L	33	A	PE1		25.5										
33µg/L	33	B	PE1		25.3										
33µg/L	33	C	PE1		25.4										
33µg/L	33	D	PE1		25.5										
100µg/L	100	A	PE1		25.4										
100µg/L	100	B	PE1		25.4										
100µg/L	100	C	PE1		25.3										
100µg/L	100	D	PE1		25.4										
(Ctrl) 0.0µg/L	0.0	A	PE2		25.5	7.7	7.5	659							
(Ctrl) 0.0µg/L	0.0	B	PE2		25.4	7.7	7.5	663							
(Ctrl) 0.0µg/L	0.0	C	PE2		25.5	7.7	7.5	658							
(Ctrl) 0.0µg/L	0.0	D	PE2		25.5	7.7	7.5	673							
3.6µg/L	3.6	A	PE2		25.4	7.7	7.5	685							

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
3.6µg/L	3.6	B	PE2		25.4	7.7	7.6	712							
3.6µg/L	3.6	C	PE2		25.5	7.7	7.6	694							
3.6µg/L	3.6	D	PE2		25.5	7.7	7.4	705							
10.9µg/L	10.9	A	PE2		25.6	7.6	7.4	690							
10.9µg/L	10.9	B	PE2		25.5	7.7	7.4	741							
10.9µg/L	10.9	C	PE2		25.5	7.7	7.4	752							
10.9µg/L	10.9	D	PE2		25.4	7.7	7.4	815							
33µg/L	33	A	PE2		25.5	7.6	7.4	801							
33µg/L	33	B	PE2		25.4	7.6	7.4	773							
33µg/L	33	C	PE2		25.6	7.6	7.4	716							
33µg/L	33	D	PE2		25.4	7.7	7.4	690							
100µg/L	100	A	PE2		25.4	7.7	7.4	657							
100µg/L	100	B	PE2		25.4	7.7	7.4	649							
100µg/L	100	C	PE2		25.5	7.7	7.4	680							
100µg/L	100	D	PE2		25.4	7.7	7.4	702							
(Ctrl) 0.0µg/L	0.0	A	PE3		25.5										
(Ctrl) 0.0µg/L	0.0	B	PE3		25.5										
(Ctrl) 0.0µg/L	0.0	C	PE3		25.6										
(Ctrl) 0.0µg/L	0.0	D	PE3		25.5										
3.6µg/L	3.6	A	PE3		25.6										
3.6µg/L	3.6	B	PE3		25.5										
3.6µg/L	3.6	C	PE3		25.5										
3.6µg/L	3.6	D	PE3		25.6										
10.9µg/L	10.9	A	PE3		25.5										
10.9µg/L	10.9	B	PE3		25.5										
10.9µg/L	10.9	C	PE3		25.5										
10.9µg/L	10.9	D	PE3		25.6										
33µg/L	33	A	PE3		25.5										
33µg/L	33	B	PE3		25.6										
33µg/L	33	C	PE3		25.6										
33µg/L	33	D	PE3		25.6										
100µg/L	100	A	PE3		25.5										
100µg/L	100	B	PE3		25.6										
100µg/L	100	C	PE3		25.5										
100µg/L	100	D	PE3		25.5										
(Ctrl) 0.0µg/L	0.0	A	PE4		25.5										
(Ctrl) 0.0µg/L	0.0	B	PE4		25.6										
(Ctrl) 0.0µg/L	0.0	C	PE4		25.5										
(Ctrl) 0.0µg/L	0.0	D	PE4		25.5										
3.6µg/L	3.6	A	PE4		25.4										
3.6µg/L	3.6	B	PE4		25.5										
3.6µg/L	3.6	C	PE4		25.5										
3.6µg/L	3.6	D	PE4		25.6										
10.9µg/L	10.9	A	PE4		25.6										
10.9µg/L	10.9	B	PE4		25.4										

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
10.9µg/L	10.9	C	PE4		25.5										
10.9µg/L	10.9	D	PE4		25.5										
33µg/L	33	A	PE4		25.4										
33µg/L	33	B	PE4		25.4										
33µg/L	33	C	PE4		25.5										
33µg/L	33	D	PE4		25.5										
100µg/L	100	A	PE4		25.6										
100µg/L	100	B	PE4		25.6										
100µg/L	100	C	PE4		25.4										
100µg/L	100	D	PE4		25.5										
(Ctrl) 0.0µg/L	0.0	A	PE5		25.5	7.7	7.4	660							
(Ctrl) 0.0µg/L	0.0	B	PE5		25.4	7.7	7.4	731							
(Ctrl) 0.0µg/L	0.0	C	PE5		25.4	7.7	7.3	823							
(Ctrl) 0.0µg/L	0.0	D	PE5		25.5	7.7	7.3	857							
3.6µg/L	3.6	A	PE5		25.5	7.7	7.2	861							
3.6µg/L	3.6	B	PE5		25.4	7.7	7.2	728							
3.6µg/L	3.6	C	PE5		25.4	7.7	7.2	798							
3.6µg/L	3.6	D	PE5		25.5	7.7	7.2	955							
10.9µg/L	10.9	A	PE5		25.4	7.7	7.2	925							
10.9µg/L	10.9	B	PE5		25.5	7.7	7.2	810							
10.9µg/L	10.9	C	PE5		25.4	7.8	7.1	787							
10.9µg/L	10.9	D	PE5		25.5	7.7	7.1	701							
33µg/L	33	A	PE5		25.5	7.7	7.1	730							
33µg/L	33	B	PE5		25.4	7.7	7.1	793							
33µg/L	33	C	PE5		25.5	7.7	7.1	839							
33µg/L	33	D	PE5		25.5	7.7	7.1	947							
100µg/L	100	A	PE5		25.4	7.7	7.0	799							
100µg/L	100	B	PE5		25.5	7.7	7.0	820							
100µg/L	100	C	PE5		25.5	7.7	7.0	931							
100µg/L	100	D	PE5		25.6	7.7	7.0	825							
(Ctrl) 0.0µg/L	0.0	A	PE6		25.5										
(Ctrl) 0.0µg/L	0.0	B	PE6		25.6										
(Ctrl) 0.0µg/L	0.0	C	PE6		25.5										
(Ctrl) 0.0µg/L	0.0	D	PE6		25.6										
3.6µg/L	3.6	A	PE6		25.4										
3.6µg/L	3.6	B	PE6		25.5										
3.6µg/L	3.6	C	PE6		25.4										
3.6µg/L	3.6	D	PE6		25.5										
10.9µg/L	10.9	A	PE6		25.6										
10.9µg/L	10.9	B	PE6		25.6										
10.9µg/L	10.9	C	PE6		25.5										
10.9µg/L	10.9	D	PE6		25.5										
33µg/L	33	A	PE6		25.4										
33µg/L	33	B	PE6		25.4										
33µg/L	33	C	PE6		25.5										

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Analytical Water Quality

BATT01-00404

Name of Treatment Group	Nominal Treatment Concentration (µg/L)	Replicate ID	Test Day (0 to 21)	Measured Treatment Concentration (µg/L)	Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
33µg/L	33	D	PE6		25.4										
100µg/L	100	A	PE6		25.6										
100µg/L	100	B	PE6		25.4										
100µg/L	100	C	PE6		25.4										
100µg/L	100	D	PE6		25.5										
(Ctrl) 0.0µg/L	0.0	A	PE7		25.5										
(Ctrl) 0.0µg/L	0.0	B	PE7		25.6										
(Ctrl) 0.0µg/L	0.0	C	PE7		25.6										
(Ctrl) 0.0µg/L	0.0	D	PE7		25.5										
3.6µg/L	3.6	A	PE7		25.6										
3.6µg/L	3.6	B	PE7		25.5										
3.6µg/L	3.6	C	PE7		25.5										
3.6µg/L	3.6	D	PE7		25.6										
10.9µg/L	10.9	A	PE7		25.5										
10.9µg/L	10.9	B	PE7		25.6										
10.9µg/L	10.9	C	PE7		25.6										
10.9µg/L	10.9	D	PE7		25.4										
33µg/L	33	A	PE7		25.5										
33µg/L	33	B	PE7		25.5										
33µg/L	33	C	PE7		25.6										
33µg/L	33	D	PE7		25.5										
100µg/L	100	A	PE7		25.5										
100µg/L	100	B	PE7		25.4										
100µg/L	100	C	PE7		25.5										
100µg/L	100	D	PE7		25.6										
Facility Water Chemistry			PE8			7.1	6.7		132	48	750	<0.05	<0.06		
(Ctrl) 0.0µg/L	0.0	A	PE8		25.5	7.8	7.5	928	132	48					$DeCl_2$ Tap Water
(Ctrl) 0.0µg/L	0.0	B	PE8		25.4	7.8	7.4	941							
(Ctrl) 0.0µg/L	0.0	C	PE8		25.5	7.8	7.4	973							
(Ctrl) 0.0µg/L	0.0	D	PE8		25.6	7.8	7.2	933							
3.6µg/L	3.6	A	PE8		25.5	7.8	7.3	877							
3.6µg/L	3.6	B	PE8		25.6	7.8	7.4	883							
3.6µg/L	3.6	C	PE8		25.5	7.8	7.3	905							
3.6µg/L	3.6	D	PE8		25.5	7.8	7.4	880							
10.9µg/L	10.9	A	PE8		25.6	7.8	7.3	741							
10.9µg/L	10.9	B	PE8		25.6	7.8	7.4	756							
10.9µg/L	10.9	C	PE8		25.5	7.8	6.8	777							
10.9µg/L	10.9	D	PE8		25.5	7.8	7.3	737							
33µg/L	33	A	PE8		25.6	7.8	7.4	650							
33µg/L	33	B	PE8		25.5	7.8	7.4	691							
33µg/L	33	C	PE8		25.4	7.8	7.3	730							
33µg/L	33	D	PE8		25.4	7.8	7.3	706							
100µg/L	100	A	PE8		25.5	7.8	7.3	875							
100µg/L	100	B	PE8		25.6	7.8	7.3	893							
100µg/L	100	C	PE8		25.5	7.8	7.3	936							

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
100µg/L	100	D	PE8		25.4	7.9	7.3	901							
(Ctrl) 0.0µg/L	0.0	A	PE9		25.5										
(Ctrl) 0.0µg/L	0.0	B	PE9		25.6										
(Ctrl) 0.0µg/L	0.0	C	PE9		25.6										
(Ctrl) 0.0µg/L	0.0	D	PE9		25.5										
3.6µg/L	3.6	A	PE9		25.5										
3.6µg/L	3.6	B	PE9		25.6										
3.6µg/L	3.6	C	PE9		25.5										
3.6µg/L	3.6	D	PE9		25.4										
10.9µg/L	10.9	A	PE9		25.6										
10.9µg/L	10.9	B	PE9		25.5										
10.9µg/L	10.9	C	PE9		25.4										
10.9µg/L	10.9	D	PE9		25.6										
33µg/L	33	A	PE9		25.5										
33µg/L	33	B	PE9		25.5										
33µg/L	33	C	PE9		25.6										
33µg/L	33	D	PE9		25.5										
100µg/L	100	A	PE9		25.5										
100µg/L	100	B	PE9		25.5										
100µg/L	100	C	PE9		25.6										
100µg/L	100	D	PE9		25.6										
(Ctrl) 0.0µg/L	0.0	A	PE10		25.4										
(Ctrl) 0.0µg/L	0.0	B	PE10		25.5										
(Ctrl) 0.0µg/L	0.0	C	PE10		25.5										
(Ctrl) 0.0µg/L	0.0	D	PE10		25.4										
3.6µg/L	3.6	A	PE10		25.5										
3.6µg/L	3.6	B	PE10		25.6										
3.6µg/L	3.6	C	PE10		25.5										
3.6µg/L	3.6	D	PE10		25.4										
10.9µg/L	10.9	A	PE10		25.5										
10.9µg/L	10.9	B	PE10		25.6										
10.9µg/L	10.9	C	PE10		25.4										
10.9µg/L	10.9	D	PE10		25.5										
33µg/L	33	A	PE10		25.6										
33µg/L	33	B	PE10		25.5										
33µg/L	33	C	PE10		25.5										
33µg/L	33	D	PE10		25.5										
100µg/L	100	A	PE10		25.4										
100µg/L	100	B	PE10		25.5										
100µg/L	100	C	PE10		25.4										
100µg/L	100	D	PE10		25.6										
(Ctrl) 0.0µg/L	0.0	A	PE11		25.5										
(Ctrl) 0.0µg/L	0.0	B	PE11		25.5										
(Ctrl) 0.0µg/L	0.0	C	PE11		25.4										
(Ctrl) 0.0µg/L	0.0	D	PE11		25.5										

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
3.6µg/L	3.6	A	PE11		25.5										
3.6µg/L	3.6	B	PE11		25.4										
3.6µg/L	3.6	C	PE11		25.5										
3.6µg/L	3.6	D	PE11		25.5										
10.9µg/L	10.9	A	PE11		25.4										
10.9µg/L	10.9	B	PE11		25.5										
10.9µg/L	10.9	C	PE11		25.5										
10.9µg/L	10.9	D	PE11		25.4										
33µg/L	33	A	PE11		25.5										
33µg/L	33	B	PE11		25.4										
33µg/L	33	C	PE11		25.5										
33µg/L	33	D	PE11		25.5										
100µg/L	100	A	PE11		25.4										
100µg/L	100	B	PE11		25.5										
100µg/L	100	C	PE11		25.4										
100µg/L	100	D	PE11		25.4										
Facility Dilution Water			0											8.9	
(Ctrl) 0.0µg/L	0.0	A	0	0.106	25.5	7.6	7.3	972	132	48					MQL=0.212µg/L, For results <MQL 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	B	0	0.106	25.4	7.5	7.4	999							MQL=0.212µg/L, For results <MQL 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	C	0	0.106	25.5	7.5	7.2	984							MQL=0.212µg/L, For results <MQL 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	D	0	0.106	25.5	7.5	7.0	867							MQL=0.212µg/L, For results <MQL 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
3.6µg/L	3.6	A	0	4.38	25.5	7.6	7.0	747							
3.6µg/L	3.6	B	0	4.05	25.4	7.6	7.0	855							
3.6µg/L	3.6	C	0	3.47	25.4	7.5	7.0	815							
3.6µg/L	3.6	D	0	3.44	25.5	7.5	7.0	878							
10.9µg/L	10.9	A	0	12.3	25.5	7.5	7.3	948							
10.9µg/L	10.9	B	0	11.4	25.5	7.5	7.2	973							
10.9µg/L	10.9	C	0	10.9	25.4	7.5	7.2	911							
10.9µg/L	10.9	D	0	14.4	25.5	7.5	7.4	984							
33µg/L	33	A	0	42.8	25.4	7.5	7.1	980							
33µg/L	33	B	0	38.8	25.3	7.5	7.2	902							
33µg/L	33	C	0	34.2	25.4	7.5	7.3	935							
33µg/L	33	D	0	39.7	25.5	7.5	7.3	979							

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
100µg/L	100	A	0	130	25.5	7.5	7.0	987	132	48					
100µg/L	100	B	0	114	25.4	7.5	7.2	977							
100µg/L	100	C	0	128	25.5	7.5	7.2	945							
100µg/L	100	D	0	120	25.5	7.5	7.1	665							
(Ctrl) 0.0µg/L	0.0	A	1		25.5										
(Ctrl) 0.0µg/L	0.0	B	1		25.4										
(Ctrl) 0.0µg/L	0.0	C	1		25.5										
(Ctrl) 0.0µg/L	0.0	D	1		25.4										
3.6µg/L	3.6	A	1		25.5										
3.6µg/L	3.6	B	1		25.5										
3.6µg/L	3.6	C	1		25.4										
3.6µg/L	3.6	D	1		25.5										
10.9µg/L	10.9	A	1		25.4										
10.9µg/L	10.9	B	1		25.5										
10.9µg/L	10.9	C	1		25.5										
10.9µg/L	10.9	D	1		25.4										
33µg/L	33	A	1		25.5										
33µg/L	33	B	1		25.5										
33µg/L	33	C	1		25.4										
33µg/L	33	D	1		25.4										
100µg/L	100	A	1		25.5										
100µg/L	100	B	1		25.4										
100µg/L	100	C	1		25.5										
100µg/L	100	D	1		25.4										
(Ctrl) 0.0µg/L	0.0	A	2		25.5										
(Ctrl) 0.0µg/L	0.0	B	2		25.5										
(Ctrl) 0.0µg/L	0.0	C	2		25.5										
(Ctrl) 0.0µg/L	0.0	D	2		25.4										
3.6µg/L	3.6	A	2		25.4										
3.6µg/L	3.6	B	2		25.4										
3.6µg/L	3.6	C	2		25.5										
3.6µg/L	3.6	D	2		25.5										
10.9µg/L	10.9	A	2		25.4										
10.9µg/L	10.9	B	2		25.5										
10.9µg/L	10.9	C	2		25.5										
10.9µg/L	10.9	D	2		25.4										
33µg/L	33	A	2		25.5										
33µg/L	33	B	2		25.4										
33µg/L	33	C	2		25.5										
33µg/L	33	D	2		25.5										
100µg/L	100	A	2		25.4										
100µg/L	100	B	2		25.5										
100µg/L	100	C	2		25.5										
100µg/L	100	D	2		25.4										
(Ctrl) 0.0µg/L	0.0	A	3		25.5	7.5	7.3	789							

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
(Ctrl) 0.0µg/L	0.0	B	3		25.5	7.4	7.3	793							
(Ctrl) 0.0µg/L	0.0	C	3		25.4	7.4	7.3	801							
(Ctrl) 0.0µg/L	0.0	D	3		25.5	7.4	7.2	800							
3.6µg/L	3.6	A	3		25.5	7.4	7.3	735							
3.6µg/L	3.6	B	3		25.5	7.4	7.3	798							
3.6µg/L	3.6	C	3		25.4	7.4	7.2	750							
3.6µg/L	3.6	D	3		25.5	7.4	7.2	701							
10.9µg/L	10.9	A	3		25.4	7.4	7.1	920							
10.9µg/L	10.9	B	3		25.5	7.4	7.1	973							
10.9µg/L	10.9	C	3		25.5	7.5	7.0	901							
10.9µg/L	10.9	D	3		25.4	7.4	7.0	825							
33µg/L	33	A	3		25.5	7.4	7.0	620							
33µg/L	33	B	3		25.6	7.3	6.8	757							
33µg/L	33	C	3		25.5	7.4	6.8	720							
33µg/L	33	D	3		25.5	7.4	6.8	698							
100µg/L	100	A	3		25.4	7.4	6.9	810							
100µg/L	100	B	3		25.5	7.4	6.9	945							
100µg/L	100	C	3		25.4	7.3	7.0	900							
100µg/L	100	D	3		25.4	7.4	7.0	833							
(Ctrl) 0.0µg/L	0.0	A	4		25.5										
(Ctrl) 0.0µg/L	0.0	B	4		25.5										
(Ctrl) 0.0µg/L	0.0	C	4		25.4										
(Ctrl) 0.0µg/L	0.0	D	4		25.5										
3.6µg/L	3.6	A	4		25.4										
3.6µg/L	3.6	B	4		25.5										
3.6µg/L	3.6	C	4		25.5										
3.6µg/L	3.6	D	4		25.4										
10.9µg/L	10.9	A	4		25.5										
10.9µg/L	10.9	B	4		25.4										
10.9µg/L	10.9	C	4		25.5										
10.9µg/L	10.9	D	4		25.5										
33µg/L	33	A	4		25.5										
33µg/L	33	B	4		25.4										
33µg/L	33	C	4		25.4										
33µg/L	33	D	4		25.4										
100µg/L	100	A	4		25.5										
100µg/L	100	B	4		25.4										
100µg/L	100	C	4		25.5										
100µg/L	100	D	4		25.5										
(Ctrl) 0.0µg/L	0.0	A	5		25.5										
(Ctrl) 0.0µg/L	0.0	B	5		25.4										
(Ctrl) 0.0µg/L	0.0	C	5		25.5										
(Ctrl) 0.0µg/L	0.0	D	5		25.5										
3.6µg/L	3.6	A	5		25.4										
3.6µg/L	3.6	B	5		25.5										

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
3.6µg/L	3.6	C	5		25.4										
3.6µg/L	3.6	D	5		25.5										
10.9µg/L	10.9	A	5		25.5										
10.9µg/L	10.9	B	5		25.4										
10.9µg/L	10.9	C	5		25.5										
10.9µg/L	10.9	D	5		25.5										
33µg/L	33	A	5		25.5										
33µg/L	33	B	5		25.4										
33µg/L	33	C	5		25.5										
33µg/L	33	D	5		25.4										
100µg/L	100	A	5		25.5										
100µg/L	100	B	5		25.4										
100µg/L	100	C	5		25.5										
100µg/L	100	D	5		25.5										
(Ctrl) 0.0µg/L	0.0	A	6		25.5										
(Ctrl) 0.0µg/L	0.0	B	6		25.5										
(Ctrl) 0.0µg/L	0.0	C	6		25.4										
(Ctrl) 0.0µg/L	0.0	D	6		25.5										
3.6µg/L	3.6	A	6		25.4										
3.6µg/L	3.6	B	6		25.5										
3.6µg/L	3.6	C	6		25.5										
3.6µg/L	3.6	D	6		25.4										
10.9µg/L	10.9	A	6		25.5										
10.9µg/L	10.9	B	6		25.4										
10.9µg/L	10.9	C	6		25.4										
10.9µg/L	10.9	D	6		25.4										
33µg/L	33	A	6		25.5										
33µg/L	33	B	6		25.5										
33µg/L	33	C	6		25.4										
33µg/L	33	D	6		25.4										
100µg/L	100	A	6		25.5										
100µg/L	100	B	6		25.5										
100µg/L	100	C	6		25.4										
100µg/L	100	D	6		25.4										
(Ctrl) 0.0µg/L	0.0	A	7	0.106	25.4				136	52					MQL=0.212µg/L. For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	B	7	0.106	25.4										MQL=0.212µg/L. For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
(Ctrl) 0.0µg/L	0.0	C	7	0.106	25.5										MQL=0.212µg/L, For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	D	7	0.106	25.4										MQL=0.212µg/L, For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
3.6µg/L	3.6	A	7	2.66	25.5										
3.6µg/L	3.6	B	7	2.41	25.4										
3.6µg/L	3.6	C	7	2.26	25.5										
3.6µg/L	3.6	D	7	2.30	25.4										
10.9µg/L	10.9	A	7	10.0	25.5										
10.9µg/L	10.9	B	7	9.32	25.4										
10.9µg/L	10.9	C	7	9.26	25.4										
10.9µg/L	10.9	D	7	9.09	25.5										
33µg/L	33	A	7	29.5	25.4										
33µg/L	33	B	7	25.6	25.5										
33µg/L	33	C	7	26.2	25.4										
33µg/L	33	D	7	25.9	25.4										
100µg/L	100	A	7	99.1	25.5										
100µg/L	100	B	7	92.7	25.5				136	48					
100µg/L	100	C	7	92.4	25.4										
100µg/L	100	D	7	87.9	25.5										
(Ctrl) 0.0µg/L	0.0	A	8		25.5	7.3	7.6	698							
(Ctrl) 0.0µg/L	0.0	B	8		25.4	7.3	7.6	733							
(Ctrl) 0.0µg/L	0.0	C	8		25.5	7.3	7.1	747							
(Ctrl) 0.0µg/L	0.0	D	8		25.4	7.3	7.5	788							
3.6µg/L	3.6	A	8		25.5	7.3	7.3	873							
3.6µg/L	3.6	B	8		25.4	7.4	6.8	825							
3.6µg/L	3.6	C	8		25.5	7.3	6.5	898							
3.6µg/L	3.6	D	8		25.4	7.3	7.4	910							
10.9µg/L	10.9	A	8		25.5	7.3	7.4	831							
10.9µg/L	10.9	B	8		25.4	7.3	7.6	810							
10.9µg/L	10.9	C	8		25.5	7.3	7.3	790							
10.9µg/L	10.9	D	8		25.4	7.3	7.2	756							
33µg/L	33	A	8		25.4	7.5	7.1	741							
33µg/L	33	B	8		25.5	7.3	6.8	739							
33µg/L	33	C	8		25.5	7.3	6.9	825							
33µg/L	33	D	8		25.4	7.3	7.4	801							
100µg/L	100	A	8		25.4	7.5	7.4	721							
100µg/L	100	B	8		25.4	7.3	7.5	703							
100µg/L	100	C	8		25.5	7.3	7.6	786							
100µg/L	100	D	8		25.5	7.3	7.6	750							
(Ctrl) 0.0µg/L	0.0	A	9		25.4										

DEST

BATT01-00404

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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
(Ctrl) 0.0µg/L	0.0	B	9		25.5										
(Ctrl) 0.0µg/L	0.0	C	9		25.5										
(Ctrl) 0.0µg/L	0.0	D	9		25.4										
3.6µg/L	3.6	A	9		25.5										
3.6µg/L	3.6	B	9		25.4										
3.6µg/L	3.6	C	9		25.5										
3.6µg/L	3.6	D	9		25.4										
10.9µg/L	10.9	A	9		25.4										
10.9µg/L	10.9	B	9		25.5										
10.9µg/L	10.9	C	9		25.4										
10.9µg/L	10.9	D	9		25.5										
33µg/L	33	A	9		25.4										
33µg/L	33	B	9		25.4										
33µg/L	33	C	9		25.4										
33µg/L	33	D	9		25.5										
100µg/L	100	A	9		25.5										
100µg/L	100	B	9		25.4										
100µg/L	100	C	9		25.4										
100µg/L	100	D	9		25.5										
(Ctrl) 0.0µg/L	0.0	A	10		25.5										
(Ctrl) 0.0µg/L	0.0	B	10		25.4										
(Ctrl) 0.0µg/L	0.0	C	10		25.5										
(Ctrl) 0.0µg/L	0.0	D	10		25.5										
3.6µg/L	3.6	A	10		25.4										
3.6µg/L	3.6	B	10		25.4										
3.6µg/L	3.6	C	10		25.4										
3.6µg/L	3.6	D	10		25.5										
10.9µg/L	10.9	A	10		25.5										
10.9µg/L	10.9	B	10		25.4										
10.9µg/L	10.9	C	10		25.5										
10.9µg/L	10.9	D	10		25.4										
33µg/L	33	A	10		25.5										
33µg/L	33	B	10		25.4										
33µg/L	33	C	10		25.5										
33µg/L	33	D	10		25.4										
100µg/L	100	A	10		25.5										
100µg/L	100	B	10		25.4										
100µg/L	100	C	10		25.5										
100µg/L	100	D	10		25.4										
(Ctrl) 0.0µg/L	0.0	A	11		25.5	7.5	7.4	660							
(Ctrl) 0.0µg/L	0.0	B	11		25.4	7.5	7.5	747							
(Ctrl) 0.0µg/L	0.0	C	11		25.5	7.5	7.3	873							
(Ctrl) 0.0µg/L	0.0	D	11		25.4	7.5	7.1	929							
3.6µg/L	3.6	A	11		25.5	7.6	7.0	596							
3.6µg/L	3.6	B	11		25.5	7.6	6.9	612							

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BATT01-00404

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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
3.6µg/L	3.6	C	11		25.4	7.6	7.1	748							
3.6µg/L	3.6	D	11		25.4	7.6	7.2	889							
10.9µg/L	10.9	A	11		25.5	7.6	7.1	794							
10.9µg/L	10.9	B	11		25.5	7.6	7.1	995							
10.9µg/L	10.9	C	11		25.4	7.6	7.1	816							
10.9µg/L	10.9	D	11		25.5	7.6	7.1	840							
33µg/L	33	A	11		25.4	7.6	7.1	968							
33µg/L	33	B	11		25.5	7.6	7.1	942							
33µg/L	33	C	11		25.5	7.6	7.2	784							
33µg/L	33	D	11		25.5	7.6	6.9	856							
100µg/L	100	A	11		25.4	7.6	7.0	644							
100µg/L	100	B	11		25.4	7.6	7.5	804							
100µg/L	100	C	11		25.5	7.5	7.0	952							
100µg/L	100	D	11		25.5	7.5	7.1	883							
(Ctrl) 0.0µg/L	0.0	A	12		25.5										
(Ctrl) 0.0µg/L	0.0	B	12		25.4										
(Ctrl) 0.0µg/L	0.0	C	12		25.5										
(Ctrl) 0.0µg/L	0.0	D	12		25.5										
3.6µg/L	3.6	A	12		25.5										
3.6µg/L	3.6	B	12		25.4										
3.6µg/L	3.6	C	12		25.5										
3.6µg/L	3.6	D	12		25.5										
10.9µg/L	10.9	A	12		25.5										
10.9µg/L	10.9	B	12		25.5										
10.9µg/L	10.9	C	12		25.5										
10.9µg/L	10.9	D	12		25.4										
33µg/L	33	A	12		25.5										
33µg/L	33	B	12		25.5										
33µg/L	33	C	12		25.5										
33µg/L	33	D	12		25.4										
100µg/L	100	A	12		25.5										
100µg/L	100	B	12		25.4										
100µg/L	100	C	12		25.5										
100µg/L	100	D	12		25.5										
(Ctrl) 0.0µg/L	0.0	A	13		25.5										
(Ctrl) 0.0µg/L	0.0	B	13		25.5										
(Ctrl) 0.0µg/L	0.0	C	13		25.5										
(Ctrl) 0.0µg/L	0.0	D	13		25.4										
3.6µg/L	3.6	A	13		25.5										
3.6µg/L	3.6	B	13		25.5										
3.6µg/L	3.6	C	13		25.4										
3.6µg/L	3.6	D	13		25.5										
10.9µg/L	10.9	A	13		25.4										
10.9µg/L	10.9	B	13		25.5										
10.9µg/L	10.9	C	13		25.5										

DEST

BATT01-00404

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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
10.9µg/L	10.9	D	13		25.4										
33µg/L	33	A	13		25.5										
33µg/L	33	B	13		25.5										
33µg/L	33	C	13		25.5										
33µg/L	33	D	13		25.4										
100µg/L	100	A	13		25.4										
100µg/L	100	B	13		25.5										
100µg/L	100	C	13		25.4										
100µg/L	100	D	13		25.5										
(Ctrl) 0.0µg/L	0.0	A	14	0.106	25.5	7.7	7.1	698							MQL=0.212µg/L, For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	B	14	0.106	25.6	7.7	7.0	725							MQL=0.212µg/L, For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	C	14	0.106	25.5	7.7	7.0	795	140	52					MQL=0.212µg/L, For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	D	14	0.106	25.6	7.7	6.9	837							MQL=0.212µg/L, For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
3.6µg/L	3.6	A	14	3.36	25.5	7.7	6.9	901							
3.6µg/L	3.6	B	14	3.15	25.5	7.7	7.0	935							
3.6µg/L	3.6	C	14	2.88	25.4	7.6	7.1	980							
3.6µg/L	3.6	D	14	2.95	25.5	7.7	6.9	961							
10.9µg/L	10.9	A	14	13.1	25.5	7.7	6.9	842							
10.9µg/L	10.9	B	14	11.6	25.4	7.7	7.0	820							
10.9µg/L	10.9	C	14	11.4	25.5	7.6	7.1	763							
10.9µg/L	10.9	D	14	12.2	25.4	7.6	7.2	798							
33µg/L	33	A	14	38.5	25.5	7.6	7.1	679							
33µg/L	33	B	14	33.0	25.4	7.6	6.8	731							
33µg/L	33	C	14	33.5	25.5	7.7	6.9	767							
33µg/L	33	D	14	32.6	25.4	7.6	6.9	809							
100µg/L	100	A	14	106	25.5	7.6	6.9	710							
100µg/L	100	B	14	95.6	25.5	7.6	7.0	798							
100µg/L	100	C	14	93.1	25.6	7.6	7.0	856	140	44					
100µg/L	100	D	14	91.4	25.5	7.6	7.0	731							
(Ctrl) 0.0µg/L	0.0	A	15		25.5										
(Ctrl) 0.0µg/L	0.0	B	15		25.5										
(Ctrl) 0.0µg/L	0.0	C	15		25.6										
(Ctrl) 0.0µg/L	0.0	D	15		25.5										

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
3.6µg/L	3.6	A	15		25.4										
3.6µg/L	3.6	B	15		25.5										
3.6µg/L	3.6	C	15		25.5										
3.6µg/L	3.6	D	15		25.6										
10.9µg/L	10.9	A	15		25.5										
10.9µg/L	10.9	B	15		25.5										
10.9µg/L	10.9	C	15		25.6										
10.9µg/L	10.9	D	15		25.5										
33µg/L	33	A	15		25.5										
33µg/L	33	B	15		25.6										
33µg/L	33	C	15		25.5										
33µg/L	33	D	15		25.5										
100µg/L	100	A	15		25.4										
100µg/L	100	B	15		25.5										
100µg/L	100	C	15		25.5										
100µg/L	100	D	15		25.5										
(Ctrl) 0.0µg/L	0.0	A	16		25.4										
(Ctrl) 0.0µg/L	0.0	B	16		25.5										
(Ctrl) 0.0µg/L	0.0	C	16		25.5										
(Ctrl) 0.0µg/L	0.0	D	16		25.5										
3.6µg/L	3.6	A	16		25.5										
3.6µg/L	3.6	B	16		25.4										
3.6µg/L	3.6	C	16		25.4										
3.6µg/L	3.6	D	16		25.5										
10.9µg/L	10.9	A	16		25.5										
10.9µg/L	10.9	B	16		25.4										
10.9µg/L	10.9	C	16		25.4										
10.9µg/L	10.9	D	16		25.5										
33µg/L	33	A	16		25.5										
33µg/L	33	B	16		25.4										
33µg/L	33	C	16		25.5										
33µg/L	33	D	16		25.5										
100µg/L	100	A	16		25.4										
100µg/L	100	B	16		25.5										
100µg/L	100	C	16		25.4										
100µg/L	100	D	16		25.5										
(Ctrl) 0.0µg/L	0.0	A	17		25.5										
(Ctrl) 0.0µg/L	0.0	B	17		25.5										
(Ctrl) 0.0µg/L	0.0	C	17		25.4										
(Ctrl) 0.0µg/L	0.0	D	17		25.4										
3.6µg/L	3.6	A	17		25.3										
3.6µg/L	3.6	B	17		25.5										
3.6µg/L	3.6	C	17		25.5										
3.6µg/L	3.6	D	17		25.6										
10.9µg/L	10.9	A	17		25.5										

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BATT01-00404

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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
10.9µg/L	10.9	B	17		25.5										
10.9µg/L	10.9	C	17		25.4										
10.9µg/L	10.9	D	17		25.4										
33µg/L	33	A	17		25.5										
33µg/L	33	B	17		25.5										
33µg/L	33	C	17		25.4										
33µg/L	33	D	17		25.5										
100µg/L	100	A	17		25.4										
100µg/L	100	B	17		25.5										
100µg/L	100	C	17		25.5										
100µg/L	100	D	17		25.5										
(Ctrl) 0.0µg/L	0.0	A	18		25.4	7.7	6.9	822							
(Ctrl) 0.0µg/L	0.0	B	18		25.4	7.7	6.6	887							
(Ctrl) 0.0µg/L	0.0	C	18		25.5	7.7	6.7	853							
(Ctrl) 0.0µg/L	0.0	D	18		25.4	7.7	7.3	846							
3.6µg/L	3.6	A	18		25.5	7.7	6.9	967							
3.6µg/L	3.6	B	18		25.4	7.7	7.0	971							
3.6µg/L	3.6	C	18		25.4	7.7	6.8	968							
3.6µg/L	3.6	D	18		25.5	7.7	7.2	953							
10.9µg/L	10.9	A	18		25.4	7.6	6.9	926							
10.9µg/L	10.9	B	18		25.5	7.6	6.8	879							
10.9µg/L	10.9	C	18		25.4	7.6	7.0	901							
10.9µg/L	10.9	D	18		25.4	7.6	7.0	899							
33µg/L	33	A	18		25.4	7.6	6.9	751							
33µg/L	33	B	18		25.5	7.6	7.0	811							
33µg/L	33	C	18		25.4	7.6	6.9	819							
33µg/L	33	D	18		25.4	7.6	6.7	800							
100µg/L	100	A	18		25.5	7.6	6.9	710							
100µg/L	100	B	18		25.4	7.6	6.7	834							
100µg/L	100	C	18		25.4	7.6	6.7	791							
100µg/L	100	D	18		25.4	7.6	6.8	755							
(Ctrl) 0.0µg/L	0.0	A	19		25.4										
(Ctrl) 0.0µg/L	0.0	B	19		25.4										
(Ctrl) 0.0µg/L	0.0	C	19		25.4										
(Ctrl) 0.0µg/L	0.0	D	19		25.3										
3.6µg/L	3.6	A	19		25.4										
3.6µg/L	3.6	B	19		25.4										
3.6µg/L	3.6	C	19		25.4										
3.6µg/L	3.6	D	19		25.3										
10.9µg/L	10.9	A	19		25.4										
10.9µg/L	10.9	B	19		25.4										
10.9µg/L	10.9	C	19		25.4										
10.9µg/L	10.9	D	19		25.3										
33µg/L	33	A	19		25.3										
33µg/L	33	B	19		25.4										

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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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
33µg/L	33	C	19		25.4										
33µg/L	33	D	19		25.4										
100µg/L	100	A	19		25.3										
100µg/L	100	B	19		25.4										
100µg/L	100	C	19		25.3										
100µg/L	100	D	19		25.4										
(Ctrl) 0.0µg/L	0.0	A	20		25.5										
(Ctrl) 0.0µg/L	0.0	B	20		25.4										
(Ctrl) 0.0µg/L	0.0	C	20		25.4										
(Ctrl) 0.0µg/L	0.0	D	20		25.4										
3.6µg/L	3.6	A	20		25.5										
3.6µg/L	3.6	B	20		25.4										
3.6µg/L	3.6	C	20		25.5										
3.6µg/L	3.6	D	20		25.4										
10.9µg/L	10.9	A	20		25.5										
10.9µg/L	10.9	B	20		25.5										
10.9µg/L	10.9	C	20		25.4										
10.9µg/L	10.9	D	20		25.5										
33µg/L	33	A	20		25.5										
33µg/L	33	B	20		25.6										
33µg/L	33	C	20		25.5										
33µg/L	33	D	20		25.4										
100µg/L	100	A	20		25.4										
100µg/L	100	B	20		25.5										
100µg/L	100	C	20		25.5										
100µg/L	100	D	20		25.5										
(Ctrl) 0.0µg/L	0.0	A	21	0.106	25.4	7.7	7.4	695							MQL=0.212µg/L, For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	B	21	0.106	25.5	7.7	7.4	630							MQL=0.212µg/L, For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	C	21	0.106	25.5	7.6	7.2	697							MQL=0.212µg/L, For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
(Ctrl) 0.0µg/L	0.0	D	21	0.106	25.4	7.7	7.2	710	132	44					MQL=0.212µg/L, For results <MQL, 1/2 MQL is reported to facilitate calculation of mean measured concentrations for control and each treatment.
3.6µg/L	3.6	A	21	2.87	25.5	7.9	7.4	730							
3.6µg/L	3.6	B	21	2.55	25.4	7.8	7.4	750							
3.6µg/L	3.6	C	21	2.49	25.5	7.8	7.4	870							

FEL

DEST

BATT01-00404

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)	pH	Dissolved Oxygen (mg/L)	Light Intensity (Lux)	Total Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µS/cm)	Residual Chlorine Oxidants (mg/L)	Ammonia (mg/L)	Iodide (µg/L)	Comments
3.6µg/L	3.6	D	21	2.46	25.4	7.8	7.6	723							
10.9µg/L	10.9	A	21	9.70	25.4	7.7	7.5	901							
10.9µg/L	10.9	B	21	8.17	25.5	7.7	7.5	975							
10.9µg/L	10.9	C	21	8.61	25.5	7.8	7.4	827							
10.9µg/L	10.9	D	21	8.61	25.4	7.8	7.2	854							
33µg/L	33	A	21	43.9	25.5	7.8	7.4	873							
33µg/L	33	B	21	32.5	25.5	7.8	7.5	891							
33µg/L	33	C	21	34.2	25.5	7.8	7.4	899							
33µg/L	33	D	21	33.4	25.4	7.8	7.4	855							
100µg/L	100	A	21	104	25.5	7.8	7.4	792							
100µg/L	100	B	21	93.9	25.4	7.8	7.4	836							
100µg/L	100	C	21	115	25.4	7.8	7.4	847							
100µg/L	100	D	21	110	25.5	7.8	7.4	823	136	44					
Facility Dilution Water			21											9.3	
Facility Water Chemistry		N/A				7.0	6.4		132	40	736	<0.05	<0.06		Facility Water Chemistry bimonthly values determined on 2/28/17, one day after study takedown.

Survival Reproduction (Daily)

DEST

BATT01-00404

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
(Ctrl) 0.0µg/L	A	0	5	5	88	85	
(Ctrl) 0.0µg/L	B	0	5	5	74	70	
(Ctrl) 0.0µg/L	C	0	5	5	79	75	
(Ctrl) 0.0µg/L	D	0	5	5	65	63	
3.6µg/L	A	0	5	5	76	73	
3.6µg/L	B	0	5	5	84	81	
3.6µg/L	C	0	5	5	70	68	
3.6µg/L	D	0	5	5	69	65	
10.9µg/L	A	0	5	5	66	63	
10.9µg/L	B	0	5	5	74	70	
10.9µg/L	C	0	5	5	69	65	
10.9µg/L	D	0	5	5	76	74	
33µg/L	A	0	5	5	0	0	
33µg/L	B	0	5	5	57	54	
33µg/L	C	0	5	5	66	63	
33µg/L	D	0	5	5	75	72	
100µg/L	A	0	5	5	76	74	
100µg/L	B	0	5	5	71	68	
100µg/L	C	0	5	5	73	70	
100µg/L	D	0	5	5	69	66	
(Ctrl) 0.0µg/L	A	1	5	5	76	73	
(Ctrl) 0.0µg/L	B	1	5	5	59	55	
(Ctrl) 0.0µg/L	C	1	5	5	66	64	
(Ctrl) 0.0µg/L	D	1	5	5	79	76	
3.6µg/L	A	1	5	5	73	70	
3.6µg/L	B	1	5	5	78	75	
3.6µg/L	C	1	5	5	63	61	
3.6µg/L	D	1	5	5	57	55	
10.9µg/L	A	1	5	5	71	67	
10.9µg/L	B	1	5	5	68	65	
10.9µg/L	C	1	5	5	77	74	
10.9µg/L	D	1	5	5	55	52	

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Survival Reproduction (Daily)

DEST

BATT01-00404

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
33µg/L	A	1	5	5	72	69	
33µg/L	B	1	5	5	58	55	
33µg/L	C	1	5	5	59	55	
33µg/L	D	1	5	5	62	58	
100µg/L	A	1	5	5	54	51	
100µg/L	B	1	5	5	59	52	
100µg/L	C	1	5	5	65	60	
100µg/L	D	1	5	5	53	50	
(Ctrl) 0.0µg/L	A	2	5	5	63	60	
(Ctrl) 0.0µg/L	B	2	5	5	62	59	
(Ctrl) 0.0µg/L	C	2	5	5	69	68	
(Ctrl) 0.0µg/L	D	2	5	5	68	65	
3.6µg/L	A	2	5	5	64	62	
3.6µg/L	B	2	5	5	70	67	
3.6µg/L	C	2	5	5	59	57	
3.6µg/L	D	2	5	5	71	68	
10.9µg/L	A	2	5	5	58	56	
10.9µg/L	B	2	5	5	61	59	
10.9µg/L	C	2	5	5	63	60	
10.9µg/L	D	2	5	5	59	56	
33µg/L	A	2	5	5	81	77	
33µg/L	B	2	5	5	60	57	
33µg/L	C	2	5	5	62	58	
33µg/L	D	2	5	5	59	55	
100µg/L	A	2	5	5	61	57	
100µg/L	B	2	5	5	56	50	
100µg/L	C	2	5	5	54	50	
100µg/L	D	2	5	5	50	44	
(Ctrl) 0.0µg/L	A	3	5	5	74	72	
(Ctrl) 0.0µg/L	B	3	5	5	71	68	
(Ctrl) 0.0µg/L	C	3	5	5	73	70	
(Ctrl) 0.0µg/L	D	3	5	5	54	52	

DEST

Survival Reproduction (Daily)

BATT01-00404

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
3.6µg/L	A	3	5	5	57	55	
3.6µg/L	B	3	5	5	64	61	
3.6µg/L	C	3	5	5	69	66	
3.6µg/L	D	3	5	5	68	65	
10.9µg/L	A	3	5	5	60	56	
10.9µg/L	B	3	5	5	58	55	
10.9µg/L	C	3	5	5	62	59	
10.9µg/L	D	3	5	5	66	63	
33µg/L	A	3	5	5	64	62	
33µg/L	B	3	5	5	62	59	
33µg/L	C	3	5	5	57	54	
33µg/L	D	3	5	5	61	58	
100µg/L	A	3	5	5	59	50	
100µg/L	B	3	5	5	38	31	
100µg/L	C	3	5	5	43	38	
100µg/L	D	3	5	5	48	40	
(Ctrl) 0.0µg/L	A	4	5	5	72	69	
(Ctrl) 0.0µg/L	B	4	5	5	57	54	
(Ctrl) 0.0µg/L	C	4	5	5	66	63	
(Ctrl) 0.0µg/L	D	4	5	5	57	53	
3.6µg/L	A	4	5	5	68	64	
3.6µg/L	B	4	5	5	57	54	
3.6µg/L	C	4	5	5	62	58	
3.6µg/L	D	4	5	5	70	66	
10.9µg/L	A	4	5	5	55	53	
10.9µg/L	B	4	5	5	64	60	
10.9µg/L	C	4	5	5	71	68	
10.9µg/L	D	4	5	5	74	70	
33µg/L	A	4	5	5	69	66	
33µg/L	B	4	5	5	52	50	
33µg/L	C	4	5	5	61	58	
33µg/L	D	4	5	5	59	56	

DEST

Survival Reproduction (Daily)

BATT01-00404

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
100µg/L	A	4	5	5	53	45	
100µg/L	B	4	5	5	32	22	
100µg/L	C	4	5	5	36	31	
100µg/L	D	4	5	5	38	28	
(Ctrl) 0.0µg/L	A	5	5	5	68	64	
(Ctrl) 0.0µg/L	B	5	5	5	69	66	
(Ctrl) 0.0µg/L	C	5	5	5	71	68	
(Ctrl) 0.0µg/L	D	5	5	5	70	66	
3.6µg/L	A	5	5	5	70	66	
3.6µg/L	B	5	5	5	59	56	
3.6µg/L	C	5	5	5	68	64	
3.6µg/L	D	5	5	5	71	65	
10.9µg/L	A	5	5	5	68	65	
10.9µg/L	B	5	5	5	66	63	
10.9µg/L	C	5	5	5	67	63	
10.9µg/L	D	5	5	5	68	65	
33µg/L	A	5	5	5	58	54	
33µg/L	B	5	5	5	56	53	
33µg/L	C	5	5	5	60	56	
33µg/L	D	5	5	5	57	53	
100µg/L	A	5	5	5	41	35	
100µg/L	B	5	5	5	39	33	
100µg/L	C	5	5	5	34	28	
100µg/L	D	5	5	5	39	27	
(Ctrl) 0.0µg/L	A	6	5	5	78	74	
(Ctrl) 0.0µg/L	B	6	5	5	71	69	
(Ctrl) 0.0µg/L	C	6	5	5	72	69	
(Ctrl) 0.0µg/L	D	6	5	5	66	63	
3.6µg/L	A	6	5	5	59	56	
3.6µg/L	B	6	5	5	67	64	
3.6µg/L	C	6	5	5	70	67	
3.6µg/L	D	6	5	5	58	56	

Survival Reproduction (Daily)

DEST

BATT01-00404

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
10.9µg/L	A	6	5	5	54	52	
10.9µg/L	B	6	5	5	68	65	
10.9µg/L	C	6	5	5	70	66	
10.9µg/L	D	6	5	5	58	55	
33µg/L	A	6	5	5	63	60	
33µg/L	B	6	5	5	52	50	
33µg/L	C	6	5	5	57	53	
33µg/L	D	6	5	5	65	62	
100µg/L	A	6	5	5	40	32	
100µg/L	B	6	5	5	35	27	
100µg/L	C	6	5	5	38	30	
100µg/L	D	6	5	5	28	20	
(Ctrl) 0.0µg/L	A	7	5	5	59	56	
(Ctrl) 0.0µg/L	B	7	5	5	73	70	
(Ctrl) 0.0µg/L	C	7	5	5	64	62	
(Ctrl) 0.0µg/L	D	7	5	5	69	66	
3.6µg/L	A	7	5	5	73	70	
3.6µg/L	B	7	5	5	58	54	
3.6µg/L	C	7	5	5	66	63	
3.6µg/L	D	7	5	5	62	58	
10.9µg/L	A	7	5	5	66	63	
10.9µg/L	B	7	5	5	71	68	
10.9µg/L	C	7	5	5	57	53	
10.9µg/L	D	7	5	5	64	60	
33µg/L	A	7	5	5	56	54	
33µg/L	B	7	5	5	65	62	
33µg/L	C	7	5	5	60	50	
33µg/L	D	7	5	5	68	64	
100µg/L	A	7	5	5	35	29	
100µg/L	B	7	5	5	29	20	
100µg/L	C	7	5	5	28	20	
100µg/L	D	7	5	5	31	21	

DEST

Survival Reproduction (Daily)

BATT01-00404

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
(Ctrl) 0.0µg/L	A	8	5	5	68	65	
(Ctrl) 0.0µg/L	B	8	5	5	69	67	
(Ctrl) 0.0µg/L	C	8	5	5	68	66	
(Ctrl) 0.0µg/L	D	8	5	5	65	63	
3.6µg/L	A	8	5	5	66	63	
3.6µg/L	B	8	5	5	68	64	
3.6µg/L	C	8	5	5	71	68	
3.6µg/L	D	8	5	5	73	71	
10.9µg/L	A	8	5	5	59	56	
10.9µg/L	B	8	5	5	68	65	
10.9µg/L	C	8	5	5	54	51	
10.9µg/L	D	8	5	5	69	66	
33µg/L	A	8	5	5	50	45	
33µg/L	B	8	5	5	53	45	
33µg/L	C	8	5	5	54	46	
33µg/L	D	8	5	5	50	42	
100µg/L	A	8	5	5	32	21	
100µg/L	B	8	5	5	33	23	
100µg/L	C	8	5	5	29	20	
100µg/L	D	8	5	5	27	18	
(Ctrl) 0.0µg/L	A	9	5	5	73	70	
(Ctrl) 0.0µg/L	B	9	5	5	75	71	
(Ctrl) 0.0µg/L	C	9	5	5	71	67	
(Ctrl) 0.0µg/L	D	9	5	5	57	54	
3.6µg/L	A	9	5	5	58	55	
3.6µg/L	B	9	5	5	73	70	
3.6µg/L	C	9	5	5	62	60	
3.6µg/L	D	9	5	5	65	62	
10.9µg/L	A	9	5	5	57	54	
10.9µg/L	B	9	5	5	69	66	
10.9µg/L	C	9	5	5	59	56	
10.9µg/L	D	9	5	5	74	70	

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DEST

BATT01-00404

Survival Reproduction (Daily)

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
33µg/L	A	9	5	5	45	40	
33µg/L	B	9	5	5	43	37	
33µg/L	C	9	5	5	49	44	
33µg/L	D	9	5	5	29	15	
100µg/L	A	9	5	5	24	15	
100µg/L	B	9	5	5	32	21	
100µg/L	C	9	5	5	22	12	
100µg/L	D	9	5	5	30	15	
(Ctrl) 0.0µg/L	A	10	5	5	64	61	
(Ctrl) 0.0µg/L	B	10	5	5	70	67	
(Ctrl) 0.0µg/L	C	10	5	5	70	67	
(Ctrl) 0.0µg/L	D	10	5	5	60	57	
3.6µg/L	A	10	5	5	59	56	
3.6µg/L	B	10	5	5	56	54	
3.6µg/L	C	10	5	5	64	61	
3.6µg/L	D	10	5	5	58	55	
10.9µg/L	A	10	5	5	67	65	
10.9µg/L	B	10	5	5	60	57	
10.9µg/L	C	10	5	5	63	61	
10.9µg/L	D	10	5	5	58	55	
33µg/L	A	10	5	5	43	38	
33µg/L	B	10	5	5	50	36	
33µg/L	C	10	5	5	45	40	
33µg/L	D	10	5	5	26	16	
100µg/L	A	10	5	5	30	20	
100µg/L	B	10	5	5	28	17	
100µg/L	C	10	5	5	24	11	
100µg/L	D	10	5	5	0	0	
(Ctrl) 0.0µg/L	A	11	5	5	68	65	
(Ctrl) 0.0µg/L	B	11	5	5	64	61	
(Ctrl) 0.0µg/L	C	11	5	5	66	63	
(Ctrl) 0.0µg/L	D	11	5	5	69	66	

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
3.6µg/L	A	11	5	5	62	59	
3.6µg/L	B	11	5	5	52	50	
3.6µg/L	C	11	5	5	68	64	
3.6µg/L	D	11	5	5	71	67	
10.9µg/L	A	11	5	5	59	56	
10.9µg/L	B	11	5	5	54	50	
10.9µg/L	C	11	5	5	58	56	
10.9µg/L	D	11	5	5	68	64	
33µg/L	A	11	5	5	40	28	
33µg/L	B	11	5	5	37	21	
33µg/L	C	11	5	5	39	30	
33µg/L	D	11	5	5	20	10	
100µg/L	A	11	5	5	17	3	
100µg/L	B	11	5	5	23	14	
100µg/L	C	11	5	5	20	9	
100µg/L	D	11	5	5	0	0	
(Ctrl) 0.0µg/L	A	12	5	5	72	68	
(Ctrl) 0.0µg/L	B	12	5	5	73	70	
(Ctrl) 0.0µg/L	C	12	5	5	71	69	
(Ctrl) 0.0µg/L	D	12	5	5	58	55	
3.6µg/L	A	12	5	5	65	62	
3.6µg/L	B	12	5	5	58	55	
3.6µg/L	C	12	5	5	71	68	
3.6µg/L	D	12	5	5	70	66	
10.9µg/L	A	12	5	5	64	61	
10.9µg/L	B	12	5	5	60	56	
10.9µg/L	C	12	5	5	65	62	
10.9µg/L	D	12	5	5	69	66	
33µg/L	A	12	5	5	39	20	
33µg/L	B	12	5	5	26	15	
33µg/L	C	12	5	5	40	26	
33µg/L	D	12	5	5	17	4	

Survival Reproduction (Daily)

DEST

BATT01-00404

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
100µg/L	A	12	5	5	0	0	
100µg/L	B	12	5	5	26	10	
100µg/L	C	12	5	5	23	7	
100µg/L	D	12	5	5	0	0	
(Ctrl) 0.0µg/L	A	13	5	5	67	63	
(Ctrl) 0.0µg/L	B	13	5	5	71	69	
(Ctrl) 0.0µg/L	C	13	5	5	58	55	
(Ctrl) 0.0µg/L	D	13	5	5	67	63	
3.6µg/L	A	13	5	5	69	66	
3.6µg/L	B	13	5	5	62	60	
3.6µg/L	C	13	5	5	58	56	
3.6µg/L	D	13	5	5	67	63	
10.9µg/L	A	13	5	5	62	58	
10.9µg/L	B	13	5	5	57	53	
10.9µg/L	C	13	5	5	62	58	
10.9µg/L	D	13	5	5	70	65	
33µg/L	A	13	5	5	23	10	
33µg/L	B	13	5	5	24	12	
33µg/L	C	13	5	5	29	20	
33µg/L	D	13	5	5	0	0	
100µg/L	A	13	5	5	0	0	
100µg/L	B	13	5	5	14	5	
100µg/L	C	13	5	5	0	0	
100µg/L	D	13	5	5	0	0	
(Ctrl) 0.0µg/L	A	14	5	5	75	71	
(Ctrl) 0.0µg/L	B	14	5	5	58	56	
(Ctrl) 0.0µg/L	C	14	5	5	63	60	
(Ctrl) 0.0µg/L	D	14	5	5	74	71	
3.6µg/L	A	14	5	5	77	72	
3.6µg/L	B	14	5	5	64	61	
3.6µg/L	C	14	5	5	59	55	
3.6µg/L	D	14	5	5	71	67	

Survival Reproduction (Daily)

DEST

BATT01-00404

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
10.9µg/L	A	14	5	5	57	53	
10.9µg/L	B	14	5	5	54	50	
10.9µg/L	C	14	5	5	56	53	
10.9µg/L	D	14	5	5	64	62	
33µg/L	A	14	5	5	19	9	
33µg/L	B	14	5	5	0	0	
33µg/L	C	14	5	5	23	12	
33µg/L	D	14	5	5	0	0	
100µg/L	A	14	5	5	0	0	
100µg/L	B	14	5	5	0	0	
100µg/L	C	14	5	5	0	0	
100µg/L	D	14	5	5	0	0	
(Ctrl) 0.0µg/L	A	15	5	5	63	60	
(Ctrl) 0.0µg/L	B	15	5	5	74	70	
(Ctrl) 0.0µg/L	C	15	5	5	58	55	
(Ctrl) 0.0µg/L	D	15	5	5	76	73	
3.6µg/L	A	15	5	5	68	65	
3.6µg/L	B	15	5	5	66	63	
3.6µg/L	C	15	5	5	56	53	
3.6µg/L	D	15	5	5	70	66	
10.9µg/L	A	15	5	5	52	49	
10.9µg/L	B	15	5	5	58	55	
10.9µg/L	C	15	5	5	52	48	
10.9µg/L	D	15	5	5	61	57	
33µg/L	A	15	5	5	0	0	
33µg/L	B	15	5	5	0	0	
33µg/L	C	15	5	5	15	3	
33µg/L	D	15	5	5	0	0	
100µg/L	A	15	5	5	0	0	
100µg/L	B	15	5	5	0	0	
100µg/L	C	15	5	5	0	0	
100µg/L	D	15	5	5	0	0	

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Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
(Ctrl) 0.0µg/L	A	16	5	5	61	59	
(Ctrl) 0.0µg/L	B	16	5	5	70	67	
(Ctrl) 0.0µg/L	C	16	5	5	61	58	
(Ctrl) 0.0µg/L	D	16	5	5	60	56	
3.6µg/L	A	16	5	5	57	54	
3.6µg/L	B	16	5	5	70	67	
3.6µg/L	C	16	5	5	65	62	
3.6µg/L	D	16	5	5	73	70	
10.9µg/L	A	16	5	5	58	55	
10.9µg/L	B	16	5	5	56	51	
10.9µg/L	C	16	5	5	54	50	
10.9µg/L	D	16	5	5	59	55	
33µg/L	A	16	5	5	0	0	
33µg/L	B	16	5	5	0	0	
33µg/L	C	16	5	5	0	0	
33µg/L	D	16	5	5	0	0	
100µg/L	A	16	5	5	0	0	
100µg/L	B	16	5	5	0	0	
100µg/L	C	16	5	5	0	0	
100µg/L	D	16	5	5	0	0	
(Ctrl) 0.0µg/L	A	17	5	5	72	68	
(Ctrl) 0.0µg/L	B	17	5	5	66	62	
(Ctrl) 0.0µg/L	C	17	5	5	59	55	
(Ctrl) 0.0µg/L	D	17	5	5	55	51	
3.6µg/L	A	17	5	5	63	60	
3.6µg/L	B	17	5	5	73	69	
3.6µg/L	C	17	5	5	68	64	
3.6µg/L	D	17	5	5	56	52	
10.9µg/L	A	17	5	5	55	51	
10.9µg/L	B	17	5	5	51	47	
10.9µg/L	C	17	5	5	53	48	
10.9µg/L	D	17	5	5	57	54	

FEL

Survival Reproduction (Daily)

DEST

BATT01-00404

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
33µg/L	A	17	5	5	0	0	
33µg/L	B	17	5	5	0	0	
33µg/L	C	17	5	5	0	0	
33µg/L	D	17	5	5	0	0	
100µg/L	A	17	5	5	0	0	
100µg/L	B	17	5	5	0	0	
100µg/L	C	17	5	5	0	0	
100µg/L	D	17	5	5	13	0	
(Ctrl) 0.0µg/L	A	18	5	5	63	60	
(Ctrl) 0.0µg/L	B	18	5	5	71	68	
(Ctrl) 0.0µg/L	C	18	5	5	73	69	
(Ctrl) 0.0µg/L	D	18	5	5	66	63	
3.6µg/L	A	18	5	5	61	58	
3.6µg/L	B	18	5	5	60	56	
3.6µg/L	C	18	5	5	64	61	
3.6µg/L	D	18	5	5	66	63	
10.9µg/L	A	18	5	5	53	50	
10.9µg/L	B	18	5	5	60	56	
10.9µg/L	C	18	5	5	57	53	
10.9µg/L	D	18	5	5	56	53	
33µg/L	A	18	5	5	0	0	
33µg/L	B	18	5	5	0	0	
33µg/L	C	18	5	5	0	0	
33µg/L	D	18	5	5	0	0	
100µg/L	A	18	5	5	0	0	
100µg/L	B	18	5	5	0	0	
100µg/L	C	18	5	5	0	0	
100µg/L	D	18	5	5	0	0	
(Ctrl) 0.0µg/L	A	19	5	5	56	52	
(Ctrl) 0.0µg/L	B	19	5	5	66	64	
(Ctrl) 0.0µg/L	C	19	5	5	71	68	
(Ctrl) 0.0µg/L	D	19	5	5	72	68	

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DEST

BATT01-00404

Survival Reproduction (Daily)

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
3.6µg/L	A	19	5	5	63	60	
3.6µg/L	B	19	5	5	53	50	
3.6µg/L	C	19	5	5	55	51	
3.6µg/L	D	19	5	5	59	56	
10.9µg/L	A	19	5	5	56	53	
10.9µg/L	B	19	5	5	54	51	
10.9µg/L	C	19	5	5	52	49	
10.9µg/L	D	19	5	5	61	57	
33µg/L	A	19	5	5	0	0	
33µg/L	B	19	5	5	0	0	
33µg/L	C	19	5	5	0	0	
33µg/L	D	19	5	5	0	0	
100µg/L	A	19	5	5	0	0	
100µg/L	B	19	5	5	0	0	
100µg/L	C	19	5	5	0	0	
100µg/L	D	19	5	5	0	0	
(Ctrl) 0.0µg/L	A	20	5	5	68	64	
(Ctrl) 0.0µg/L	B	20	5	5	70	66	
(Ctrl) 0.0µg/L	C	20	5	5	65	61	
(Ctrl) 0.0µg/L	D	20	5	5	73	70	
3.6µg/L	A	20	5	5	57	54	
3.6µg/L	B	20	5	5	56	53	
3.6µg/L	C	20	5	5	62	58	
3.6µg/L	D	20	5	5	65	63	
10.9µg/L	A	20	5	5	63	60	
10.9µg/L	B	20	5	5	59	56	
10.9µg/L	C	20	5	5	57	54	
10.9µg/L	D	20	5	5	65	61	
33µg/L	A	20	5	5	0	0	
33µg/L	B	20	5	5	0	0	
33µg/L	C	20	5	5	0	0	
33µg/L	D	20	5	5	0	0	

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Number of Surviving Males	Number of Surviving Females	Number of Eggs	Number of Fertilized Eggs	Comments
100µg/L	A	20	5	5	0	0	
100µg/L	B	20	5	5	0	0	
100µg/L	C	20	5	5	0	0	
100µg/L	D	20	5	5	0	0	
(Ctrl) 0.0µg/L	A	21	5	5	74	70	
(Ctrl) 0.0µg/L	B	21	5	5	65	61	
(Ctrl) 0.0µg/L	C	21	5	5	72	69	
(Ctrl) 0.0µg/L	D	21	5	5	59	56	
3.6µg/L	A	21	5	5	66	62	
3.6µg/L	B	21	5	5	68	65	
3.6µg/L	C	21	5	5	57	54	
3.6µg/L	D	21	5	5	63	60	
10.9µg/L	A	21	5	5	57	54	
10.9µg/L	B	21	5	5	60	56	
10.9µg/L	C	21	5	5	65	61	
10.9µg/L	D	21	5	5	70	65	
33µg/L	A	21	5	5	0	0	
33µg/L	B	21	5	5	0	0	
33µg/L	C	21	5	5	0	0	
33µg/L	D	21	5	5	0	0	
100µg/L	A	21	5	5	0	0	
100µg/L	B	21	5	5	0	0	
100µg/L	C	21	5	5	0	0	
100µg/L	D	21	5	5	0	0	

DEST BATT01-00404 Clinical Signs (Daily)

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Secondary Sex Characteristics	Clinical Signs	Number of Males Affected	Number of Females Affected
(Ctrl) 0.0µg/L	A	0	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	0	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	0	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	0	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	0	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	0	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	0	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	0	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	0	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	0	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	0	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	0	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	0	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	0	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	0	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	0	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	0	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	0	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	0	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	0	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	1	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	1	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	1	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	1	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	1	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	1	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	1	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	1	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	1	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	1	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	1	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	1	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	1	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	1	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	1	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	1	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	1	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	1	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	1	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	1	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	2	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	2	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	2	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	2	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	2	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	2	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	2	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	2	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	2	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	2	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	2	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	2	Present	None, Normal Appearance and Behavior	5	5

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Secondary Sex Characteristics	Clinical Signs	Number of Males Affected	Number of Females Affected
33µg/L	A	2	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	2	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	2	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	2	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	2	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	2	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	2	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	2	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	3	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	3	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	3	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	3	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	3	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	3	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	3	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	3	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	3	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	3	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	3	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	3	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	3	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	3	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	3	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	3	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	3	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	3	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	3	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	3	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	4	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	4	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	4	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	4	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	4	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	4	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	4	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	4	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	4	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	4	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	4	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	4	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	4	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	4	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	4	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	4	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	4	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	4	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	4	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	4	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	5	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	5	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	5	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	5	Present	None, Normal Appearance and Behavior	5	5

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Secondary Sex Characteristics	Clinical Signs	Number of Males Affected	Number of Females Affected
3.6µg/L	A	5	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	5	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	5	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	5	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	5	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	5	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	5	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	5	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	5	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	5	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	5	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	5	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	5	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	5	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	5	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	5	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	6	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	6	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	6	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	6	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	6	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	6	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	6	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	6	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	6	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	6	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	6	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	6	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	6	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	6	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	6	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	6	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	6	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	6	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	6	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	6	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	7	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	7	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	7	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	7	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	7	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	7	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	7	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	7	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	7	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	7	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	7	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	7	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	7	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	7	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	7	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	7	Present	None, Normal Appearance and Behavior	5	5

DEST BATT01-00404 Clinical Signs (Daily)

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Secondary Sex Characteristics	Clinical Signs	Number of Males Affected	Number of Females Affected
100µg/L	A	7	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	7	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	7	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	7	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	8	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	8	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	8	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	8	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	8	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	8	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	8	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	8	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	8	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	8	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	8	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	8	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	8	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	8	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	8	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	8	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	8	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	8	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	8	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	8	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	9	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	9	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	9	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	9	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	9	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	9	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	9	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	9	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	9	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	9	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	9	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	9	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	9	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	9	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	9	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	9	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	9	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	9	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	9	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	9	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	10	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	10	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	10	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	10	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	10	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	10	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	10	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	10	Present	None, Normal Appearance and Behavior	5	5

DEST BATT01-00404 Clinical Signs (Daily)

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Secondary Sex Characteristics	Clinical Signs	Number of Males Affected	Number of Females Affected
10.9µg/L	A	10	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	10	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	10	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	10	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	10	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	10	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	10	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	10	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	10	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	10	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	10	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	10	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	11	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	11	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	11	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	11	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	11	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	11	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	11	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	11	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	11	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	11	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	11	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	11	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	11	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	11	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	11	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	11	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	11	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	11	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	11	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	11	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	12	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	12	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	12	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	12	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	12	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	12	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	12	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	12	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	12	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	12	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	12	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	12	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	12	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	12	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	12	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	12	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	12	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	12	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	12	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	12	Present	None, Normal Appearance and Behavior	5	5

DEST BATT01-00404 Clinical Signs (Daily)

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Secondary Sex Characteristics	Clinical Signs	Number of Males Affected	Number of Females Affected
(Ctrl) 0.0µg/L	A	13	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	13	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	13	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	13	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	13	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	13	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	13	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	13	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	13	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	13	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	13	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	13	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	13	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	13	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	13	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	13	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	13	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	13	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	13	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	13	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	14	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	14	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	14	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	14	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	14	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	14	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	14	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	14	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	14	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	14	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	14	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	14	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	14	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	14	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	14	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	14	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	14	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	14	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	14	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	14	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	15	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	15	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	15	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	15	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	15	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	15	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	15	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	15	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	15	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	15	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	15	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	15	Present	None, Normal Appearance and Behavior	5	5

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Secondary Sex Characteristics	Clinical Signs	Number of Males Affected	Number of Females Affected
33µg/L	A	15	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	15	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	15	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	15	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	15	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	15	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	15	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	15	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	16	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	16	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	16	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	16	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	16	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	16	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	16	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	16	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	16	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	16	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	16	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	16	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	16	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	16	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	16	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	16	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	16	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	16	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	16	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	16	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	17	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	17	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	17	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	17	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	17	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	17	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	17	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	17	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	17	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	17	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	17	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	17	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	17	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	17	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	17	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	17	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	17	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	17	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	17	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	17	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	18	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	18	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	18	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	18	Present	None, Normal Appearance and Behavior	5	5

DEST BATT01-00404 Clinical Signs (Daily)

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Secondary Sex Characteristics	Clinical Signs	Number of Males Affected	Number of Females Affected
3.6µg/L	A	18	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	18	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	18	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	18	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	18	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	18	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	18	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	18	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	18	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	18	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	18	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	18	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	18	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	18	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	18	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	18	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	19	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	19	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	19	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	19	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	19	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	19	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	19	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	19	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	19	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	19	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	19	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	19	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	19	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	19	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	19	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	19	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	19	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	19	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	19	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	19	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	20	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	20	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	20	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	20	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	20	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	20	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	20	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	20	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	20	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	20	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	20	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	20	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	20	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	20	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	20	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	20	Present	None, Normal Appearance and Behavior	5	5

DEST BATT01-00404 Clinical Signs (Daily)

Name of Treatment Group	Replicate ID	Test Day (0 to 21)	Secondary Sex Characteristics	Clinical Signs	Number of Males Affected	Number of Females Affected
100µg/L	A	20	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	20	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	20	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	20	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	A	21	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	B	21	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	C	21	Present	None, Normal Appearance and Behavior	5	5
(Ctrl) 0.0µg/L	D	21	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	A	21	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	B	21	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	C	21	Present	None, Normal Appearance and Behavior	5	5
3.6µg/L	D	21	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	A	21	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	B	21	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	C	21	Present	None, Normal Appearance and Behavior	5	5
10.9µg/L	D	21	Present	None, Normal Appearance and Behavior	5	5
33µg/L	A	21	Present	None, Normal Appearance and Behavior	5	5
33µg/L	B	21	Present	None, Normal Appearance and Behavior	5	5
33µg/L	C	21	Present	None, Normal Appearance and Behavior	5	5
33µg/L	D	21	Present	None, Normal Appearance and Behavior	5	5
100µg/L	A	21	Present	None, Normal Appearance and Behavior	5	5
100µg/L	B	21	Present	None, Normal Appearance and Behavior	5	5
100µg/L	C	21	Present	None, Normal Appearance and Behavior	5	5
100µg/L	D	21	Present	None, Normal Appearance and Behavior	5	5

DEST BATT01-00404 Individual Observations (Day21)

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Genotypic Sex (M or F)	Phenotypic Sex (M or F)	Body Weight (mg)	Body Length (mm)	Gonad Weight (mg)	GSI (%)	Papillae	Secondary Sex Characteristics and Other Clinical Signs	Comments
(Ctrl) 0.0µg/L	A	1	M	M	407.8	27.9			93		
(Ctrl) 0.0µg/L	A	2	M	M	362.5	25.8			77		
(Ctrl) 0.0µg/L	A	3	M	M	369.8	28.0			62		
(Ctrl) 0.0µg/L	A	4	F	F	503.8	29.7			0		
(Ctrl) 0.0µg/L	A	5	F	F	402.6	26.5			0		
(Ctrl) 0.0µg/L	A	6	F	F	340.4	25.5			0		
(Ctrl) 0.0µg/L	A	7		M	349.0	25.0	1.8	0.52%	88		
(Ctrl) 0.0µg/L	A	8		M	362.1	26.8	2.1	0.58%	76		
(Ctrl) 0.0µg/L	A	9		F	349.8	24.8	25.2	7.20%	0		
(Ctrl) 0.0µg/L	A	10		F	345.1	23.3	19.1	5.53%	0		
(Ctrl) 0.0µg/L	B	1	M	M	573.5	31.2			97		
(Ctrl) 0.0µg/L	B	2	M	M	360.1	26.3			67		
(Ctrl) 0.0µg/L	B	3	M	M	421.9	26.6			94		
(Ctrl) 0.0µg/L	B	4	F	F	463.5	30.2			0		
(Ctrl) 0.0µg/L	B	5	F	F	364.5	26.9			0		
(Ctrl) 0.0µg/L	B	6	F	F	372.1	26.5			0		
(Ctrl) 0.0µg/L	B	7		M	360.5	27.2	4.0	1.11%	78		
(Ctrl) 0.0µg/L	B	8		M	337.2	26.5	5.1	1.51%	92		
(Ctrl) 0.0µg/L	B	9		F	331.1	24.6	18.6	5.62%	0		
(Ctrl) 0.0µg/L	B	10		F	326.0	21.4	42.5	13.04%	0		
(Ctrl) 0.0µg/L	C	1	M	M	458.5	25.8			80		
(Ctrl) 0.0µg/L	C	2	M	M	410.3	27.0			93		
(Ctrl) 0.0µg/L	C	3	M	M	403.6	25.6			89		
(Ctrl) 0.0µg/L	C	4	F	F	528.3	30.2			0		
(Ctrl) 0.0µg/L	C	5	F	F	344.7	26.5			0		
(Ctrl) 0.0µg/L	C	6	F	F	327.8	26.6			0		
(Ctrl) 0.0µg/L	C	7		M	347.8	27.8	2.0	0.58%	62		
(Ctrl) 0.0µg/L	C	8		M	404.9	25.4	4.0	0.99%	95		
(Ctrl) 0.0µg/L	C	9		F	323.6	22.0	14.8	4.57%	0		
(Ctrl) 0.0µg/L	C	10		F	313.2	25.2	15.0	4.79%	0		
(Ctrl) 0.0µg/L	D	1	M	M	474.2	29.0			90		
(Ctrl) 0.0µg/L	D	2	M	M	371.0	26.5			71		
(Ctrl) 0.0µg/L	D	3	M	M	354.1	25.8			73		
(Ctrl) 0.0µg/L	D	4	F	F	350.1	26.1			0		
(Ctrl) 0.0µg/L	D	5	F	F	310.9	24.0			0		
(Ctrl) 0.0µg/L	D	6	F	F	301.1	25.5			0		
(Ctrl) 0.0µg/L	D	7		M	333.3	26.6	1.9	0.57%	98		
(Ctrl) 0.0µg/L	D	8		M	317.9	22.8	2.1	0.66%	84		
(Ctrl) 0.0µg/L	D	9		F	358.4	24.8	24.5	6.84%	0		
(Ctrl) 0.0µg/L	D	10		F	361.2	25.6	31.7	8.78%	0		
3.6µg/L	A	1	M	M	581.2	30.8			103		
3.6µg/L	A	2	M	M	410.2	26.3			89		
3.6µg/L	A	3	M	M	390.2	27.1			78		
3.6µg/L	A	4	F	F	360.4	26.1			0		
3.6µg/L	A	5	F	F	340.9	26.4			0		
3.6µg/L	A	6	F	F	329.7	24.0			0		
3.6µg/L	A	7		M	348.7	26.6	1.5	0.43%	96		
3.6µg/L	A	8		M	365.8	27.0	3.0	0.82%	91		
3.6µg/L	A	9		F	323.1	23.6	25.7	7.95%	0		
3.6µg/L	A	10		F	316.3	23.7	24.1	7.62%	0		
3.6µg/L	B	1	M	M	527.5	30.2			109		
3.6µg/L	B	2	M	M	445.7	27.3			67		
3.6µg/L	B	3	M	M	359.7	27.8			87		
3.6µg/L	B	4	F	F	472.1	26.8			0		
3.6µg/L	B	5	F	F	392.2	27.3			0		
3.6µg/L	B	6	F	F	327.3	23.4			0		
3.6µg/L	B	7		M	355.6	27.7	3.3	0.93%	85		
3.6µg/L	B	8		M	349.8	24.5	3.1	0.89%	74		

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Genotypic Sex (M or F)	Phenotypic Sex (M or F)	Body Weight (mg)	Body Length (mm)	Gonad Weight (mg)	GSI (%)	Papillae	Secondary Sex Characteristics and Other Clinical Signs	Comments
3.6µg/L	B	9		F	347.6	22.6	31.8	9.15%	0		
3.6µg/L	B	10		F	345.5	25.5	30.3	8.77%	0		
3.6µg/L	C	1	M	M	472.6	28.8			76		
3.6µg/L	C	2	M	M	451.0	28.6			62		
3.6µg/L	C	3	M	M	464.3	28.4			81		
3.6µg/L	C	4	F	F	413.1	27.4			0		
3.6µg/L	C	5	F	F	412.3	27.6			0		
3.6µg/L	C	6	F	F	399.3	27.2			0		
3.6µg/L	C	7		M	394.7	26.7	3.6	0.91%	77		
3.6µg/L	C	8		M	389.1	27.7	6.1	1.57%	69		
3.6µg/L	C	9		F	381.4	27.3	48.1	12.61%	0		
3.6µg/L	C	10		F	375.2	27.2	40.7	10.85%	0		
3.6µg/L	D	1	M	M	658.6	31.3			85		
3.6µg/L	D	2	M	M	522.0	29.4			93		
3.6µg/L	D	3	M	M	411.1	28.5			73		
3.6µg/L	D	4	F	F	470.1	28.2			0		
3.6µg/L	D	5	F	F	506.3	28.9			0		
3.6µg/L	D	6	F	F	319.8	26.1			0		
3.6µg/L	D	7		M	379.6	27.4	2.0	0.53%	85		
3.6µg/L	D	8		M	431.6	28.8	4.5	1.04%	64		
3.6µg/L	D	9		F	428.3	28.6	25.7	6.00%	0		
3.6µg/L	D	10		F	318.8	25.6	30.4	9.54%	0		
10.9µg/L	A	1	M	M	679.2	32.9			89		
10.9µg/L	A	2	M	M	534.0	30.4			86		
10.9µg/L	A	3	M	M	456.1	28.3			101		
10.9µg/L	A	4	F	F	517.8	30.0			0		
10.9µg/L	A	5	F	F	360.8	27.2			0		
10.9µg/L	A	6	F	F	327.6	25.2			0		
10.9µg/L	A	7		M	333.9	27.2	1.9	0.57%	75		
10.9µg/L	A	8		M	352.1	25.2	2.2	0.62%	85		
10.9µg/L	A	9		F	312.1	26.0	24.5	7.85%	0		
10.9µg/L	A	10		F	299.1	24.5	16.3	5.45%	0		
10.9µg/L	B	1	M	M	435.7	29.7			79		
10.9µg/L	B	2	M	M	375.0	28.1			86		
10.9µg/L	B	3	M	M	401.9	27.2			74		
10.9µg/L	B	4	F	F	465.9	29.0			0		
10.9µg/L	B	5	F	F	351.6	25.2			0		
10.9µg/L	B	6	F	F	407.3	27.0			0		
10.9µg/L	B	7		M	320.7	27.2	3.4	1.06%	90		
10.9µg/L	B	8		M	301.3	25.7	3.7	1.23%	79		
10.9µg/L	B	9		F	329.1	25.6	46.3	14.07%	0		
10.9µg/L	B	10		F	333.3	24.8	23.3	6.99%	0		
10.9µg/L	C	1	M	M	490.7	29.1			64		
10.9µg/L	C	2	M	M	315.1	26.2			82		
10.9µg/L	C	3	M	M	443.0	29.1			77		
10.9µg/L	C	4	F	F	490.1	30.2			0		
10.9µg/L	C	5	F	F	374.1	27.1			0		
10.9µg/L	C	6	F	F	336.4	25.6			0		
10.9µg/L	C	7		M	352.1	25.4	3.2	0.91%	75		
10.9µg/L	C	8		M	330.7	24.9	3.2	0.97%	90		
10.9µg/L	C	9		F	322.8	24.4	24.0	7.43%	0		
10.9µg/L	C	10		F	359.0	24.8	23.3	6.49%	0		
10.9µg/L	D	1	M	M	519.2	31.8			70		
10.9µg/L	D	2	M	M	397.1	27.0			78		
10.9µg/L	D	3	M	M	428.0	29.4			97		
10.9µg/L	D	4	F	F	383.8	26.8			0		
10.9µg/L	D	5	F	F	403.3	26.5			0		
10.9µg/L	D	6	F	F	392.9	26.6			0		

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Genotypic Sex (M or F)	Phenotypic Sex (M or F)	Body Weight (mg)	Body Length (mm)	Gonad Weight (mg)	GSI (%)	Papillae	Secondary Sex Characteristics and Other Clinical Signs	Comments
10.9µg/L	D	7		M	436.5	28.1	4.6	1.05%	49		
10.9µg/L	D	8		M	426.5	26.7	3.6	0.84%	64		
10.9µg/L	D	9		F	331.7	25.7	22.3	6.72%	0		
10.9µg/L	D	10		F	321.6	24.9	20.5	6.37%	0		
33µg/L	A	1	M	M	495.9	29.6			63		
33µg/L	A	2	M	M	441.8	28.5			50		
33µg/L	A	3	M	M	480.7	28.4			65		
33µg/L	A	4	F	F	417.6	28.6			0		
33µg/L	A	5	F	F	396.4	28.3			0		
33µg/L	A	6	F	F	368.6	25.1			0		
33µg/L	A	7		M	396.4	28.9	2.0	0.50%	97		
33µg/L	A	8		M	382.0	26.0	2.7	0.71%	46		
33µg/L	A	9		F	354.5	28.4	33.0	9.31%	0		
33µg/L	A	10		F	321.3	25.6	26.9	8.37%	0		
33µg/L	B	1	M	M	486.0	29.5			81		
33µg/L	B	2	M	M	466.3	29.6			75		
33µg/L	B	3	M	M	372.2	28.6			84		
33µg/L	B	4	F	F	663.3	32.1			0		
33µg/L	B	5	F	F	469.0	28.5			0		
33µg/L	B	6	F	F	390.9	26.5			0		
33µg/L	B	7		M	324.1	25.2	3.6	1.11%	83		
33µg/L	B	8		M	316.7	24.4	3.7	1.17%	85		
33µg/L	B	9		F	432.4	28.3	24.0	5.55%	0		
33µg/L	B	10		F	310.1	24.5	45.0	14.51%	0		
33µg/L	C	1	M	M	495.6	28.4			65		
33µg/L	C	2	M	M	411.5	27.5			59		
33µg/L	C	3	M	M	372.9	28.0			60		
33µg/L	C	4	F	F	532.9	29.4			0		
33µg/L	C	5	F	F	429.5	27.2			0		
33µg/L	C	6	F	F	339.9	24.3			0		
33µg/L	C	7		M	322.7	23.5	3.1	0.96%	64		
33µg/L	C	8		M	321.1	22.4	2.4	0.75%	60		
33µg/L	C	9		F	318.6	24.2	23.7	7.44%	0		
33µg/L	C	10		F	317.9	21.6	23.7	7.46%	0		
33µg/L	D	1	M	M	498.3	29.6			84		
33µg/L	D	2	M	M	399.8	27.9			101		
33µg/L	D	3	M	M	423.1	28.2			53		
33µg/L	D	4	F	F	614.7	30.6			0		
33µg/L	D	5	F	F	610.9	32.1			0		
33µg/L	D	6	F	F	490.6	29.0			0		
33µg/L	D	7		M	326.6	26.0	1.8	0.55%	58		
33µg/L	D	8		M	320.7	26.6	1.6	0.50%	89		
33µg/L	D	9		F	352.5	28.9	25.7	7.29%	0		
33µg/L	D	10		F	307.9	25.3	26.1	8.48%	0		
100µg/L	A	1	M	M	405.1	29.7			88		
100µg/L	A	2	M	M	428.2	28.4			74		
100µg/L	A	3	M	M	368.2	28.6			63		
100µg/L	A	4	F	F	396.2	28.6			0		
100µg/L	A	5	F	F	374.4	27.2			0		
100µg/L	A	6	F	F	335.5	26.5			0		
100µg/L	A	7		M	325.6	26.9	2.6	0.80%	67		
100µg/L	A	8		M	310.1	22.3	2.7	0.87%	65		
100µg/L	A	9		F	319.6	26.3	23.7	7.42%	0		
100µg/L	A	10		F	309.9	25.4	16.1	5.20%	0		
100µg/L	B	1	M	M	424.2	28.1			59		
100µg/L	B	2	M	M	353.6	26.0			77		
100µg/L	B	3	M	M	327.2	24.8			48		
100µg/L	B	4	F	F	393.8	27.7			0		

DEST BATT01-00404 Individual Observations (Day21)

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Genotypic Sex (M or F)	Phenotypic Sex (M or F)	Body Weight (mg)	Body Length (mm)	Gonad Weight (mg)	GSI (%)	Papillae	Secondary Sex Characteristics and Other Clinical Signs	Comments
100µg/L	B	5	F	F	362.4	26.2			0		
100µg/L	B	6	F	F	351.7	25.9			0		
100µg/L	B	7		M	328.5	24.0	4.2	1.28%	46		
100µg/L	B	8		M	311.2	22.6	2.5	0.80%	65		
100µg/L	B	9		F	309.9	26.1	19.5	6.29%	0		
100µg/L	B	10		F	307.1	21.4	12.3	4.01%	0		
100µg/L	C	1	M	M	378.5	27.2			51		
100µg/L	C	2	M	M	372.3	27.9			63		
100µg/L	C	3	M	M	341.1	25.9			68		
100µg/L	C	4	F	F	365.3	27.1			0		
100µg/L	C	5	F	F	408.5	28.6			0		
100µg/L	C	6	F	F	377.0	27.1			0		
100µg/L	C	7		M	315.8	29.2	2.7	0.85%	57		
100µg/L	C	8		M	307.3	24.6	2.3	0.75%	56		
100µg/L	C	9		F	336.2	26.5	30.1	8.95%	0		
100µg/L	C	10		F	311.1	24.6	25.3	8.13%	0		
100µg/L	D	1	M	M	476.9	29.7			77		
100µg/L	D	2	M	M	403.1	27.9			94		
100µg/L	D	3	M	M	348.8	25.3			72		
100µg/L	D	4	F	F	481.9	29.7			0		
100µg/L	D	5	F	F	409.6	27.7			0		
100µg/L	D	6	F	F	382.4	26.2			0		
100µg/L	D	7		M	321.4	24.8	3.1	0.96%	62		
100µg/L	D	8		M	310.1	24.0	3.6	1.16%	76		
100µg/L	D	9		F	339.6	26.1	18.5	5.45%	0		
100µg/L	D	10		F	307.3	24.4	10.7	3.48%	0		

Group (µg/L)	Replicate ID	Tank ID	Day 21 Animal ID	Gonadal Staging Overall	Gonad Phenotype Score	Cystic Degeneration	Vacuolation	Testicular Degeneration	Follicular Cell Hyperplasia	Follicular Cell Hypertrophy	Inflammation, Granulomatous (Liver)	Increased Spermatagonia
0	A	A2	138-A2	1	1	2	1	1	2	1	1	1
0	A	A3	139-A3	1	1	1	1	2	1	1		1
0	B	B1	143-B1	1	1	2	1	2	2	1		
0	B	B2	144-B2	1	1		1	2	2	1		
0	B	B3	145-B3	1	1	2	1	2	2	2		
0	C	C1	149-C1	1	1			1	2	1		
0	C	C2	150-C2	1	1	1			2	1		
0	C	C3	151-C3	2	1			2	2	1		
0	D	D1	155-D1	2	1	1	1	2	2	1		
0	D	D2	156-D2	2	1		1	1	2	2		
0	D	D3	157-D3	1	1			1	1	1		
10.9	A	A1	185-A1						2	1		
10.9	A	A2	186-A2						2	1		
10.9	A	A3	187-A3						1	1		
10.9	B	B1	191-B1						2	1		
10.9	B	B2	192-B2						1	1		
10.9	B	B3	193-B3						1	2		
10.9	C	C1	197-C1						2	1		
10.9	C	C2	198-C2						1	1		
10.9	C	C3	199-C3						1	1		
10.9	D	D1	203-D1						2	1		
10.9	D	D2	204-D2						2	1		
10.9	D	D3	205-D3						2	1		
33	A	A1	209-A1						2	1		
33	A	A2	210-A2						1	1		
33	A	A3	211-A3						1			
33	B	B1	215-B1						2	2		
33	B	B2	216-B2						2	1		
33	B	B3	217-B3						1	1		
33	C	C1	221-C1						2	1		
33	C	C2	222-C2						2	1		
33	C	C3	223-C3						2	1		
33	D	D1	227-D1						2	1		
33	D	D2	228-D2						2	1		
33	D	D3	229-D3						1	1		
100	A	A1	233-A1	1	1	1		1	2	1	2	
100	A	A2	234-A2	1	1	1	1	1	2	1		
100	A	A3	235-A3	1	1	2	1	2	1	1	3	1
100	B	B1	239-B1	1	1	2		1	2	2	2	
100	B	B2	240-B2	1	1		1	2	2	1		
100	B	B3	241-B3	1	1			2	2	2		
100	C	C1	245-C1	1	1	2		1	2	1	3	
100	C	C2	246-C2	1	1	2	1	2	2	2		
100	C	C3	247-C3	1	1	1	1	1	2	1		
100	D	D1	251-D1	1	1	2	1		2	2		
100	D	D2	252-D2	1	1	2		2	2	1		
100	D	D3	253-D3	2	1		2	1	1			

FEL

DEST

BATT01-00404

Male Gonadal (Histopathology

Group (µg/L)	Replicate ID	Tank ID	Follicular Cells, Hyaline Degeneration	Bile Pigment	Cytoplasmic Inclusions, Eosinophilic	Seminoma, Benign	Follicle Size, Decreased	Pigmented Macrophage Aggregates	Interstitial Cells, Increased	Not Remarkable (Liver)	Colloid, Inspissated	Dysgerminoma	Basophilia
0	A	A2	2										
0	A	A3	1										
0	B	B1	1	1	1	P							
0	B	B2					2						
0	B	B3	2					1					
0	C	C1	1		2				1				
0	C	C2											
0	C	C3	1							X			
0	D	D1	1					1					
0	D	D2	1		1			1					
0	D	D3	1		1								
10.9	A	A1					1						
10.9	A	A2	2										
10.9	A	A3											
10.9	B	B1	1										
10.9	B	B2	1								2		
10.9	B	B3	1										
10.9	C	C1	2										
10.9	C	C2	1										
10.9	C	C3	1										
10.9	D	D1	1								1		
10.9	D	D2											
10.9	D	D3									2		
33	A	A1	2										
33	A	A2											
33	A	A3											
33	B	B1	1										
33	B	B2											
33	B	B3											
33	C	C1	2										
33	C	C2											
33	C	C3	1				2				1		
33	D	D1	2								1		
33	D	D2											
33	D	D3											
100	A	A1	1						1			P	
100	A	A2	1		1			1			1		
100	A	A3	1			P	1	1	1				
100	B	B1						1					
100	B	B2								X			
100	B	B3											
100	C	C1	1					1					
100	C	C2						1					
100	C	C3	1			P							
100	D	D1	1						1				
100	D	D2											1
100	D	D3											

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DEST

BATT01-00404

Female Gonadal (Histopathology

Group (µg/L)	Replicate ID	Tank ID	Day 21 Animal ID	Gonadal Staging Overall	Gonad Phenotype Score	Basophilia	Cystic Degeneration	Vacuolation	Pigmented Macrophage Aggregates	Cytoplasmic Inclusions, Eosinophilic	Seminoma, Malignant	Post-Ovulatory Follicles	Not Remarkable (Thyroid)	Follicular Cells, Hyaline Degeneration
0	A	A5	141-A5	3	5	2	2	1		1		1	X	
0	A	A6	142-A6	3	5	3	1	1				1		1
0	B	B4	146-B4	1	5	3	1		2	1			X	
0	C	C6	154-C6	3	5	3	2	1				1	X	
0	B	B5	147-B5	3	5	3	1	1				1	X	
0	B	B6	148-B6	2	5	3	1	1					X	
0	C	C4	152-C4	4	5	2	2	3	3			1		2
10.9	A	A4	188-A4	1	5	2	1	2						
0	C	C5	153-C5	2	5	3	2	1				1	X	
10.9	D	D4	206-D4	3	5	3		2	1			1		
0	D	D4	158-D4	2	5	3	1	1		1		2	X	
0	D	D5	159-D5	3	5	3	3	1				1	X	
0	D	D6	160-D6	2	5	3	2					1	X	
10.9	A	A5	189-A5	2	5	3	3	1				1		
10.9	A	A6	190-A6	1	5	2	1	1				1		
10.9	B	B4	194-B4	3	5	3	2	1	2	1		2		
10.9	B	B5	195-B5	1	5	1		1						
10.9	B	B6	196-B6	1	5	2		1						
10.9	C	C4	200-C4	3	5	3	2	1	1	1		1		
10.9	C	C5	201-C5	2	5	3		1		1		2		
10.9	C	C6	202-C6	3	5	3	2	1		1		1		
10.9	D	D5	207-D5	3	5	3						1		
10.9	D	D6	208-D6	3	5	3	1	1				1		
33	A	A4	212-A4	1	5			3						
33	A	A5	213-A5	1	5		1	3						
33	A	A6	214-A6	3	5	3		2	1			1		
33	B	B4	218-B4	2	5	1	2	3				2		
33	B	B5	219-B5	1	5		1	3						
33	B	B6	220-B6	1	5		1	3						
33	C	C4	224-C4	1	5		2	3						
33	C	C5	225-C5	4	5	1	1	2				2		
33	C	C6	226-C6	2	5	3	1	1				1		
33	D	D4	230-D4		5	2			1					
33	D	D5	231-D5		5		2	1		1				
33	D	D6	232-D6	2	5	3	2	1	1			1		
100	A	A4	236-A4	1	5	1	1	3				1		
100	A	A5	237-A5	3	5	3	2	2	1			1	X	
100	A	A6	238-A6	4	5	3	1	1		1		1	X	
100	B	B4	242-B4	4	5	3	1	1				1	X	
100	B	B5	243-B5	2	5	3	1					1	X	
100	B	B6	244-B6	2	5	3	2	1	1	1			X	
100	C	C4	248-C4	2	5	3	1	2				2	X	
100	C	C5	249-C5	4	5	3		2		1		1	X	
100	C	C6	250-C6	3	5	3		2	1			1	X	
100	D	D4	254-D4	1	5		1	3			P		X	
100	D	D5	255-D5	1	5	1		3					X	
100	D	D6	256-D6	3	5	1		3				1	X	

DEST

BATT01-00404

Female Gonadal (Histopathology

Group (µg/L)	Replicate ID	Tank ID	Oocyte Atresia	Egg Debrin Oviduct	Inflammation, Granulomatous (Liver)	Single Cell Necrosis	Inflammation, Granulomatous (Ovary)	Focus of Cellular Alteration	Post-Ovulatory Follicles, Vacuolation	Lymphoma/ Leukemia (Liver)	Lymphoma/ Leukemia (Ovary)	Unable to Stage	Oviduct, Fibrovascular Proliferation
0	A	A5											
0	A	A6											
0	B	B4	1	3			3						
0	C	C6			1								
0	B	B5											
0	B	B6											
0	C	C4	1	2		1	1						
10.9	A	A4	2		1		2						
0	C	C5	1										
10.9	D	D4			2		2						
0	D	D4	1										
0	D	D5											
0	D	D6	1			1							
10.9	A	A5											
10.9	A	A6	1										
10.9	B	B4				1	1						
10.9	B	B5	2				1						
10.9	B	B6	1										
10.9	C	C4											
10.9	C	C5	1										
10.9	C	C6											
10.9	D	D5											
10.9	D	D6											
33	A	A4						1					
33	A	A5	1						1				
33	A	A6	1										
33	B	B4	1							P	P		
33	B	B5	1										
33	B	B6	1										
33	C	C4	1										
33	C	C5											
33	C	C6										P	3
33	D	D4	4				2					P	2
33	D	D5	4				1						
100	A	D6	1			1			1				
100	A	A4	2										
100	A	A5						3					
100	A	A6											
100	B	B4	2										
100	B	B5	1				1						
100	B	B6	2										
100	C	C4	1										
100	C	C5						3					
100	C	C6	1										
100	D	D4	2										
100	D	D5	3										
100	D	D6											

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Female Gonadal (Histo)pathology

DEST

Group (µg/L)	Replicate ID	Tank ID	Perifollicular Cell Hyperplasia-Hypertrophy	Follicular Cell Hyperplasia
0	A	A5		
0	A	A6		
0	B	B4		
0	C	C6		
0	B	B5		
0	B	B6		
0	C	C4		
10.9	A	A4		
0	C	C5		
10.9	D	D4		
0	D	D4		
0	D	D5		
0	D	D6		
10.9	A	A5		
10.9	A	A6		
10.9	B	B4		
10.9	B	B5		
10.9	B	B6		
10.9	C	C4		
10.9	C	C5		
10.9	C	C6		
10.9	D	D5		
10.9	D	D6		
33	A	A4		
33	A	A5		
33	A	A6		
33	B	B4		
33	B	B5		
33	B	B6		
33	C	C4		
33	C	C5		
33	C	C6		
33	D	D4		
33	D	D5	1	
33	D	D6		
100	A	A4		1
100	A	A5		
100	A	A6		
100	B	B4		
100	B	B5		
100	B	B6		
100	C	C4		
100	C	C5		
100	C	C6		
100	D	D4		
100	D	D5		
100	D	D6		

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Sample Number	Genetic Sex (M or F)	Log10 of (copies vtg/ng total RNA)
(Ctrl) 0.0µg/L	A	7	497	M	1.47
(Ctrl) 0.0µg/L	A	8	498	M	1.53
(Ctrl) 0.0µg/L	A	9	499	F	5.27
(Ctrl) 0.0µg/L	A	10	500	F	2.61
(Ctrl) 0.0µg/L	B	7	501	M	1.59
(Ctrl) 0.0µg/L	B	8	502	M	2.01
(Ctrl) 0.0µg/L	B	9	503	F	5.16
(Ctrl) 0.0µg/L	B	10	504	F	2.81
(Ctrl) 0.0µg/L	C	7	505	M	2.20
(Ctrl) 0.0µg/L	C	8	506	M	1.37
(Ctrl) 0.0µg/L	C	9	507	F	5.20
(Ctrl) 0.0µg/L	C	10	508	F	5.35
(Ctrl) 0.0µg/L	D	7	509	M	1.90
(Ctrl) 0.0µg/L	D	8	510	M	1.81
(Ctrl) 0.0µg/L	D	9	511	F	4.92
(Ctrl) 0.0µg/L	D	10	512	F	4.68
3.6µg/L	A	7	513	M	3.44
3.6µg/L	A	8	514	M	2.41
3.6µg/L	A	9	515	F	4.17
3.6µg/L	A	10	516	F	5.30
3.6µg/L	B	7	517	M	3.00
3.6µg/L	B	8	518	M	2.98
3.6µg/L	B	9	519	F	4.01
3.6µg/L	B	10	520	F	5.54
3.6µg/L	C	7	521	M	3.67
3.6µg/L	C	8	522	M	4.17
3.6µg/L	C	9	523	F	5.30
3.6µg/L	C	10	524	F	5.37
3.6µg/L	D	7	525	M	3.97
3.6µg/L	D	8	526	M	3.70
3.6µg/L	D	9	527	F	5.55
3.6µg/L	D	10	528	F	5.01
10.9µg/L	A	7	529	M	4.32
10.9µg/L	A	8	530	M	2.85
10.9µg/L	A	9	531	F	4.25
10.9µg/L	A	10	532	F	5.54
10.9µg/L	B	7	533	M	3.89
10.9µg/L	B	8	534	M	3.08
10.9µg/L	B	9	535	F	5.06
10.9µg/L	B	10	536	F	4.66
10.9µg/L	C	7	537	M	2.41
10.9µg/L	C	8	538	M	2.47
10.9µg/L	C	9	539	F	5.59
10.9µg/L	C	10	540	F	5.17

Name of Treatment Group	Replicate ID	Day 21 Animal ID	Sample Number	Genetic Sex (M or F)	Log10 of (copies vtg/ng total RNA)
10.9µg/L	D	7	541	M	3.15
10.9µg/L	D	8	542	M	3.03
10.9µg/L	D	9	543	F	5.09
10.9µg/L	D	10	544	F	4.45
33µg/L	A	7	545	M	1.53
33µg/L	A	8	546	M	1.59
33µg/L	A	9	547	F	4.30
33µg/L	A	10	548	F	4.16
33µg/L	B	7	549	M	2.90
33µg/L	B	8	550	M	1.79
33µg/L	B	9	551	F	4.89
33µg/L	B	10	552	F	5.43
33µg/L	C	7	553	M	3.82
33µg/L	C	8	554	M	2.92
33µg/L	C	9	555	F	4.48
33µg/L	C	10	556	F	2.11
33µg/L	D	7	557	M	1.87
33µg/L	D	8	558	M	1.65
33µg/L	D	9	559	F	4.10
33µg/L	D	10	560	F	4.79
100µg/L	A	7	561	M	1.96
100µg/L	A	8	562	M	2.50
100µg/L	A	9	563	F	4.23
100µg/L	A	10	564	F	2.79
100µg/L	B	7	565	M	1.33
100µg/L	B	8	566	M	1.25
100µg/L	B	9	567	F	4.34
100µg/L	B	10	568	F	2.72
100µg/L	C	7	569	M	2.62
100µg/L	C	8	570	M	1.67
100µg/L	C	9	571	F	5.50
100µg/L	C	10	572	F	4.33
100µg/L	D	7	573	M	2.83
100µg/L	D	8	574	M	1.50
100µg/L	D	9	575	F	5.59
100µg/L	D	10	576	F	2.96

Appendix G. Statistical Analysis Report

Endocrine Disruptor Screening Program

USEPA Contract No: EP-W-11-063

Task Order No: 17

Final Statistical Analysis Report for

Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

FEL Study No. BATT01-00404

(FSTRA #404)

April 17, 2018

BATTELLE

505 King Avenue

Columbus, Ohio 43201

COMPLIANCE STATEMENT

FEL Study No. BATT01-00404

Study Title: Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka

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

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 4/17/18
Battelle Study Statistician Date

SIGNATURES

Name (Role)	Signature	Date
Po-Hsu Chen (Battelle Statistician / Report Originator)	Po-Hsu Chen	4/17/18
Ying-Liang Chou (Battelle Statistician/ Technical Reviewer)	Ying-Liang Chou	4/17/18
Vince Brown (Program Manager)	Vince Brown	4/17/2018

USEPA Contract No.: EP-W-11-063, TO 17
FEL Study No. BATT01-00404 (FSTRA #404)

Quality Assurance Statement
Battelle

USEPA Contract No.: EP-W-11-063, TO 17
Study No. BATT01-00404

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	September 18, 2017	September 18, 2017
Audit Draft Statistical Report	September 18, 2017	September 18, 2017
Audit Final Statistical Report	April 9, 2018	April 9, 2018

Quality Assurance Unit, Battelle — Date: 4-17-18

USEPA Contract No.: EP-W-11-063, TO 17
FEL Study No. BATT01-00404 (FSTRA #404)

INTRODUCTION

This report summarizes the statistical analysis of the data collected under the Fort Environmental Laboratories (FEL) Study Number BATT01-00404, "Fish Short-Term Reproduction Assay of 2-Ethylhexyl 4-Hydroxybenzoate with Japanese Medaka" (FSTRA #404).

The Fish Short-Term Reproduction Assay (FSTRA) was intended to identify and characterize a suite of histological and biochemical endpoints that reflect effects associated with disturbance to the hypothalamus-pituitary-gonadal (HPG) endocrine axis. The assay was initiated with sexually mature male and female fathead minnows traditionally, but medaka was used as a modification in the present study. FSTRA evaluated potential, inciting contributors to fish population declines by evaluating the effects, both through endocrine and non-endocrine mechanisms, from exposure to contaminants that may adversely affect populations. Thus, the primary objective of this study was to evaluate the potential reproductive toxicity of 2-Ethylhexyl 4-Hydroxybenzoate (2-EHHB) to Japanese medaka in accordance with the guidelines established in OPPTS 890.1350 (1) and OECD guideline 229 (2) with modification to use medaka *in lieu* of fathead minnow among other changes.

A fish short-term reproduction assay was performed in which Japanese medaka were exposed to different concentrations of the test substance (2-EHHB) for 21 days. Fish were exposed to one of four different concentrations of the test substance (3.6, 10.9, 33, and 100 µg/L) and dilution water control. Four replicates (exposure vessels, or tanks) were included for each treatment group and the control group. Each tank contained a total of ten fish, five male fish and five female fish, for a total of 20 males and 20 females per treatment group. All fish were impartially assigned to tanks prior to pre-exposure and then were randomly assigned to the exposure treatments within a tank after successful spawning had been established. The experimental design is presented in Table 1.

Table 1. Experimental Design

2-EHHB Treatment Group (µg/L)	Number of Replicates (tanks)	Number of Female Fish per Replicate	Number of Male Fish per Replicate	Total Number of Fish per Treatment Group
0.0 (control)	4	5	5	40
3.6	4	5	5	40
10.9	4	5	5	40
33	4	5	5	40
100	4	5	5	40
	TOTAL	100	100	200

The primary endpoints measured in this study were survival (yes/no), fecundity (the number of eggs per female per reproductive test day), fertilization success (the number of embryos divided by the total number of eggs × 100), female/male papillary processes score, female/male liver vitellogenin (VTG) concentration, female/male gonadal somatic index (GSI), female/male body weight, female/male body length, and female/male gonad weight. Data on gonad, liver, and thyroid histopathology for study termination specimens were also collected.

STATISTICAL METHODS

Statistical analyses were performed consistent with OPPTS 890.1350 (1) and OECD 229 guidelines (2). Preliminary analyses (i.e. descriptive statistics such as means, medians, standard errors of the mean, and percent coefficient of variation) were performed separately on continuous quantitative biological endpoints: fecundity, fertilization success, female/male papillary processes score, female/male VTG, female/male GSI, female/male body weight, female/male body length, and female/male gonad weight. Concentration-response monotonicity was assessed visually via plots of replicate means and the group medians of the replicate means.

To determine if significant differences in mortality existed between treatment groups, survival data were evaluated with the Cochran-Armitage test with survival results pooled across replicates within treatment or control group if data were considered to be monotonic. If monotonicity was not observed, Fisher's Exact test with a Bonferroni-Holm adjustment was performed.

The treatment effect assessment was performed using data from the control group and the four treatment groups. For continuous quantitative endpoints that followed a monotonic concentration-response, the Jonckheere-Terpstra test was applied in a step-down manner. For endpoints that were not consistent with a monotonic concentration-response, the data were evaluated for normality (Shapiro-Wilk's test) and homogeneity of variance (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. The Jonckheere-Terpstra test and Mann-Whitney-Wilcoxon test with a Bonferroni-Holm adjustment to the p-values were performed on replicate medians, and Dunnett's test was performed on replicate means.

Potential statistical outliers were assessed for all treatment and control data for all continuous quantitative endpoints. For selected endpoints (e.g., fecundity and fertilization success), the presence of outliers among the replicate means were determined, while for the other endpoints, outliers were determined among the individual animal measurements. An analysis of variance model was fit to the data separately for each endpoint. Model residuals were estimated for each observation. If the residual value was greater than the median residual plus three times the residual interquartile range (i.e., the difference between the 75^{th} and 25^{th} percentiles), then the observed value was flagged as a potential statistical outlier. Analyses were performed with and without the potential outliers. Analyses in this report present results using all data, while Appendix B contains the results when potential outliers were removed.

Histopathology data took the form of severity scores or developmental stages. A RSCABS (Rao-Scott Cochran-Armitage by Slices) test (3), which uses a step-down Rao-Scott

adjusted Cochran-Armitage trend test on severity slices in a histopathology response, was used to evaluate histopathology data. In the by-slices (BS) process, the severity scores associated with an endpoint are split into two groups based on the score being tested. One group contains all severity scores less than the target score, and the other group contains all severity scores equal to or greater than the target score. Developmental gonad staging data were analyzed as reported to detect acceleration in development and then manually inverted prior to a second analysis to detect a delay in development. Histopathological data for gonad, liver, and thyroid were collected and statistically analyzed using the RSCABS approach, per the study pathologist's recommendation. The analysis was restricted to data from control specimens and specimens selected from the three highest 2-EHHB concentration groups (10.9, 33, and 100 µg/L), to better determine the presence of any exposure-related effects.

The statistical analysis software package SAS® (version 9.4) was used in all statistical analyses on biological data, while the RSCABS analysis on histopathology data was performed using the RSCABS package (version 0.9.2) prepared for the R software package (version 3.4) (4). In the biological data analyses, statistical significance was assessed at the two-sided 0.05 level, while the RSCABS analysis was performed at a one-sided 0.05 level at each slice.

RESULTS

Table 2 summarizes the 21-day survival rate. All fish survived in the study; therefore, no statistical analysis was performed on survival rate data.

Tables 3 through 9 present descriptive statistics (replicate means, number of samples, overall treatment mean, overall treatment median, standard error of the mean, and percent coefficient of variation) by treatment group for fecundity, fertilization success, female and male papillary processes score, female and male VTG, female and male GSI, female and male body weight, female and male body length, and female and male gonad weight, respectively.

Table 10 presents results from the statistical analysis to assess the significance of treatment effects. Fecundity, fertilization success, and male papillary processes score each followed a monotonic concentration-response trend based on a visual assessment using medians of replicate and treatment means. Significant decreasing trends were seen for fecundity across all four treatment groups (3.6, 10.9, 33, and 100 µg/L), for fertilization success across the highest three treatment groups (10.9, 33, and 100 µg/L), and for male papillary processes score across the highest two treatment groups (33 and 100 µg/L).

For the ten endpoints not having a monotonic-concentration response (Table 10), data for male VTG, male GSI, female body length, male body length, and male gonad weight were normally distributed with homogeneous variance, and female GSI and female gonad weight were also normally distributed with homogeneous variance after the data were log transformed. Female VTG and male body weight data were not normally distributed but had homogeneous variance, and female body weight data were not normally distributed and had heterogeneous variance. No normalizing data transformation was deemed sufficient for these three endpoints. For nine of the ten endpoints, the pairwise comparison results showed no significant differences

existed between the treatment groups and control. For male VTG, significant difference from control occurred for both treatment groups 2 and 3 (3.6, and 10.9 µg/L).

Tables 11a through 13b present the RSCABS analysis results performed on histopathology data for the control group and the three treatment groups associated with the highest dose levels of 2-EHHB. Tables 11a and 11b include analyses for female ovary and male testes, Tables 12a and 12b represent female and male liver, and Tables 13a and 13b represent female and male thyroid, respectively. Table 11a shows that for Egg Debrin Oviduct at severity slice 1 and 2, significant treatment effects were seen at the two highest treatment groups (33 and 100 µg/L). For Oocyte Atresia at slice 2, a significant treatment effect was observed at the highest treatment group (100 µg/L). RSCABS analysis was not performed for histopathological endpoints with no findings (all not remarkable). Results from the other RSCABS tables can be interpreted in a similar manner.

Figures A-1 to A-14 in Appendix A present plots showing replicate means as well as the median of the replicate means from each group for the visual assessment of concentration-response monotonicity. As noted earlier, the above statistical analyses were repeated for endpoints having statistical outliers identified, with those outliers removed from analysis. Tables and figures in Appendix B present statistical analyses performed on the data with seven potential statistical outliers removed for five endpoints (fertilization success, female GSI, female body weight, male body weight, and female gonad weight). Table B-1 lists these potential statistical outliers. Table B-2 displays results from the statistical analysis of these five endpoints to assess the significance of treatment effects upon excluding the outliers. There was no change in the statistical significance results upon removing the potential outliers. Figure B-1 to B-5 present the plot of the replicate means as well as the median of the replicate means for the visual assessment of concentration-response monotonicity.

DISCUSSION ON RSCABS ANALYSIS

The RSCABS analysis applied to the histopathology data uses a "step-down" procedure on treatment (concentration) level. In this procedure, if a higher treatment (concentration) was not statistically significant for a specified severity slice, then any lower treatments (concentrations) were inferred to be not significant. For a specified endpoint, the RSCABS summary tables present results only for those severity slices for which specimens existed at that severity level or greater. Then for a given severity slice, only those treatment levels for which specimens exist at that severity level or greater are included in the tables. For example, in Table 12a (Basophilia), no specimens existed with severity level 1 or less in treatment group 1 (0.0 µg/L). (Note that animals in treatment group 2, 3.6 µg/L, were not histopathologically evaluated.) Thus, even though significant treatment effects were seen at group 4 and 5 (33 and 100 µg/L) for severity slice 1, no test was performed at treatment group 3 (10.9 µg/L) for severity slice 1. Due to the general complexity in utilizing RSCABS in statistical analysis for histopathology data, one should use caution when interpreting RSCABS results and should consult with a pathologist if necessary.

STUDY ARCHIVAL

Supporting data and the final report were archived at Battelle.

REFERENCES

1. OPPTS. 890.1350 Fish Short-Term Reproduction Assay, United States Environmental Protection Agency, Washington DC, EPA 740-C-09-007, October 2009.

2. OECD/OCDE 229. OECD Guideline for the Testing of Chemicals. Fish Short Term Reproduction Assay, 2 October 2012.

3. 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.

4. Swintek J. https://cran.r-project.org/web/packages/RSCABS/RSCABS.pdf, April 26, 2017.

Table 2. Summary of 21-Day Survival Rate.

		Female Survival (%)					Male Survival (%)					Overall Survival (%)				
Treatment (µg/L)	Replicate	N	Percent	Mean	SEM	CV (%)	N	Percent	Mean	SEM	CV (%)	N	Percent	Mean	SEM	CV (%)
0.0	A	5	100.0	100.0	0.0	0.0	5	100.0	100.0	0.0	0.0	10	100.0	100.0	0.0	0.0
	B	5	100.0				5	100.0				10	100.0			
	C	5	100.0				5	100.0				10	100.0			
	D	5	100.0				5	100.0				10	100.0			
3.6	A	5	100.0	100.0	0.0	0.0	5	100.0	100.0	0.0	0.0	10	100.0	100.0	0.0	0.0
	B	5	100.0				5	100.0				10	100.0			
	C	5	100.0				5	100.0				10	100.0			
	D	5	100.0				5	100.0				10	100.0			
10.9	A	5	100.0	100.0	0.0	0.0	5	100.0	100.0	0.0	0.0	10	100.0	100.0	0.0	0.0
	B	5	100.0				5	100.0				10	100.0			
	C	5	100.0				5	100.0				10	100.0			
	D	5	100.0				5	100.0				10	100.0			
33	A	5	100.0	100.0	0.0	0.0	5	100.0	100.0	0.0	0.0	10	100.0	100.0	0.0	0.0
	B	5	100.0				5	100.0				10	100.0			
	C	5	100.0				5	100.0				10	100.0			
	D	5	100.0				5	100.0				10	100.0			
100	A	5	100.0	100.0	0.0	0.0	5	100.0	100.0	0.0	0.0	10	100.0	100.0	0.0	0.0
	B	5	100.0				5	100.0				10	100.0			
	C	5	100.0				5	100.0				10	100.0			
	D	5	100.0				5	100.0				10	100.0			

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.
Mean, SEM, and CV (%) calculated from the percent survival estimate for each replicate within treatment (i.e., N = 4).

Table 3. Descriptive Statistics for Fecundity and Fertilization Success.

		Fecundity (Number of Eggs per Female per Day)						Fertilization Success (%)					
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	Median	SEM	CV (%)	N	Replicate Mean	Mean	Median	SEM	CV (%)
0.0	A	5	13.66	13.43	13.48	0.13	1.87	5	95.12	95.35	95.31	0.14	0.29
	B	5	13.56					5	95.51				
	C	5	13.40					5	95.66				
	D	5	13.09					5	95.12				
3.6	A	5	12.90	12.88	12.82	0.11	1.74	5	95.13	95.08	95.10	0.05	0.11
	B	5	12.69					5	95.20				
	C	5	12.73					5	95.06				
	D	5	13.18					5	94.94				
10.9	A	5	11.91	12.25	12.14	0.19	3.16	5	94.88	94.54	94.46	0.12	0.25
	B	5	12.15					5	94.36				
	C	5	12.13					5	94.43				
	D	5	12.81					5	94.50				
33	A	5	6.88	6.29	6.42	0.33	10.48	5	87.53	86.33	86.28	0.49	1.12
	B	5	6.08					5	86.52				
	C	5	6.76					5	85.21				
	D	5	5.46					5	86.04				
100	A	5	4.25	3.96	4.10	0.20	9.98	5	80.27	75.77	74.82	1.62	4.26
	B	5	4.23					5	73.20				
	C	5	3.96					5	75.96				
	D	5	3.40					5	73.67				

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.
Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4).

Table 4. Descriptive Statistics for Papillary Processes Score by Sex.

		Female Papillary Processes Score						Male Papillary Processes Score					
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	Median	SEM	CV (%)	N	Replicate Mean	Mean	Median	SEM	CV (%)
0.0	A	5	0.00	0.00	0.00	0.00	.	5	79.2	83.0	83.5	1.3	3.3
	B	5	0.00					5	85.6				
	C	5	0.00					5	83.8				
	D	5	0.00					5	83.2				
3.6	A	5	0.00	0.00	0.00	0.00	.	5	91.4	82.2	82.2	3.9	9.4
	B	5	0.00					5	84.4				
	C	5	0.00					5	73.0				
	D	5	0.00					5	80.0				
10.9	A	5	0.00	0.00	0.00	0.00	.	5	87.2	79.5	79.6	3.3	8.3
	B	5	0.00					5	81.6				
	C	5	0.00					5	77.6				
	D	5	0.00					5	71.6				
33	A	5	0.00	0.00	0.00	0.00	.	5	64.2	71.1	70.6	4.9	13.7
	B	5	0.00					5	81.6				
	C	5	0.00					5	61.6				
	D	5	0.00					5	77.0				
100	A	5	0.00	0.00	0.00	0.00	.	5	71.4	66.4	65.2	4.4	13.2
	B	5	0.00					5	59.0				
	C	5	0.00					5	59.0				
	D	5	0.00					5	76.2				

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.
Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4).
All female papillary processes scores were 0.

Table 5. Descriptive Statistics Liver Vitellogenin (VTG) by Sex.

		Female VTG [Log 10(copies VTG/ng total RNA]						Male VTG [Log 10(copies VTG/ng total RNA]					
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	Median	SEM	CV (%)	N	Replicate Mean	Mean	Median	SEM	CV (%)
0.0	A	2	3.940	4.500	4.393	0.325	14.456	2	1.500	1.735	1.793	0.080	9.195
	B	2	3.985					2	1.800				
	C	2	5.275					2	1.785				
	D	2	4.800					2	1.855				
3.6	A	2	4.735	5.031	5.028	0.160	6.364	2	2.925	3.418	3.413	0.266	15.595
	B	2	4.775					2	2.990				
	C	2	5.335					2	3.920				
	D	2	5.280					2	3.835				
10.9	A	2	4.895	4.976	4.878	0.137	5.512	2	3.585	3.150	3.288	0.260	16.487
	B	2	4.860					2	3.485				
	C	2	5.380					2	2.440				
	D	2	4.770					2	3.090				
33	A	2	4.230	4.283	4.338	0.385	17.958	2	1.560	2.259	2.053	0.404	35.961
	B	2	5.160					2	2.345				
	C	2	3.295					2	3.370				
	D	2	4.445					2	1.760				
100	A	2	3.510	4.058	3.903	0.337	16.598	2	2.230	1.958	2.155	0.223	22.809
	B	2	3.530					2	1.290				
	C	2	4.915					2	2.145				
	D	2	4.275					2	2.165				

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.
Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4).

Table 6. Descriptive Statistics for Gonadal Somatic Index (GSI%) by Sex.

		Female GSI (%)						Male GSI (%)					
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	Median	SEM	CV (%)	N	Replicate Mean	Mean	Median	SEM	CV (%)
0.0	A	2	6.37	7.05	7.09	0.99	28.22	2	0.55	0.82	0.70	0.17	42.28
	B	2	9.33					2	1.31				
	C	2	4.68					2	0.79				
	D	2	7.81					2	0.62				
3.6	A	2	7.79	9.06	8.37	0.93	20.58	2	0.63	0.89	0.85	0.13	29.31
	B	2	8.96					2	0.91				
	C	2	11.73					2	1.24				
	D	2	7.77					2	0.79				
10.9	A	2	6.65	7.67	6.81	0.96	24.95	2	0.60	0.91	0.94	0.11	25.20
	B	2	10.53					2	1.15				
	C	2	6.96					2	0.94				
	D	2	6.55					2	0.95				
33	A	2	8.84	8.55	8.36	0.57	13.38	2	0.61	0.78	0.73	0.14	35.51
	B	2	10.03					2	1.14				
	C	2	7.45					2	0.86				
	D	2	7.89					2	0.53				
100	A	2	6.31	6.12	5.73	0.89	29.21	2	0.84	0.93	0.94	0.07	14.48
	B	2	5.15					2	1.04				
	C	2	8.54					2	0.80				
	D	2	4.47					2	1.06				

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.
Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4).

Table 7. Descriptive Statistics for Body Weight by Sex.

		Female Body Weight (mg)						Male Body Weight (mg)					
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	Median	SEM	CV (%)	N	Replicate Mean	Mean	Median	SEM	CV (%)
0.0	A	5	388.34	365.91	369.48	10.84	5.93	5	370.24	389.00	387.63	10.93	5.62
	B	5	371.44					5	410.64				
	C	5	367.52					5	405.02				
	D	5	336.34					5	370.10				
3.6	A	5	334.08	378.99	386.60	16.33	8.62	5	419.22	435.45	426.78	16.00	7.35
	B	5	376.94					5	407.66				
	C	5	396.26					5	434.34				
	D	5	408.66					5	480.58				
10.9	A	5	363.48	371.02	371.57	3.50	1.89	5	471.06	416.44	413.89	24.10	11.57
	B	5	377.44					5	366.92				
	C	5	376.48					5	386.32				
	D	5	366.66					5	441.46				
33	A	5	371.68	421.98	420.45	25.03	11.86	5	439.36	402.72	393.38	12.38	6.15
	B	5	453.14					5	393.06				
	C	5	387.76					5	384.76				
	D	5	475.32					5	393.70				
100	A	5	347.12	358.97	353.37	9.00	5.01	5	367.44	357.86	358.19	7.03	3.93
	B	5	344.98					5	348.94				
	C	5	359.62					5	343.00				
	D	5	384.16					5	372.06				

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.
Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4).

Table 8. Descriptive Statistics for Body Length by Sex.

		Female Body Length (mm)						Male Body Length (mm)					
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	Median	SEM	CV (%)	N	Replicate Mean	Mean	Median	SEM	CV (%)
0.0	A	5	25.96	25.80	25.94	0.20	1.57	5	26.70	26.68	26.51	0.32	2.37
	B	5	25.92					5	27.56				
	C	5	26.10					5	26.32				
	D	5	25.20					5	26.14				
3.6	A	5	24.76	26.18	26.23	0.72	5.48	5	27.56	28.05	27.80	0.37	2.61
	B	5	25.12					5	27.50				
	C	5	27.34					5	28.04				
	D	5	27.48					5	29.08				
10.9	A	5	26.58	26.36	26.37	0.10	0.76	5	28.80	27.98	28.09	0.44	3.13
	B	5	26.32					5	27.58				
	C	5	26.42					5	26.94				
	D	5	26.10					5	28.60				
33	A	5	27.20	27.43	27.59	0.81	5.87	5	28.28	27.34	27.56	0.49	3.60
	B	5	27.98					5	27.46				
	C	5	25.34					5	25.96				
	D	5	29.18					5	27.66				
100	A	5	26.80	26.47	26.79	0.34	2.53	5	27.18	26.40	26.65	0.47	3.54
	B	5	25.46					5	25.10				
	C	5	26.78					5	26.96				
	D	5	26.82					5	26.34				

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.
Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4).

Table 9. Descriptive Statistics for Gonad Weight by Sex.

		Female Gonad Weight (mg)						Male Gonad Weight (mg)					
Treatment (µg/L)	Replicate	N	Replicate Mean	Mean	Median	SEM	CV (%)	N	Replicate Mean	Mean	Median	SEM	CV (%)
0.0	A	2	22.15	23.93	25.13	3.49	29.15	2	1.95	2.88	2.50	0.61	42.33
	B	2	30.55					2	4.55				
	C	2	14.90					2	3.00				
	D	2	28.10					2	2.00				
3.6	A	2	24.90	32.10	29.55	4.29	26.72	2	2.25	3.39	3.23	0.54	31.83
	B	2	31.05					2	3.20				
	C	2	44.40					2	4.85				
	D	2	28.05					2	3.25				
10.9	A	2	20.40	25.06	22.53	3.32	26.46	2	2.05	3.23	3.38	0.43	26.87
	B	2	34.80					2	3.55				
	C	2	23.65					2	3.20				
	D	2	21.40					2	4.10				
33	A	2	29.95	28.51	27.93	2.38	16.69	2	2.35	2.61	2.55	0.41	31.23
	B	2	34.50					2	3.65				
	C	2	23.70					2	2.75				
	D	2	25.90					2	1.70				
100	A	2	19.90	19.53	17.90	2.95	30.21	2	2.65	2.96	3.00	0.23	15.24
	B	2	15.90					2	3.35				
	C	2	27.70					2	2.50				
	D	2	14.60					2	3.35				

SEM Standard error of the mean.
CV(%) Coefficient of variation = (standard deviation / mean) × 100.
Mean, SEM, and CV (%) calculated from the replicate means for each treatment (i.e., N = 4).

Table 10. Statistical Analysis Results

Endpoints	Monotonicity Assessment[1]	Normality Test[2]	Homogeneity of Variance Test[3]	Jonckheere-Terpstra Test[4] (p-value)	Significant Pairwise Comparisons to Control[5] (p-value)
Fecundity	Monotonic[6]	NP	NP	Group 5 (<0.0001) Group 4 (<0.0001) Group 3 (0.0021) Group 2 (0.0433)	NP
Fertilization Success	Monotonic[6]	NP	NP	Group 5 (<0.0001) Group 4 (<0.0001) Group 3 (0.0034)	NP
Female Papillary Processes Score[7]	NP	NP	NP	NP	NP
Male Papillary Processes Score	Monotonic[6]	NP	NP	Group 5 (0.0012) Group 4 (0.0394)	NP
Female VTG[8]	Non-Monotonic	Non-Normal	Homogeneity	NP	NS
Male VTG[8]	Non-Monotonic	Normal	Homogeneity	NP	Group 3 (0.0069) Group 2 (0.0017)
Female GSI	Non-Monotonic	Log-Normal[9]	Log-Homogeneity[10]	NP	NS
Male GSI	Non-Monotonic	Normal	Homogeneity	NP	NS
Female Body Weight (mg)	Non-Monotonic	Non-Normal	Heterogeneity	NP	NS
Male Body Weight (mg)	Non-Monotonic	Non-Normal	Homogeneity	NP	NS
Female Body Length (mm)	Non-Monotonic	Normal	Homogeneity	NP	NS
Male Body Length (mm)	Non-Monotonic	Normal	Homogeneity	NP	NS
Female Gonad Weight (mg)	Non-Monotonic	Log-Normal[9]	Log-Homogeneity[10]	NP	NS
Male Gonad Weight (mg)	Non-Monotonic	Normal	Homogeneity	NP	NS

1. Monotonicity was assessed visually from the replicate means and the median of the replicate means.
2. Shapiro-Wilk test for normality.
3. Levene's test for homogeneity of variance.
4. Jonckheere-Terpstra step-down trend test was performed on monotonic concentration-response data. Only statistically significant treatment trends were listed.
5. Control is group 1 and test concentration groups are groups 2 to 5 (ordering from 3.6 to 100 µg/L). 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, non-normal, and heterogeneous variance data), Mann-Whitney-Wilcoxon test was used in the pairwise comparisons to control with Bonferroni-Holm multiple comparison adjustment.
6. Monotonic decreasing.
7. All female papillary processes scores were 0. Thus, no analysis was performed.
8. The unit for both female and male VTG was Log10(copies VTG)/ng total RNA.
9. Normal when log-transformed.
10. Homogeneous when log-transformed.

NP Statistical test or comparison was not performed.

NS No statistically significant differences were found for Jonckheere-Terpstra step-down trend test, Dunnett's test, or Mann-Whitney-Wilcoxon test with a Bonferroni-Holm adjustment.

Table 11a. Summary of RSCABS Results (p-values) for Female Ovary.

Endpoint	Severity(1)	Treatment(2)	Test Statistics	P-Value	Significant
Egg Debrin Oviduct	1	5	-1.9382	0.0263	*
	1	4	-1.7823	0.0374	*
	1	3	-1.4771	0.0698	
	2	5	-1.9382	0.0263	*
	2	4	-1.7823	0.0374	*
	2	3	-1.4771	0.0698	
	3	5	-1.3368	0.0906	
Gonadal Staging Overall(3)	2	5	-1.2789	0.1005	
	3	5	-0.6567	0.2557	
	4	5	0.8207	0.2059	
Inflammation, Granulomatous	1	5	-0.7615	0.2232	
	2	5	-0.9545	0.1699	
	3	5	-1.3368	0.0906	
Oocyte Atresia	1	5	1.4892	0.0682	
	2	5	2.4328	0.0075	*
	2	4	1.3060	0.0958	
	3	5	1.3209	0.0933	
	4	5	0.4541	0.3249	
Oviduct, Fibrovascular Proliferation	1	5	0.4541	0.3249	
	2	5	0.4541	0.3249	
	3	5	0.4379	0.3307	
Perifollicular Cell Hyperplasia-Hypertrophy	1	5	0.4379	0.3307	
Post-Ovulatory Follicles	1	5	-0.7264	0.2338	
	2	5	0.0000	0.5000	
Post-Ovulatory Follicles, Vacuolation	1	5	1.2907	0.0984	
Seminoma, Malignant	1	5	0.0000	0.5000	

Severity grade: Grade 0 = not remarkable, Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, Grade 4 = severe.

* Statistically significant treatment effect at the 0.05 level.

(1) Severity in the table indicates severity slices: 1 = severity grade 1 or greater vs severity grade 0; 2 = severity grade 2 or greater vs severity grades 0 and 1; 3 = severity grade 3 or greater vs severity grades 0, 1, and 2; etc. This table reports those severity slices for which there were observed results at that severity grade or greater.

(2) For a given severity slice, treatment levels for which there were no pathological findings were not included in table. Only control and the 3 highest treatment groups 3, 4, and 5 (10.9, 33, and 100 µg/L) were evaluated.
5=treatment groups 5 (100 µg/L), 4 (33 µg/L), 3 (10.9 µg/L), and 1 (0.0 µg/L) were included in the RSCABS analysis.
4=treatment groups 4 (33 µg/L), 3 (10.9 µg/L), and 1 (0.0 µg/L) were included in the RSCABS analysis.
3=treatment groups 3 (10.9 µg/L) and 1 (0.0 µg/L) were included in the RSCABS analysis.

(3) For female ovary staging, severity grade 0 = undeveloped, severity grade 1 = early development, severity grade 2 = mid-development, severity grade 3 = late development, severity grade 4 = late development/hydrated, severity grade 5 = Post-ovulatory.

Table 11b. Summary of RSCABS Results (p-values) for Male Testes.

Endpoint	Severity[1]	Treatment[2]	Test Statistics	P-Value	Significant
Dysgerminoma	1	5	1.3368	0.0906	
Gonad Phenotype Score[3]	1	5	0.0000	0.5000	
Gonadal Staging Overall[4]	1	5	0.0000	0.5000	
	2	5	-1.0339	0.1506	
Increased Spermatagonia	1	5	-0.2078	0.4177	
Interstitial Cells, Increased	1	5	1.0339	0.1506	
Seminoma, Benign	1	5	0.8359	0.2016	
Testicular Degeneration	1	5	0.0000	0.5000	
	2	5	-0.5492	0.2914	

Severity grade: Grade 0 = not remarkable, Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, Grade 4 = severe.

(1) Severity in the table indicates severity slices: 1 means severity grade 1 or greater vs severity grade 0; 2 means severity grade 2 or greater vs severity grades 0 and 1; 3 means severity grade 3 or greater vs severity grades 0, 1, and 2; and so on. Reporting in table was those severity slices for which there were observed results at that severity grade or greater.

(2) For a given severity slice, treatment levels for which there were no pathological findings were not included in table. Only control and the 3 highest treatment groups 3, 4, and 5 (10.9, 33, and 100 µg/L) were evaluated. 5=treatment groups 5 (100 µg/L), 4 (33 µg/L), 3 (10.9 µg/L), and 1 (0.0 µg/L) were included in the RSCABS analysis.

(3) For gonad phenotype score, severity grade 1 = testicular tissue entirely, severity grade 2 = testicular tissue predominates, severity grade 3 = amounts of testicular and ovarian tissue, severity grade 4 = ovarian tissue predominates, severity grade 5 = ovarian tissue entirely.

(4) For male testes staging, severity grade 0 = undeveloped, severity grade 1 = early spermatogenic, severity grade 2 = mid-spermatogenic, severity grade 3 = late spermatogenic, severity grade 4 = spent.

Table 12a. Summary of RSCABS Results (p-values) for Female Liver.

Endpoint	Severity[1]	Treatment[2]	Test Statistics	P-Value	Significant
Basophilia	1	5	-1.7078	0.0438	*
	1	4	-3.2863	0.0005	*
	2	5	-3.1415	0.0008	*
	2	4	-3.7477	0.0001	*
	2	3	-1.0205	0.1537	
	3	5	-2.0645	0.0195	*
	3	4	-2.8835	0.0020	*
	3	3	-0.8018	0.2113	
Cystic Degeneration	1	5	-1.5550	0.0600	
	2	5	-1.6100	0.0537	
	3	5	-1.2907	0.0984	
Cytoplasmic Inclusions, Eosinophilic	1	5	-0.7875	0.2155	
Focus of Cellular Alteration	1	5	1.8712	0.0307	*
	1	4	1.2318	0.1090	
	2	5	1.9382	0.0263	*
	3	5	1.9382	0.0263	*
Inflammation, Granulomatous	1	5	-1.3467	0.0890	
	2	5	-0.4379	0.3307	
Lymphoma Leukemia	1	5	0.4379	0.3307	
Pigmented Macrophage Aggregates	1	5	0.0000	0.5000	
	2	5	-1.8712	0.0307	*
	2	4	-1.4771	0.0698	
	3	5	-1.3368	0.0906	
Single Cell Necrosis	1	5	-1.4072	0.0797	
Vacuolation	1	5	0.6311	0.2640	
	2	5	2.7720	0.0028	*
	2	4	2.1743	0.0148	*
	2	3	0.5999	0.2743	
	3	5	1.9994	0.0228	*
	3	4	1.9148	0.0278	*
	3	3	-1.0205	0.1537	

Severity grade: Grade 0 = not remarkable, Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, Grade 4 = severe.

* Statistically significant treatment effect at the 0.05 level.

(1) Severity in the table indicates severity slices: 1 means severity grade 1 or greater vs severity grade 0; 2 means severity grade 2 or greater vs severity grades 0 and 1; 3 means severity grade 3 or greater vs severity grades 0, 1, and 2; and so on. Reporting in table was those severity slices for which there were observed results at that severity grade or greater.

(2) For a given severity slice, treatment levels for which there were no pathological findings were not included in table. Only control and the 3 highest treatment groups 3, 4, and 5 (10.9, 33, and 100 µg/L) were evaluated.
5=treatment groups 5 (100 µg/L), 4 (33 µg/L), 3 (10.9 µg/L), and 1 (0.0 µg/L) were included in the RSCABS analysis.
4=treatment groups 4 (33 µg/L), 3 (10.9 µg/L), and 1 (0.0 µg/L) were included in the RSCABS analysis.
3=treatment groups 3 (10.9 µg/L) and 1 (0.0 µg/L) were included in the RSCABS analysis.

Table 12b. Summary of RSCABS Results (p-values) for Male Liver.

Endpoint	Severity[1]	Treatment[2]	Test Statistics	P-Value	Significant
Basophilia	1	5	1.3368	0.0906	
Bile Pigment	1	5	-1.3368	0.0906	
Cystic Degeneration	1	5	0.2785	0.3903	
	2	5	1.8593	0.0315	*
	2	4	-2.0800	0.0188	*
	2	3	-1.8208	0.0343	*
Cytoplasmic Inclusions, Eosinophilic	1	5	-1.9219	0.0273	*
	1	4	-2.5981	0.0047	*
	1	3	-2.1909	0.0142	*
	2	5	-1.3368	0.0906	
Inflammation, Granulomatous	1	5	1.9219	0.0273	*
	1	4	-1.2318	0.1090	
	2	5	2.8026	0.0025	*
	3	5	1.9382	0.0263	*
Pigmented Macrophage Aggregates	1	5	1.1852	0.1180	
Vacuolation	1	5	1.3571	0.0874	
	2	5	1.3368	0.0906	

Severity grade: Grade 0 = not remarkable, Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, Grade 4 = severe.

* Statistically significant treatment effect at the 0.05 level.

(1) Severity in the table indicates severity slices: 1 means severity grade 1 or greater vs severity grade 0; 2 means severity grade 2 or greater vs severity grades 0 and 1; 3 means severity grade 3 or greater vs severity grades 0, 1, and 2; and so on. Reporting in table was those severity slices for which there were observed results at that severity grade or greater.

(2) For a given severity slice, treatment levels for which there were no pathological findings were not included in table. Only control and the 3 highest treatment groups 3, 4, and 5 (10.9, 33, and 100 µg/L) were evaluated.
5=treatment groups 5 (100 µg/L), 4 (33 µg/L), 3 (10.9 µg/L), and 1 (0.0 µg/L) were included in the RSCABS analysis.
4=treatment groups 4 (33 µg/L), 3 (10.9 µg/L), and 1 (0.0 µg/L) were included in the RSCABS analysis.
3=treatment groups 3 (10.9 µg/L) and 1 (0.0 µg/L) were included in the RSCABS analysis.

Table 13a. Summary of RSCABS Results (p-values) for Female Thyroid.

Endpoint	Severity[1]	Treatment[2]	Test Statistics	P-Value	Significant
Follicular Cell Hyperplasia	1	5	1.3368	0.0906	
Follicular Cells, Hyaline Degeneration	1	5	-1.9382	0.0263	*
	1	4	-1.7823	0.0374	*
	1	3	-1.4771	0.0698	
	2	5	-1.3368	0.0906	

Severity grade: Grade 0 = not remarkable, Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, Grade 4 = severe.

* Statistically significant treatment effect at the 0.05 level.

(1) Severity in the table indicates severity slices: 1 means severity grade 1 or greater vs severity grade 0; 2 means severity grade 2 or greater vs severity grades 0 and 1; 3 means severity grade 3 or greater vs severity grades 0, 1, and 2; and so on. Reporting in table was those severity slices for which there were observed results at that severity grade or greater.

(2) For a given severity slice, treatment levels for which there were no pathological findings were not included in table. Only control and the 3 highest treatment groups 3, 4, and 5 (10.9, 33, and 100 µg/L) were evaluated.
5=treatment groups 5 (100 µg/L), 4 (33 µg/L), 3 (10.9 µg/L), and 1 (0.0 µg/L) were included in the RSCABS analysis.
4=treatment groups 4 (33 µg/L), 3 (10.9 µg/L), and 1 (0.0 µg/L) were included in the RSCABS analysis.
3=treatment groups 3 (10.9 µg/L) and 1 (0.0 µg/L) were included in the RSCABS analysis.

Table 13b. Summary of RSCABS Results (p-values) for Male Thyroid.

Endpoint	Severity[1]	Treatment[2]	Test Statistics	P-Value	Significant
Colloid, Inspissated	1	5	0.5738	0.2831	
	2	5	-0.6461	0.2591	
Follicle Size, Decreased	1	5	0.0000	0.5000	
	2	5	-0.6450	0.2595	
Follicular Cell Hyperplasia	2	5	0.0918	0.4634	
Follicular Cell Hypertrophy	1	5	-1.2907	0.0984	
	2	5	1.0347	0.1504	
Follicular Cells, Hyaline Degeneration	1	5	-1.5336	0.0626	
	2	5	-0.9145	0.1802	

Severity grade: Grade 0 = not remarkable, Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, Grade 4 = severe.

* Statistically significant treatment effect at the 0.05 level.

(1) Severity in the table indicates severity slices: 1 means severity grade 1 or greater vs severity grade 0; 2 means severity grade 2 or greater vs severity grades 0 and 1; 3 means severity grade 3 or greater vs severity grades 0, 1, and 2; and so on. Reporting in table was those severity slices for which there were observed results at that severity grade or greater.

(2) For a given severity slice, treatment levels for which there were no pathological findings were not included in table. Only control and the 3 highest treatment groups 3, 4, and 5 (10.9, 33, and 100 µg/L) were evaluated. 5=treatment groups 5 (100 µg/L), 4 (33 µg/L), 3 (10.9 µg/L), and 1 (0.0 µg/L) were included in the RSCABS analysis.

Appendix A

Plots Used for the Visual Assessment of Concentration-Response Monotonicity

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FEL Study No. BATT01-00404 (FSTRA #404)

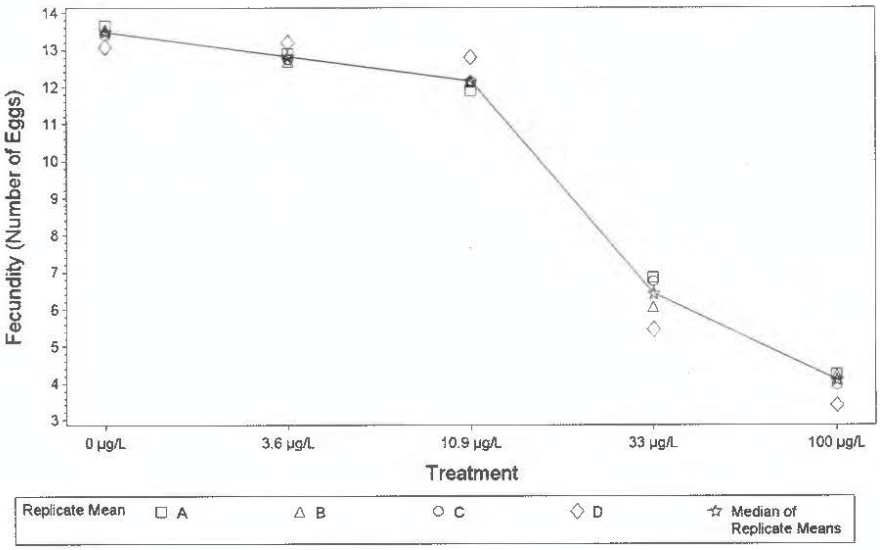

Figure A-1. Replicate Means and Median of Replicate Means for Fecundity.

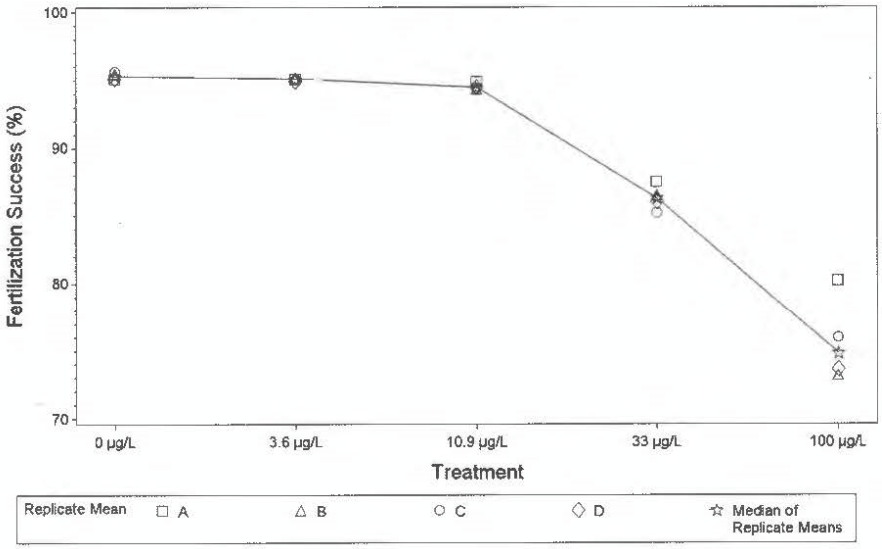

Figure A-2. Replicate Means and Median of Replicate Means for Fertilization Success (%).

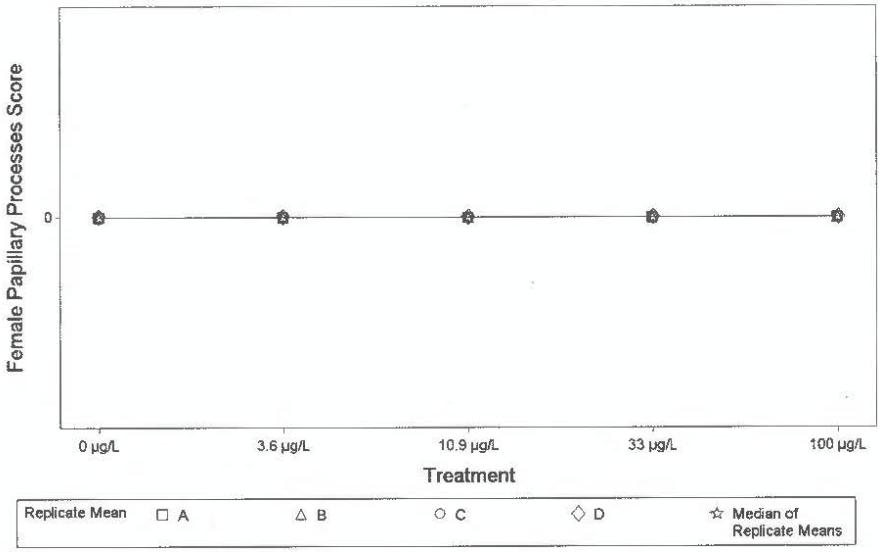

Figure A-3. Replicate Means and Median of Replicate Means for Female Papillae Processes Score.

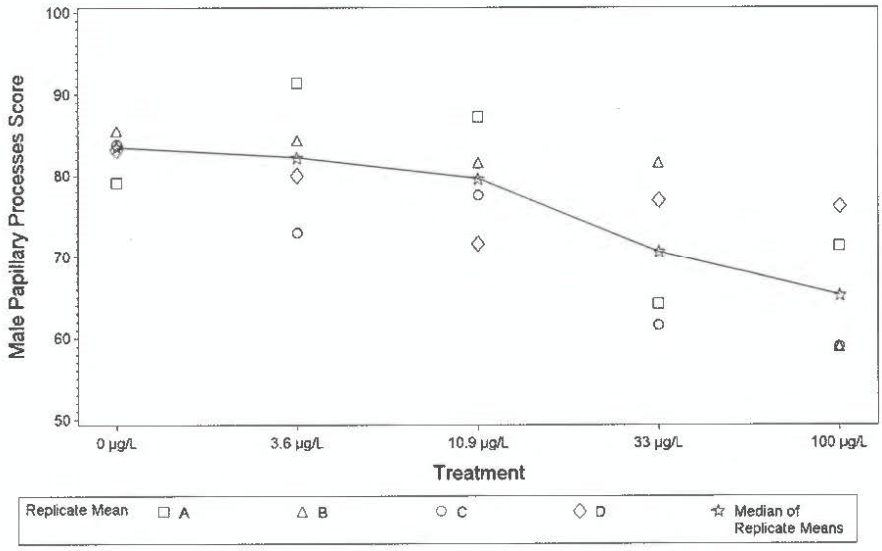

Figure A-4. Replicate Means and Median of Replicate Means for Male Papillae Process Score.

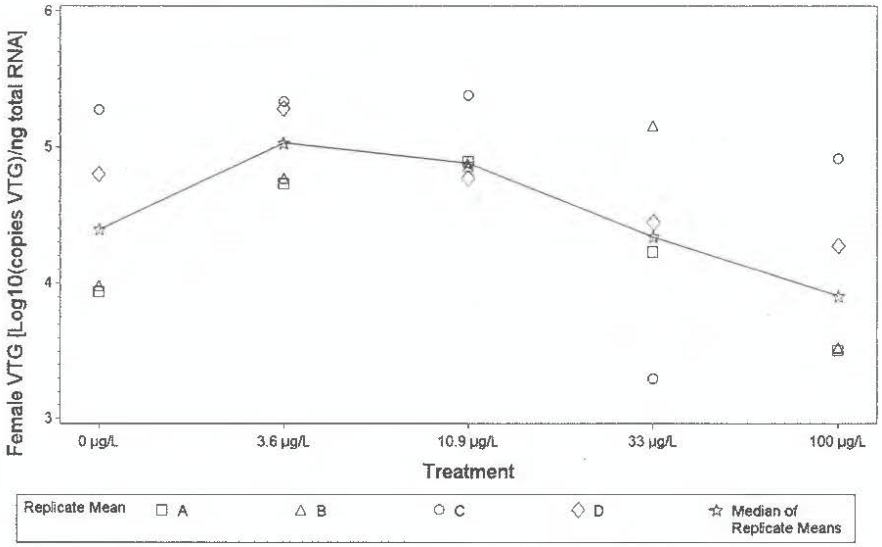

Figure A-5. Replicate Means and Median of Replicate Means for Female VTG (Log 10 (copies VTG/ng total RNA)).

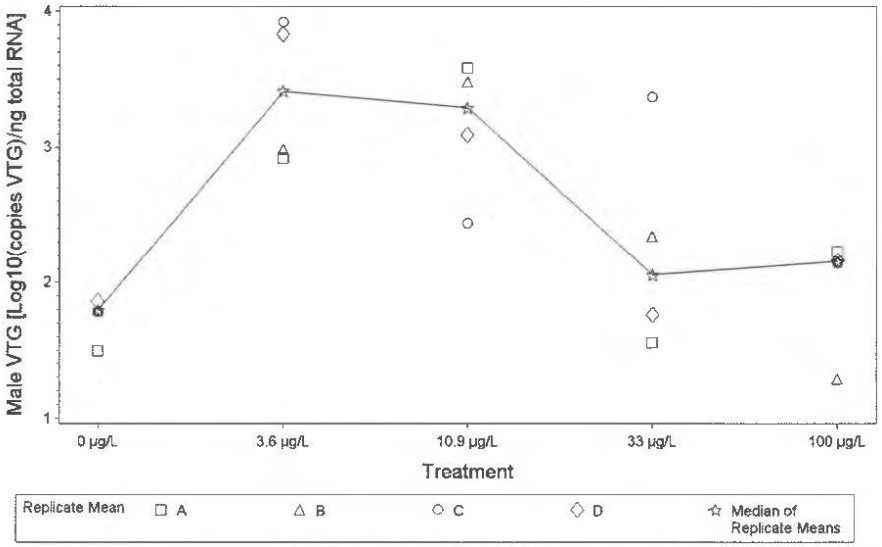

Figure A-6. Replicate Means and Median of Replicate Means for Male VTG (Log 10 (copies VTG/ng total RNA)).

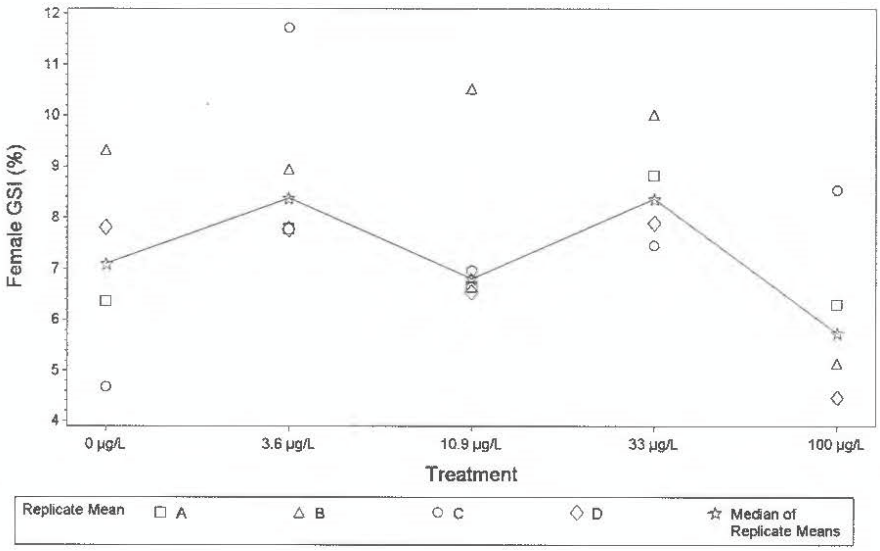

Figure A-7. Replicate Means and Median of Replicate Means for Female GSI (%).

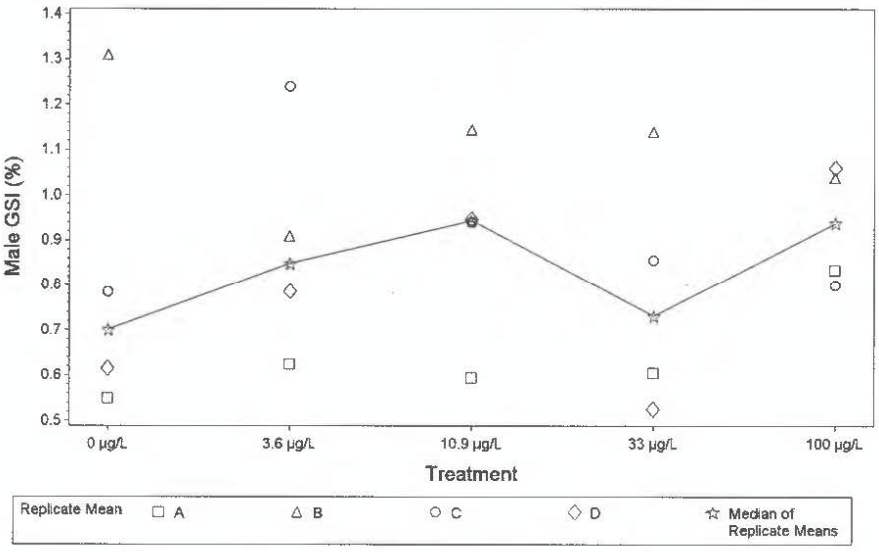

Figure A-8. Replicate Means and Median of Replicate Means for Male GSI (%).

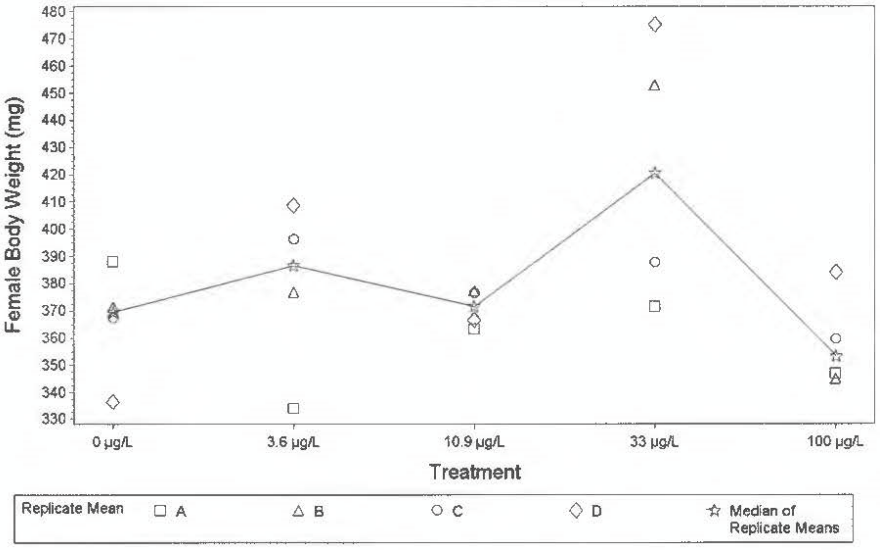

Figure A-9. Replicate Means and Median of Replicate Means for Female Body Weight (mg).

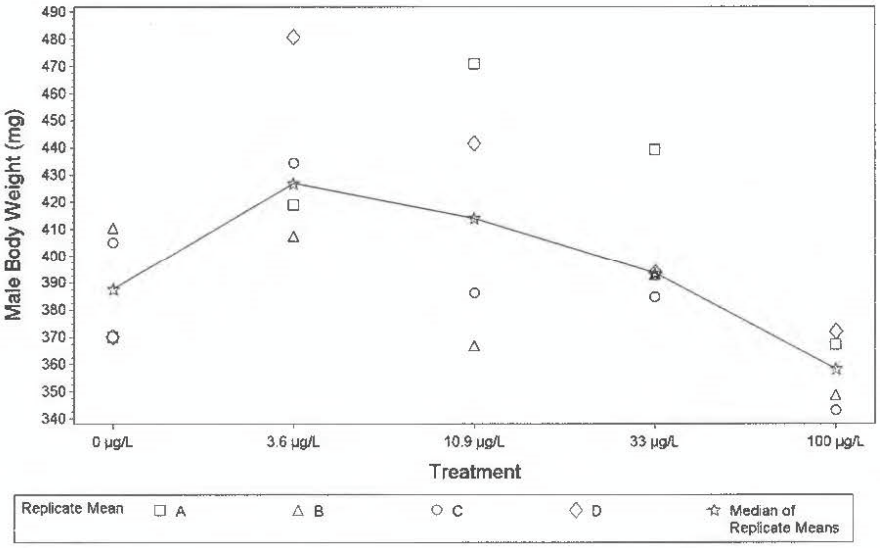

Figure A-10. Replicate Means and Median of replicate means for Male Body Weight (mg).

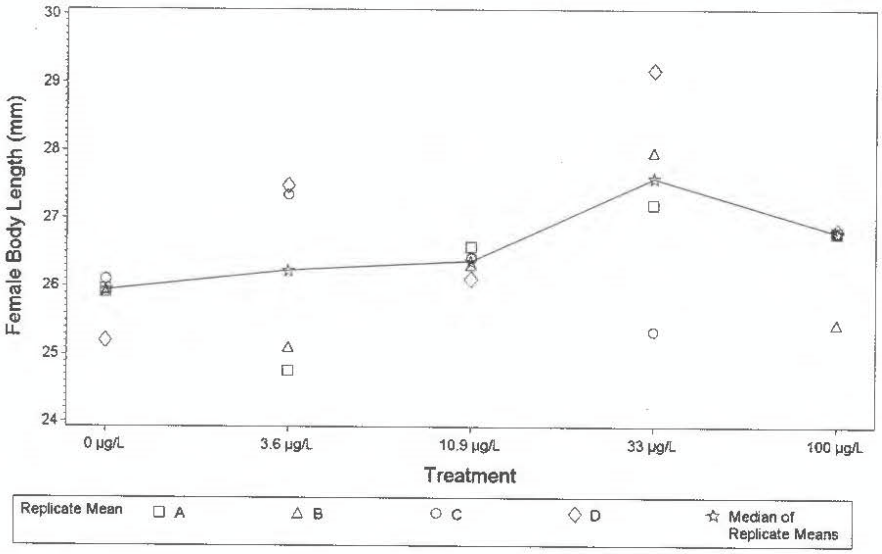

Figure A-11. Replicate Means and Median of Replicate Means for Female Body Length (mg).

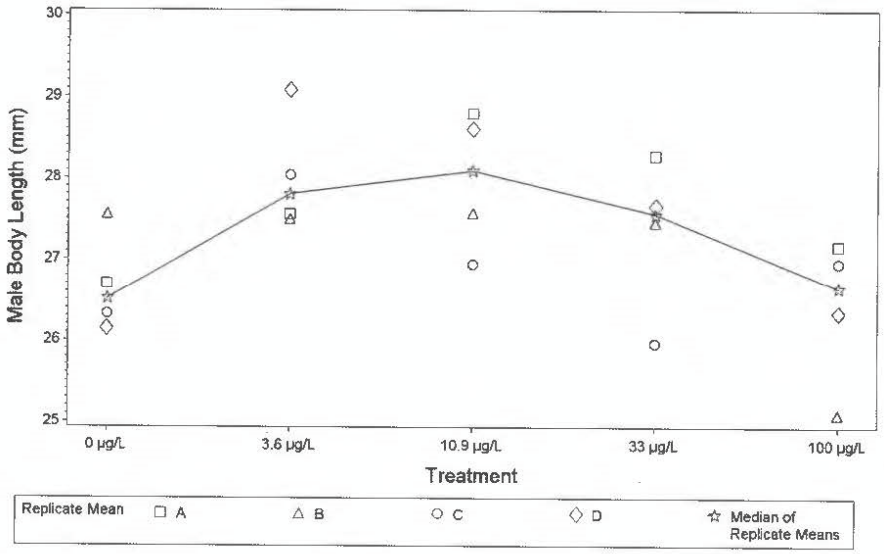

Figure A-12. Replicate Means and Median of Replicate Means for Male Body Length (mm).

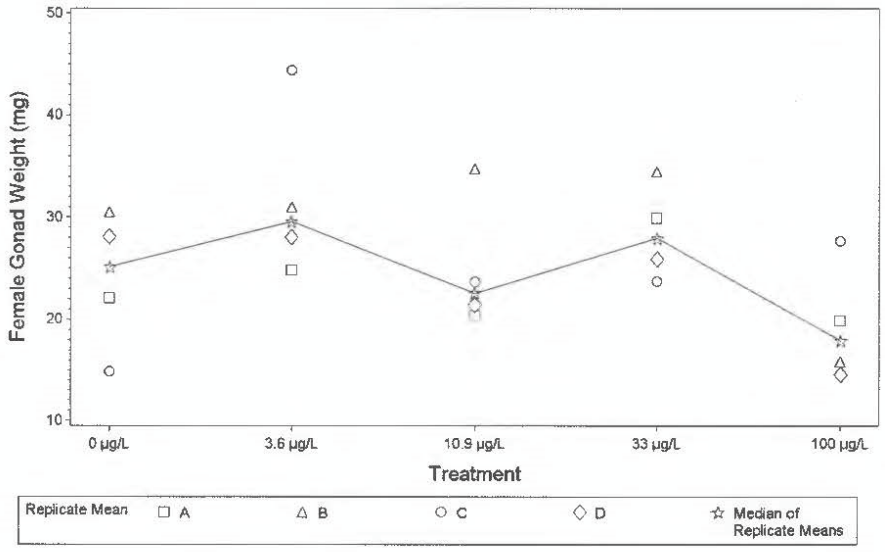

Figure A-13. Replicate Means and Median of Replicate Means for Female Gonad Weight (mg).

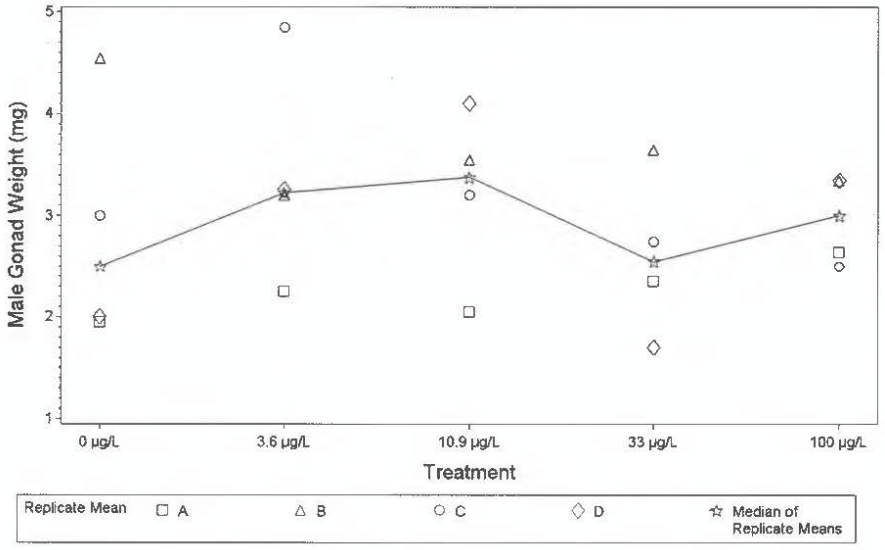

Figure A-14. Replicate Means and Median of Replicate Means for Male Gonad Weight (mg).

Appendix B

Statistical Analyses Performed on Data with Potential Statistical Outliers Removed

USEPA Contract No.: EP-W-11-063, TO 17
FEL Study No. BATT01-00404 (FSTRA #404)

Table B-1. Potential Statistical Outliers.

Endpoint	Treatment (µg/L)	Replicate	Observed Value	Predicted Value	Residual
Fertilization Success	100	A	80.269	75.775	4.494
Female GSI	0	B	13.040	7.046	5.994
Female GSI	10.9	B	14.070	7.671	6.399
Female GSI	33	B	14.510	8.551	5.959
Female Body Weight (mg)	33	B	663.300	421.975	241.325
Male Body Weight (mg)	10.9	A	679.200	416.440	262.760
Female Gonad Weight (mg)	10.9	B	46.300	25.063	21.237

Note: The potential statistical outlier for fertilization success was calculated at replicate level. For other four endpoints (female GSI, female body weight, male body weight, female gonad weight), the potential statistical outliers were calculated at the individual animal level.

Table B-2. Statistical Analysis Results, Potential Statistical Outliers Removed.

Endpoint	Monotonicity Assessment[1]	Normality Test[2]	Homogeneity of Variance Test[3]	Jonckheere-Terpstra Test[4] (p-value)	Significant Pairwise Comparisons to Control[5] (p-value)
Fertilization Success	Monotonic[6]	NP	NP	Group 5 (<0.0001) Group 4 (<0.0001) Group 3 (0.0034)	NP
Female GSI	Non-Monotonic	Normal	Homogeneity	NP	NS
Female Body Weight (mg)	Non-Monotonic	Non-Normal	Heterogeneity	NP	NS
Male Body Weight (mg)	Non-Monotonic	Non-Normal	Homogeneity	NP	NS
Female Gonad Weight (mg)	Non-Monotonic	Log-Normal[7]	Log-Homogeneity[8]	NP	NS

1. Monotonicity was assessed visually from the replicate means and the median of the replicate means.
2. Shapiro-Wilk test for normality.
3. Levene's test for homogeneity of variance.
4. Jonckheere-Terpstra step-down test was performed on monotonic concentration-response data. Only statistically significant treatment trends were listed.
5. Control is group 1 and test concentration groups are groups 2 to 5 (ordering from 3.6 to 100 µg/L). 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, non-normal, and heterogeneous variance data), Mann-Whitney-Wilcoxon test was used in the pairwise comparisons to control with Bonferroni-Holm multiple comparison adjustment.
6. Monotonic decreasing.
7. Normal when log-transformed.
8. Homogeneous when log-transformed.

NP Statistical test or comparison was not performed.

NS No statistically significant differences were found for Jonckheere-Terpstra step-down trend test, Dunnett's test, or Mann-Whitney-Wilcoxon test with a Bonferroni-Holm adjustment.

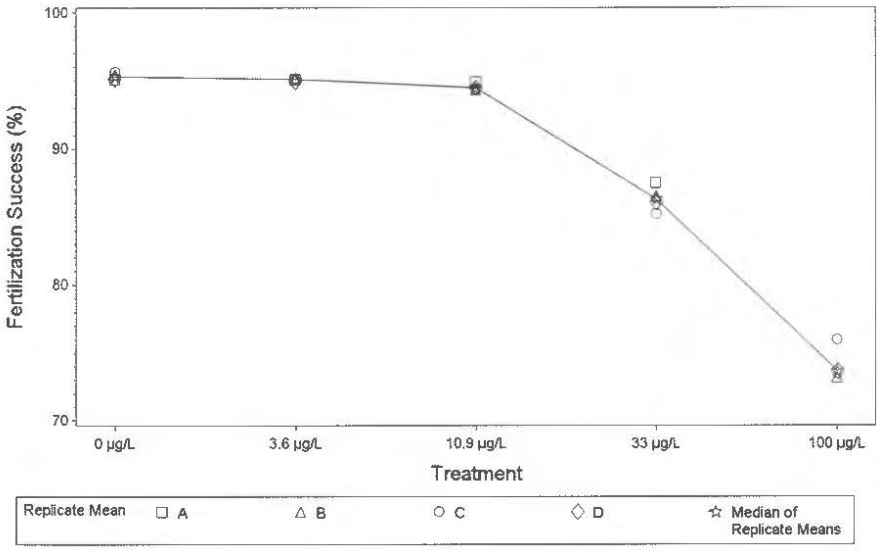

Figure B-1. Replicate Means and Median of Replicate Means for Fertilization Success (%).

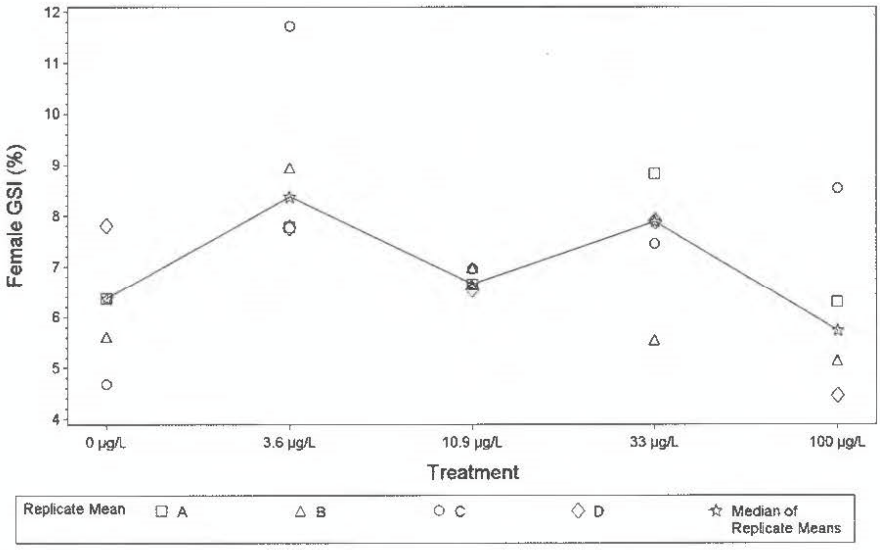

Figure B-2. Replicate Means and Median of Replicate Means for Female GSI (%).

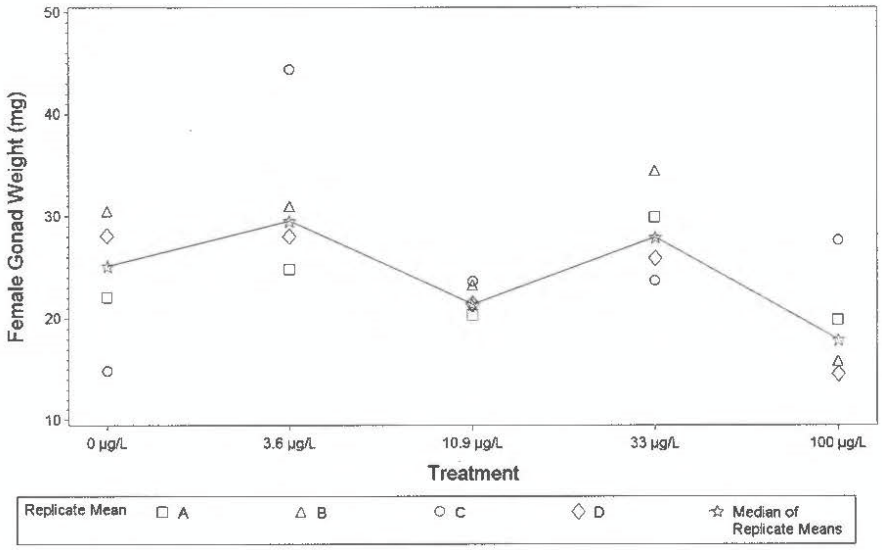

Figure B-5. Replicate Means and Median of Replicate Means for Female Gonad Weight (mg).

Appendix H. 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 - FSTRA - O. Latipes

Analysis Date	Tech Initials	Sample ID	Temp (C)	pH (su)	DO (mg/L)
10/03/16	DJF	Control Renewal	25.3	7.4	7.4
10/03/16	DJF	0.001 mg/L Renewal	25.3	7.2	7.3
10/03/16	DJF	0.01 mg/L Renewal	25.4	7.3	7.3
10/03/16	DJF	0.1 mg/L Renewal	25.4	7.3	7.2
10/03/16	DJF	1.0 mg/L Renewal	25.5	7.3	7.1
10/03/16	DJF	5.0 mg/L Renewal	25.5	7.4	7.3
10/04/16	DJF	Control Spent	25.4	7.2	6.7
10/04/16	DJF	0.001 mg/L Spent	25.4	7.3	6.5
10/04/16	DJF	0.01 mg/L Spent	25.4	7.4	6.3
10/04/16	DJF	0.1 mg/L Spent	25.6	7.4	6.3
10/04/16	DJF	1.0 mg/L Spent	25.5	7.3	6.2
10/04/16	DJF	5.0 mg/L Spent	25.6	7.2	5.7
10/04/16	DJF	Control Renewal	25.4	7.3	7.0
10/04/16	DJF	0.001 mg/L Renewal	25.5	7.3	6.9
10/04/16	DJF	0.01 mg/L Renewal	25.5	7.5	6.9
10/04/16	DJF	0.1 mg/L Renewal	25.6	7.4	7.0
10/04/16	DJF	1.0 mg/L Renewal	25.5	7.4	7.1
10/05/16	DJF	Control Spent	25.4	7.3	6.6
10/05/16	DJF	0.001 mg/L Spent	25.7	7.4	6.3
10/05/16	DJF	0.01 mg/L Spent	25.6	7.2	6.3
10/05/16	DJF	0.1 mg/L Spent	25.6	7.5	6.0
10/05/16	DJF	1.0 mg/L Spent	25.6	7.5	5.4
10/05/16	DJF	Control Renewal	25.4	7.3	6.9
10/05/16	DJF	0.001 mg/L Renewal	25.6	7.3	7.0
10/05/16	DJF	0.01 mg/L Renewal	25.3	7.4	7.0
10/05/16	DJF	0.1 mg/L Renewal	25.4	7.4	7.1
10/06/16	DJF	Control Spent	25.5	7.2	6.1
10/06/16	DJF	0.001 mg/L Spent	25.5	7.3	6.2
10/06/16	DJF	0.01 mg/L Spent	25.4	7.3	6.0
10/06/16	DJF	0.1 mg/L Spent	25.6	7.4	5.6
10/06/16	DJF	Control Renewal	25.5	7.4	7.4
10/06/16	DJF	0.001 mg/L Renewal	25.6	7.3	7.3
10/06/16	DJF	0.01 mg/L Renewal	25.5	7.3	7.0
10/06/16	DJF	0.1 mg/L Renewal	25.4	7.2	7.1
10/07/16	DJF	Control Spent	25.5	7.2	6.2
10/07/16	DJF	0.001 mg/L Spent	25.6	7.3	6.0
10/07/16	DJF	0.01 mg/L Spent	25.6	7.4	6.0
10/07/16	DJF	0.1 mg/L Spent	25.5	7.4	5.5
10/07/16	DJF	Dechlor Water	-	7.5	-

FEL

CULTURE SURVIVAL/STAGE DATA SHEET

Client/Project-WO No: **BATT01-00385** Test Type: **FSTRA RF**

Sample No. / ID: **2-EHHB** Test No.: **1** Species: ***O. Latipes***

	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.:	10	10	10	10	10	10	
Test Day	**Test Date**	**Tech Init.**	**No. Survived (Rep A / Rep B)**					**Observations**
0	10/03/16	DJF	5 / 5	5 / 5	5 / 5	5 / 5	5 / 5	5 / 5	Normal behaving and no abnormalities in Control - 5.0 mg/L
1	10/04/16	DJF	5 / 5	5 / 5	5 / 5	5 / 5	2 / 1	0 / 0	Normal behaving and no abnormalities in Control - 1.0 mg/L
2	10/05/16	DJF	5 / 5	5 / 5	5 / 5	5 / 5	0 / 0	0 / 0	Normal behaving and no abnormalities in Control - 0.1 mg/L
3	10/06/16	DJF	5 / 5	5 / 5	5 / 5	5 / 5	0 / 0	0 / 0	Normal behaving and no abnormalities in Control - 0.1 mg/L
4	10/07/16	DJF	5 / 5	5 / 5	5 / 5	5 / 5	0 / 0	0 / 0	Normal behaving and no abnormalities in Control - 0.1 mg/L