Appendix A
WA 3-11 Quality Assurance Statement

Quality Assurance Statement

The overall objectives of the Quality Assurance Unit (QAU) are to ensure that all laboratory procedures performed at Fort Environmental Laboratories, Inc. (FEL) meet the highest standards set forth in the FEL Quality Management Program (QMP). Specifically, the goals of the QAU are to ensure the accuracy and precision of laboratory operations, reports, and written work projects generated by FEL. These objectives are achieved through routine technical systems audits (TSAs) of personnel training records, equipment records, standard operating procedures, study protocols, and study implementation; audits of data quality (ADQs) of generated data; and audits of project reports.

Documents, data, and the final report generated by FEL for Work Assignment (WA) 3-11, Amphibian Metamorphosis Assay Demonstration Study, have been reviewed by our QAU and meet the criteria set forth in USEPA's Endocrine Disruptor Screening Program (EDSP) QMP and the following study specific guidance documents: Quality Assurance Project Plan (QAPP), Study Plan, and Study Protocol. Although it was not requested that this study meet the requirements of Good Laboratory Practice (GLP) (40 CFR Part 160, July 1, 1999), the study was conducted in accordance with the principles set forth in the GLP regulations. QA documentation for this study, including; QAU inspection schedule, TSAs, ADQs, and the study completion form, are on file at FEL.

Robert Rogers Date Signed:_____
Quality Assurance Manager

Douglas J. Fort Date Signed:_____

Study Director

Appendix B
WA 3-11 / WA 5-8 Study Guidance Documents:

WA 3-11 Study Plan / Protocol with Amendments

WA 3-11 QAPP with Amendments

WA 5-8 Study Plan

WA 5-8 QAPP

Teleconference Records

EPA Technical Directives

Protocol Deviations

Study Guidance Documents:

WA 3-11 Study Plan / Protocol with Amendments

FINAL STUDY PLAN

ON

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 3-11, TASK 3

JANUARY 7, 2004

Prepared for

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1.0 INTRODUCTION

A number of man-made environmental pollutants have the potential to interfere with endocrine function (1-6), and there is evidence of reproductive or endocrine dysfunction in wildlife species that have been exposed environmentally to such endocrine-disrupting contaminants (EDCs) (7-13). While there have been recent reports of alterations of the stress hormone axis in amphibians (14,15), there is little or no published evidence of reproductive dysfunction or developmental effects in this group as a result of exposure to EDCs. A recent special report on environmental endocrine disruption by the U.S. Environmental Protection Agency cited no reports of such effects in amphibians, although it concluded that "this class of vertebrate represents a unique sentinel animal model for laboratory and field exposure studies" (16).

In spite of increasing alarm over global population declines in amphibian species over the last 20 years, only recently has evidence of reproductive toxicity due to xenobiotic exposure been provided. Habitat destruction, fragmentation, or disturbance may be sufficient to explain some declines, but not all. Other potential causes include habitat acidification, predation, competition by introduced species (17), increased ultraviolet (UV) radiation resulting from atmospheric ozone depletion (18), and exposure to toxic environmental contaminants (19,20). Amphibians may be particularly vulnerable to waterborne environmental contaminants due to their largely aquatic life histories and their highly permeable skin (21).

Metamorphosis is a period of substantial morphological change in which an organism alters its mode of living and occurs in all major chordate groups with the exception of amniotes (15). In fact, metamorphosis is developmentally comparable to post-embryonic organogenesis in mammals (15). Three primary characteristics define metamorphosis, (1) change in non-reproductive structures between a post-hatch or larval state and sexual maturity, (2) form of the larvae enable it to occupy a unique ecological niche different from that used by the adult life stage, and (3) the morphological changes that occur at the conclusion of larval development depend on some environmental stimulus, either external (i.e., temperature or food supply) or internal (hormonal changes). Each of the three classes of amphibians, anurans, urodeles, and caecilians undergo metamorphosis, although not all species within each class metamorphose. For example, obligatory neotenic urodeles do not metamorphose, and reproduce as an aquatic "adult larvae". Three primary morphological changes occur during metamorphosis, (1) resorption or regression of tissue or organ systems that have primary function only in the larval life stage, (2) the remodeling of larval organ systems to their adult form, which are suitable only for the adult, and (3) *de novo* development of tissues in the adult that are not required by the larvae. These changes are most marked in anuran species, and less obvious in urodeles and caecilians. In each of the three classes of amphibians, metamorphosis is controlled by thyroid hormone (TH), although less is currently known about the role of TH in the metamorphosis of caecilian species. Amphibian metamorphosis has been most widely studied in anurans, primarily due to the dramatic nature of metamorphosis and the ease in use of anuran species in research. However, within the anurans, metamorphosis has been reasonably well studied in only three species, *Xenopus laevis* (South African clawed frog), *Rana catesbeiana* (bull frog), and *R. pipiens* (Northern Leopard frog). Anuran metamorphosis is separated into three distinct periods, premetamorphosis, prometamorphosis, and metamorphic climax. Premetamorphosis refers to a

period of embryonic and early larvae development that takes place without thyroid hormone. Some advanced morphological developments occur during this stage including hind limb bud development. More specific morphogenesis, such as differentiation of the toes and rapid growth (elongation) of the hind limbs, occurs during prometamorphosis. Biochemically, prometamorphosis is characterized by rising concentrations of endogenous TH. The final period is metamorphic climax in which a surge of TH triggers the final processes associated with metamorphosis, including forelimb development and resorption of the tail. Drastic internal transformations at the organ system, tissue, and biochemical levels are also taking place during prometamorphosis and metamorphic climax.

From an evolutionary standpoint, amphibians are distinctively separated phylogenetically from other vertebrates. However, specific aspects of the thyroid axis are conserved amongst most chordates at both the morphological and molecular levels. Thus, the conserved nature of the thyroid axis enhances the ability to use an amphibian, particularly an anuran, as a general model for evaluating thyroid disruption that can be extrapolated to other vertebrate species.

The thyroid axis represents one potential target for environmental chemicals. Environmental agents, toxicants, natural products, and complex mixtures can alter metamorphosis by interacting with the thyroid axis. Further, the complexity of the thyroid axis yields many different possible mechanisms of inhibiting metamorphic processes in amphibians at differing biochemical and molecular levels. Thus, from this end, use of amphibians to screen for thyroid disrupting chemicals or chemical mixtures as a representative chordate is not unreasonable.

To date, the debate on endocrine disruptors has mostly revolved around gonadal steroids including estrogens and androgens, because of controversy regarding their possible link to infertility, breast cancer, and lower sperm counts. Thus, the thyroid has received comparatively little attention. The effects of synthetic chemicals in the environment on thyroid function have been surveyed recently (16). This review confirms the hypothesis of thyroid disruption by environmental chemicals in wildlife and supports the need for human population and laboratory animal studies on compounds already identified as thyroid disruptors. The effects of more than 40 pesticides and 45 industrial chemicals on the thyroid axis are described.

The U.S. Environmental Protection Agency is implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is an amphibian metamorphosis screening assay designed to detect substances that interact with the thyroid systems of vertebrates. It is thought that the inclusion of the amphibian metamorphosis assay in Tier 1 is important because amphibian metamorphosis provides a well-studied thyroid dependent process on which to build an assay. Additionally, the amphibian assay is considered essential in the overall screening battery to detect substances that may disrupt the vertebrate thyroid axis.

A previous work assignment in this contract (WA 2-20) was initiated to provide a detailed review of amphibian metamorphosis assays suitable for use in the EDSP. One of the candidate protocols described was a Prometamorphosis Assay with *Xenopus*. The recommended protocol further outlined a method beginning with Nieuwkoop and Faber (21) stage 51 or 54 and

continuing for 14-21 days with key endpoints based on changes in developmental stage and thyroid histology. This approach already has been utilized for several compounds at the U.S. EPA Office of Research and Development/Mid-Continent Ecological Division (ORD/MED) laboratory (U.S. EPA ORD/MED staff, personal communication). The purpose of this work assignment is to perform a multiple chemical demonstration and evaluation of the amphibian prometamorphosis assay as developed by the U.S. EPA ORD/MED.

The primary experimental objective is to determine the differential sensitivity of beginning exposure with stage 51 and 54 larvae and to establish the necessary duration of exposure. The primary endpoints will be growth, mortality, and developmental stage. To achieve this objective, two concurrent studies (one cohort exposed from stage 51; another cohort exposed from stage 54) will be conducted for each chemical with individuals from the same cohort of organisms. One will be initiated with stage 51 larvae and conducted for 21 days and the other with stage 54 larvae, conducted for 14 days.

2.0 STUDY DESIGN

Adult Care and Breeding

Adult care and breeding will be conducted in accordance with standard guidelines (22). The *X. laevis* larvae used for these studies will be obtained exclusively from in-house cultures. To induce breeding, adult female and male breeding stock will be injected with human chorionic gonadotropin (hCG) to induce reproduction. Essentially, 4 to 6 hours prior to breeding, female and male specimens will be injected with approximately 700 IU and 500 IU of hCG dissolved in amphibian saline, respectively.

Test Specimens

The embryos are held at 21°C ± 1°C for four days to allow for hatching, at which time they are divided into groups of 250/50 L (5 larvae/L, which is the same density as will be used in the actual test) and maintained in a constant flow (50 mL/minute) of dilution water at approximately 21°C until they reach the desired developmental stages used in the studies. Larvae are to be fed a blended mixture of TetraFin® (Tetra Sales, Blacksburg, VA, USA), *Spirulina* algae discs (The Wardley Corporation, Secaucus, N.J., USA), Silver Cup Trout Starter (Nelson & Sons Inc., Murray Utah, USA) along with live brine shrimp (Bio-Marine® Brand, Bio-Marine Inc., Hawthorne CA, USA) twice/day on weekdays and once/day on the weekends, regardless of treatment.

Test Initiation

A summary of the experimental conditions to be used in the present study are provided in Table 1. At 14-17 days post-hatch, larvae will be anesthetized using 100 mg/L of MS-222 buffered with 200 mg/L of sodium bicarbonate, sorted by stage (21), and allowed to recover from anesthesia in dechlorinated tap water. After recovery, 200 stage 51 tadpoles will be counted and randomly divided into tanks containing 4 L of test solution (20 tadpoles/tank, with a density of 5

tadpoles/L). Remaining tadpoles will be placed back in clean water until they reach stage 54 when they will be used to start the stage 54 portion of each study (using similar methods used for the stage 51 tadpoles). Stage 51 and 54 larvae will be exposed to four test concentrations and a control. Each test concentration and control will be replicated with 20 organisms per replicate.

Table 1. Experimental Conditions

Test Type	Metamorphosis Assay
Test Animal	*X. laevis* Larvae
Initial Larval Stages	Stage 51 and Stage 54
Test Duration	21 Days for Stage 51 and 14 Days for Stage 54
Number of Test Substances	6
Dilution Media	Dechlorinated (aged) Tap Water
Test Solution Volume/Tank	4.0 L
Larvae/Tank	20
Photoperiod	12 hr Dark and 12 hr Light
Light Intensity at Water Surface	61 to 139 lumens
Water Temperature	21°C ± 1°C
Number of Test Concentrations	4
Number of Controls	1
Replicates/Test Concentration and Control	2

Test Termination

Once in the test system, mortality observations will be made daily and any dead larvae will be removed. On exposure day 21 and 14 for the stage 51 and stage 54 experiments, respectively, all organisms in the study will be anesthetized in MS-222, evaluated for developmental stage, weighed, and preserved in Bouin's solution for possible histological analysis of the thyroid glands.

Endpoints

The primary endpoints that will be evaluated in the present study will be mortality, developmental stage, growth, and thyroid histology. These endpoints including the measurement format and the frequency of measurement during the proposed studies are described in Table 2. Gross morphology will be measured as a secondary endpoint.

Table 2. Primary and Secondary Endpoints

Primary Endpoint	Frequency of Measurement	Measurement Format
Mortality	Daily	% at Test Conclusion
Developmental Stage	At Test Conclusion	Individual NF Stage Distribution
Growth	At Test Conclusion	Weight
Thyroid Histology	At Test Conclusion	Pathology Summary
Secondary Endpoint		
Gross Morphology	At Test Conclusion	% External Abnormality

Test Materials

Six test chemicals, which are summarized in Table 3, will be evaluated in the present study, including: 6-n-propylthiouracil (PTU), phenobarbital, pregnenolone-16-a-carbonitrile, L-thyroxine (T4), dexamethasone, and methimazole. All chemicals should be confirmed to be at least 98% pure. Primary stock solutions for PTU (700 mg/L) will be made in glass or Teflon™ coated containers by dissolving the chemical in dechlorinated tap water heated to 40 °C and mixed using a high-speed magnetic stirrer. The working stock solution (20.0 mg/L) will be made by diluting the primary stock solution with dechlorinated tap water. Primary stock solutions for T4 (4.04 mg/L) will be made in glass or Teflon™ coated containers by dissolving chemical in 200 mL of 50 millimolar sodium hydroxide (NaOH) solution and diluting with dechlorinated tap water. This solution will then be added to dechlorinated tap water to make the working stock solution (4.0 µg/L). Primary stock solutions for methimazole (2,500 mg/L) will be made in glass or Teflon™ coated containers by dissolving the chemical in dechlorinated tap water and mixed using a high-speed magnetic stirrer. The working solution (50 mg/L) will be made by diluting the primary stock solution with dechlorinated tap water. Stock solution preparation for the remaining three chemicals will be determined in consultation with the Work Assignment Manager (WAM) and the U.S. EPA ORD/MED technical experts. While U.S. EPA ORD/MED experts have experience with PTU, T4, and methimazole, it is likely that additional background work and/or range-finding studies will be needed to assist in the ultimate selection of test concentrations for the other three chemicals. At least four test exposure concentrations and a control should be run in each assay, with a minimum of two replicates per treatment. Exposure concentrations should be 2.5, 5.0, 10.0, and 20.0 mg/L for PTU , 0.5, 1.0, 2.0, and 4.0 µg/L for T4, and 6.25, 12.5, 25, and 50 mg/L for methimazole.

Table 3. Test Substances

Test Substance	Primary Stock Concentration	Working Stock Concentration	Exposure Concentrations
PTU	700 mg/L	20.0 mg/L	2.5, 5.0, 10.0, 20.0 mg/L
Phenobarbital	To Be Determined (TBD)	TBD	TBD
Pregnenolone-16-∝-carbonitrile	TBD	TBD	TBD
T4	4.04 mg/L	4.0 µg/L	0.5, 1.0, 2.0, 4.0 µg/L
Dexamethasone	TBD	TBD	TBD
Methimazole	2,500 mg/L	50 mg/L	6.25, 12.5, 25, 50 mg/L

Exposure System

A flow-through exposure system will be used for all studies. The system, containing components constructed of glass and silicone, stainless steel, and/or Teflon™, will be capable of generating five replicated exposure concentrations for each chemical with a dilution factor of 0.5 for test compounds, as well as replicated controls. Exposure tanks will be equipped with standpipes, which should provide an actual tank volume of 4.0 L. The flow rate to each tank will be 50 ml/minute. Fluorescent lamps within the laboratory will provide a photoperiod of 12 hr light:12 hr dark at an intensity that ranges from 61 to 139 lumens at the surface of the water.

Chemical Analysis

Diluter system performance will be monitored daily and exposure concentrations will be analyzed at test initiation, at least weekly thereafter, and at test termination. Analytical procedures for testing these chemicals will be obtained from U.S. EPA ORD/MED, if available. For those analytical methods not provided by U.S. EPA ORD/MED, the contractor will survey available methods for chemical analysis.

Histological Procedures

Histological preparation of thyroid tissue samples and pathological interpretation will be performed by Experimental Pathology Laboratories, Inc. under the direction of pathologist Dr. Marilyn Wolfe. Five randomly selected larvae from each of the two replicates from each of the four test concentrations for each of the six test substances and the control (300 specimens total) will be allocated for thyroid gland histology.

The following procedure will be followed for sectioning and evaluating the thyroid glands of *X. laevis*:

1. The head of the frog will be cut from the body in a transverse plane just caudal to the eyes.
2. The head will be processed on an automatic tissue processor and then embedded in

paraffin with the cut side down.

3. The microtomist will rough-cut the frog head until the thyroid gland is observed bi-laterally in the section.

4. When the thyroid glands are reached, the microtomist will take three step sections with approximately 32 microns between each step. These steps will be mounted on one slide, if possible.

5. The slides will be stained with hematoxylin and eosin.

6. The thyroid glands will be evaluated by an ACVP-certified veterinary pathologist.

7. The histopathologic evaluation of the thyroid glands will include such changes in the glands as hypertrophy of follicular cells, hyperplasia of thyroid follicles, size of thyroid follicles and degree of colloid accumulation in the thyroid follicles, a qualitative assessment as to size of the thyroid gland as might be possible, and any other relevant changes.

8. A Pathology Summary, which will include the materials and methods and the results comparing the thyroid tissue from control frogs with exposed frogs, will be written. Appropriate Individual Animal Tables and Summary Incidence Tables will accompany the Pathology Summary.

9. Digital photographs will be taken to illustrate changes, if any, in the thyroid glands of exposed frogs as compared to thyroid glands of control frogs.

Statistics

Developmental stage will be analyzed based on the distribution of stages after 14 and 21 days of exposure for both tests conducted with stage 51 and 54 larvae. The non-parametric Kruskal-Wallis one-way analysis of variance will be conducted on the entire data set for each stage. Pairwise comparisons between treated and control treatments will be conducted using the Mann-Whitney U test, with a significance level of $p \leq 0.05$. Wet weight will be analyzed by analysis of variance followed by pairwise comparisons between treated and control using the Bonferroni method, with a significance level of $p \leq 0.05$.

3.0 REFERENCES

1. Tulner, W.W. Uterotrophic action of the insecticide methoxychlor. Science 133:647-648, 1961.

2. Welch, R.M., Levin, W. and Conney, A.H. Estrogenic action of DDT and its analogues. Toxicol. Appl. Parmacol. 14:358-367, 1969.

3. Jobling, S. and Sumpter, J.P. Detergent components in sewage effluent are weakly oestrogenic to fish: an *in vitro* study using rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquat. Toxicol. 27:361-372, 1993.

4. Palmiter, R.D. and Mulvihill, E.R. Estrogenic activity of the insecticide kepone on the chicken oviduct. Science 201:356-358, 1978.

5. Gray, L.E.J., Ostby, J.S. and Kelce, W.R. Developmental effects of an environmental antiandrogen: the fungicide vinclosolin alters sex differentiation of the male rat. Toxicol. Appl. Pharmacol. 129:46-52, 1994.

6. Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Kemppainen J.A. and Wilson, E.M. Persistent DDT metabolite p,p'-DEE is a potent androgen receptor antagonist. Nature 375:581-585, 1995.

7. Guillette, L.J. Jr., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F. and Woodward, A.R. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect. 102:680-688, 1994.

8. Guillette, L.J. Jr., Pickford, D.B., Crain, D.A., Rooney, A.A. and Percival, H.F. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. Gen. Comp. Endocrinol. 101:32-42, 1996.

9. Crain, D.A., Guillette, L.J. Jr., Rooney, A.A. and Pickford, D.B. Alterations in steroidogenesis in alligators (Alligator mississippiensis) exposed naturally and experimentally to environmental contaminants. Environ. Health Perspect. 105:528-533, 1997.

10. Crain, D.A., Guillette, L.J. Jr., Pickford, D.B., Percival, H.F. and Woodward, A.R. Sex steroid and thyroid hormone concentrations in juvenile alligators (Alligator mississippiensis) from contaminated and reference lakes in Florida, USA. Environ. Toxicol. Chem. 17:446-452, 1998.

11. Folmar, L.C., Denslow, N.D., Rao, V., Chow, M., Crain, D.A., Enblom, J., Marcino, J. and Guillette, L.J. Jr. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environ. Health Perspect. 104:1096-1101, 1996.

12. Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Taylor, C.R. and Sumpter, J.P. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 8:275-285, 1994.

13. Van der Kraak, G.J., Munkittrick, K.R., McMaster, M.E., Portt, C.B. and Chang, J.P. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. Toxicol. Appl. Pharmacol. 115:224-233, 1992.

14. Gendron, A.D., Bishop, C.A., Fortin, R. and Hontela, A. *In vivo* testing of the functional integrity of the corticosterone-producing axis in mudpuppy (Amphibia) exposed to chlorinated hydrocarbons in the wild. Environ. Toxicol. Chem. 16:1694-1706, 1997.

15. Hayes, T.B., Wu, T.H. and Gill, T.N. DDT-like effects as a result of corticosterone treatment in an anuran amphibian: is DDT a corticoid mimic or a stressor? Environ. Toxicol. Chem. 16:1948-1953, 1997.

16. U.S. EPA. Special report on environmental endocrine disruption: an effects assessment and analysis. EPA/630/R-96/012. Washington, DC: U.S. Environmental Protection Agency, 1997.

17. Wake, D.B. Declining amphibian populations. Science 253:860, 1991.

18. Blaustein, A.R., Hoffman, P.D., Hokit, K.G., Kiesecker, J.M., Walls, S.C. and Hays, J.B. UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines. Proc. Natl. Acad. Sci. USA 91:1791-1795, 1994.

19. Hall, R.J. and Henry, P.F.P. Assessing effects of pesticides on amphibians and reptiles: status and needs. Herpetol. J. 2:65-71, 1992.

20. Carey, C. and Bryant, C.J. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environ. Health Perspect. 103(suppl 4):13-17, 1995.

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

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

APPENDIX A

STUDY PROTOCOL

FEL Protocol No. BATT02-1

Work Assignment 3-11

Effective Date: January 7, 2004

EPA Contract No. 68-W-01-023

Title:
Frog Metamorphosis Assay Demonstration

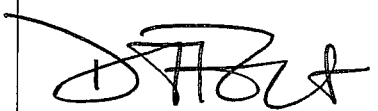
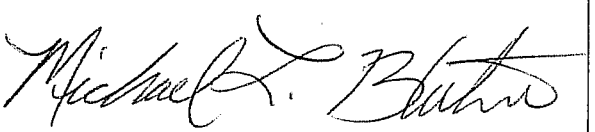
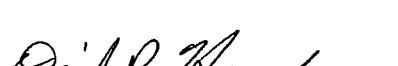
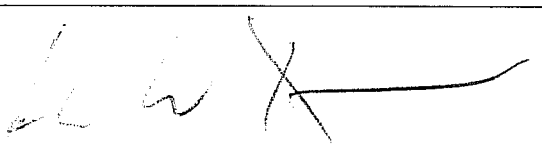
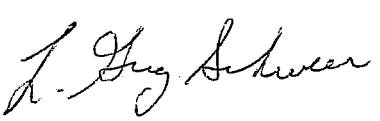
Sponsor:
U.S. Environmental Protection Agency
Endocrine Disruptor Screening Program
Washington, D.C.

Study Facility:
Fort Environmental Laboratories, Inc.
1414 South Sangre Road
Stillwater, Oklahoma 74074

Amendments:

Number	Date	Section(s)	Page(s)
1	2/17/2004	EXPOSURE SYSTEM	5
2	3/5/2004	CHEMICAL ANALYSIS	5
3	11/4/2004	MULTIPLE SECTIONS	3, 5, 7
4			

Approvals:

Title/Name	Signature	Date
Fort Environmental Labs Principal Investigator: Douglas J. Fort, Ph.D.		1/19/04
Battelle Work Assignment Leader: Michael L. Blanton		1/21/04
Battelle Program Manager: David P. Houchens, Ph.D.		1/6/2004
Fort Environmental Labs Study Team Leader: John Thomas		1/19/04
Fort Environmental Labs QAU: Robert Rogers		1/19/04
EPA Work Assignment Manager: Les Touart, Ph.D.		1-27-04
EPA Project Officer: L. Greg Schweer		1-27-04

OBJECTIVE

The primary experimental objective of the amphibian metamorphosis assay is to determine the differential sensitivity of beginning chemical exposure with stage 51 and 54 *Xenopus laevis* larvae and to establish the necessary duration of exposure. To achieve this objective, two concurrent studies (one test group exposed from stage 51 and another from stage 54) will be conducted for each test substance with all larvae originating from the same clutch of eggs.

STUDY SCHEDULE

The studies are scheduled to start in January 2004, after EPA approval of the Final Study Plan and the Final Quality Assurance Project Plan. The studies will conclude after metamorphosis assays on six test chemicals have been completed, data are analyzed, and a draft of the final report will be submitted to EPA no later than August 31, 2004.

EXPERIMENTAL DESIGN

Test Substances

Six test chemicals will be utilized in the studies including: 6-n-propylthiouracil (PTU), phenobarbital, pregnenolone-16-∝-carbonitrile, L-thyroxine (T4), dexamethasone, and methimazole (Table 1). All chemicals should be at least 98% pure, as confirmed by analyses conducted by the Contractor under the Chemical Repository Work Assignment (i.e., WA 3-2 or its follow-on work assignment in Option Year 3). Primary stock solutions for PTU (700 mg/L) will be made in glass or Teflon™ coated containers by dissolving the chemical in dechlorinated tap water heated to 40 °C and mixed using a high-speed magnetic stirrer. The working stock solution (20.0 mg/L) will be made by diluting the primary stock solution with dechlorinated tap water. Primary stock solutions for T4 (4.04 mg/L) will be made in glass or Teflon™ coated containers by dissolving chemical in 200 mL of 50 millimolar sodium hydroxide (NaOH) solution and diluting with dechlorinated tap water. This solution will then be added to dechlorinated tap water to make the working stock solution (4.0 µg/L). Primary stock solutions for methimazole (2,500 mg/L) will be made in glass or Teflon™ coated containers by dissolving the chemical in dechlorinated tap water and mixed using a high-speed magnetic stirrer. The working solution (50 mg/L) will be made by diluting the primary stock solution with dechlorinated tap water. Stock solution preparation for the remaining three chemicals will be determined in consultation with the Work Assignment Manager (WAM) and the U.S. EPA Office of Research and Development/Mid-Continent Ecological Division (ORD/MED) technical experts. While U.S. EPA ORD/MED experts have experience with PTU, T4, and methimazole, it is likely that additional background work and/or range-finding studies will be needed to assist

in the ultimate selection of test concentrations for the other three chemicals. At least four test exposure concentrations and a control should be run in each assay, with a minimum of two replicates per treatment. Exposure concentrations for PTU should be 2.5, 5.0, 10.0, and 20.0 mg/L and 0.5, 1.0, 2.0, and 4.0 µg/L for T4. Exposure concentrations for methimazole should be 6.25, 12.5, 25, and 50 mg/L.

Table 1. Test Substances

Test Substance	Primary Stock Concentration	Working Stock Concentration	Exposure Concentrations
PTU	700 mg/L	20.0 mg/L	2.5, 5.0, 10.0, 20.0 mg/L
Phenobarbital	To Be Determined (TBD)	TBD	TBD
Pregnenolone-16-∝-carbonitrile	TBD	TBD	TBD
T4	4.04 mg/L	4.0 µg/L	0.5, 1.0, 2.0, 4.0 µg/L
Dexamethasone	TBD	TBD	TBD
Methimazole	2,500 mg/L	50 mg/L	6.25, 12.5, 25, 50 mg/L

Test Animals

The test species will be the South African Clawed Frog (*X. laevis)*, from which stage 51 and 54 larvae will be used in the metamorphosis assay (Nieuwkoop and Faber, 1974). *X. laevis* is a well-studied and well-understood laboratory animal that exhibits ease of use in the laboratory, rapid development, and simplicity of observation due to transparency during embryo-larval development (ASTM E1439-98, 1998).

Origin, Handling, and Feeding

The *X. laevis* larvae used for these studies will be obtained from an in-house culture (originally purchased from *Xenopus* I, Dexter, MI.) where adults will be injected with human chorionic gonadotropin (HCG) to induce reproduction. Details for adult frog care and breeding, specific to Fort Environmental Laboratories (FEL), are found in Standard Operating Procedures (SOP) 8.2 and 8.3, respectively. Fertilized egg collection will be performed as described in ASTM E1439-98 and the *Atlas of Abnormalities*, (Bantle, et al., 1998). The embryos will be held at approximately 21°C for four days to allow for hatching, at which time they will be divided into groups of 250/50 L and maintained in a constant flow (50 mL/minute) of dilution water at approximately 21°C until they reach the desired developmental stages used in the studies. Larvae are to be fed a blended mixture of TetraFin® (Tetra Sales, Blacksburg, VA, USA), *Spirulina*

algae discs (The Wardley Corporation, Secaucus, N.J., USA), Silver Cup Trout Starter (Nelson & Sons Inc., Murray Utah, USA), along with live brine shrimp (Bio-Marine® Brand, Bio-Marine Inc., Hawthorne CA, USA) twice daily on weekdays and once daily on weekends, regardless of treatment.

Exposure System

A flow-through exposure system will be used for all studies. The system, whose components should be glass and silicone, stainless steel, and/or Teflon™, will be capable of generating five replicated exposure concentrations for each chemical with a dilution factor of 0.5 for test compounds, as well as replicated controls. Exposure tanks will be equipped with standpipes, which should provide an actual tank volume of 4.0 L. The flow rate to each tank will be 50 mL/minute. If dissolved oxygen (DO) levels can not be maintained => than 4.0 mg/L, each exposure tank will be equipped with an aeration system to provide adequate DO. Fluorescent lamps will provide a photoperiod of 12 hr light:12 hr dark at an intensity that should range from 61 to 139 lumens at the water surface.

Chemical Analysis

Temperature, pH, and DO will be measured daily to monitor diluter system performance. Exposure concentrations will be analyzed at test initiation, at least weekly thereafter, and at test termination.

Test Method

At 14-17 days post-hatch, larvae will be anesthetized using 100 mg/L of MS-222 buffered with 200 mg/L of sodium bicarbonate, sorted by stage (Nieuwkoop and Faber, 1974), and allowed to recover from anesthesia in 100% dilution water. After recovery, 200 stage 51 larvae will be counted and randomly divided into tanks containing 4.0 L of test solution (20 larvae/tank, with a density of 5 larvae/L). Remaining larvae will be placed back in dilution water until they reach stage 54 when they will be used to start the stage 54 portion of each study (using similar methods used for the stage 51 tadpoles). Stage 51 and 54 larvae will be exposed to four test concentrations and a dilution water control. Each test concentration and control will be replicated with 20 organisms per replicate. Once in the test system, mortality observations will be made daily and any dead larvae will be removed. On exposure day 21 and 14 for the stage 51 and stage 54 experiments, respectively, all organisms in the study will be anesthetized in MS-222, evaluated for developmental stage, weighed, and preserved in Bouin's solution for possible histological analysis of the thyroid glands. Experimental conditions of the assay are presented in Table 2.

Table 2. Experimental Conditions

Test Type	Metamorphosis Assay
Test Animal	*X. laevis* Larvae
Initial Larval Stages	Stage 51 and Stage 54
Test Duration	21 Days for Stage 51 and 14 Days for Stage 54
Number of Test Substances	6
Dilution Media	Dechlorinated (aged) Tap Water
Test Solution Volume/Tank	4.0 L
Larvae/Tank	20
Photoperiod	12 hr Dark and 12 hr Light
Light Intensity at Water Surface	61 to 139 lumens
Water Temperature	21°C ± 1°C
Number of Test Concentrations	4
Number of Controls	1
Replicates/Test Concentration and Control	2

Data Collection and Endpoints

Test data and daily observations will be recorded in study journals. Study journals will include study tracking sheets, calendars identifying major events, study journal logs for recording detailed observations and comments, daily mortality data, weekly developmental stage data, and growth (weight) data. The primary endpoints of the assay will be mortality, developmental stage, growth, and thyroid histology (Table 3). Gross morphology will be a secondary endpoint.

Table 3. Primary and Secondary Endpoints

Primary Endpoint	Frequency of Measurement	Measurement Format
Mortality	Daily	% at Test Conclusion
Developmental Stage	At Test Conclusion	Individual NF Stage
Growth	At Test Conclusion	Weight
Thyroid Histology	At Test Conclusion	Pathology Summary
Secondary Endpoint		
Gross Morphology	At Test Conclusion	% External Abnormality

Thyroid Histology

Experimental Pathology Laboratories in Herndon, VA, will perform the histology and pathology interpretation. Five randomly selected larvae from each of the two replicates from each of the four test concentrations for each of the six test substances and the control (300 specimens total) will be allocated for thyroid gland histology.

Histological Procedure

The following procedure will be followed for sectioning and evaluating the thyroid glands of *X. laevis*:

1. The head of the frog will be cut from the body in a transverse plane just caudal to the eyes.

2. The head will be processed on an automatic tissue processor and then embedded in paraffin with the cut side down.

3. The microtomist will rough-cut the frog head until the thyroid gland is observed bi-laterally in the section.

4. When the thyroid glands are reached, the microtomist will take three step sections with approximately 32 microns between each step. These steps will be mounted on one slide, if possible.

5. The slides will be stained with hematoxylin and eosin.

6. The thyroid glands will be evaluated by an ACVP-certified veterinary pathologist.

7. The histopathologic evaluation of the thyroid glands will include such changes in the glands as hypertrophy of follicular cells, hyperplasia of thyroid follicles, size of thyroid follicles and degree of colloid accumulation in the thyroid follicles, a qualitative assessment as to size of the thyroid gland as might be possible, and any other relevant changes.

8. A Pathology Summary, which will include the materials and methods and the results comparing the thyroid tissue from control frogs with exposed frogs, will be written. Appropriate Individual Animal Tables and Summary Incidence Tables will accompany the Pathology Summary.

9. Digital photographs will be taken to illustrate changes, if any, in the thyroid glands of exposed frogs as compared to thyroid glands of control frogs.

Statistical Analyses

Developmental stage will be analyzed based on the distribution of stages after 14 and 21 days of exposure for both tests conducted with stage 51 and 54 larvae. The non-parametric Kruskal-Wallis one-way analysis of variance will be conducted on the entire data set for each stage. Pairwise comparisons between treated and control treatments will be conducted using the Mann-Whitney U test, with a significance level of $p \leq 0.05$. Wet weight will be analyzed by analysis of variance followed by pairwise comparisons between treated and control using the Bonferroni method, with a significance level of $p \leq 0.05$.

RECORDS TO BE MAINTAINED

Raw data, derived data, QA reports, correspondence, and draft and final reports will be electronically maintained in computer files. Printed copies of this material also will be kept in designated file cabinets located in a secured file room at the study facility.

REPORTING

Monthly progress reports and quarterly progress reports will be provided to EPA throughout the actual studies. A draft of the final report will be sent to EPA with pertinent raw and statistical data attached. The final report will be sent after EPA approval of the draft report.

CHANGES TO PROTOCOL

Permanent changes to the finalized study protocol will require that written amendments be prepared and submitted to the Principal Investigator and QAU Director prior to incorporation. The amendment will be reviewed to determine the potential impact on the study. If accepted, 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 reviewed by the Principal Investigator and QAU Director to assess the potential impact on the study. If accepted, deviations will be recorded in the study journal, dated, and initialed by the Principal Investigator.

TEST SUBSTANCE WASTE DISPOSAL

Disposal of test substance waste will be performed in accordance with those requirements provided in the Material Safety Data Sheets (MSDS).

ARCHIVAL

Data files, correspondence, QA records, and reports will be archived indefinitely. Storage of archived files will be maintained in a two-tier manner. Archived files will first be kept in secure file cabinets, located in the study facility laboratory, for a period of one year or until the final report has been reviewed and accepted by the client. After that time, the files will be transferred to storage file boxes and archived off premises at a secured commercial storage facility. The preserved test specimens will be labeled and archived in the laboratory until such time as specimen degradation renders the samples useless.

REFERENCES

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

ASTM E1439-98, *Standard guide for conducting the frog embryo teratogenesis assay-Xenopus (FETAX)*, 1998.

Bantle, J.A., Dumont, J.N., Finch, R.A., and Linder, G., Fort, D.J., *Atlas of abnormalities: a guide for the performance of FETAX*, 2nd Ed., Oklahoma State Publications Department, 1998.

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

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

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Frog Metamorphosis Assay Demonstration	
Amendment Number: 1	**Document ID Number**: WA 3-11
Submitted By: John Henry Flowers	**Date**: 2/17/04
Amendment Relating To:	
[ ] Protocol [X] Study Plan [ ] QAPP [ ] QMP [ ] SOP	
[ ] Other (describe):	

Original Specifications:

Page 6, Paragraph 1, Line 6. "The flow rate to each tank will be 50 mL/minute."

Planned Change:

Change flow rate for assay to 25 mL/minute. The flow rate for rearing the pre-test tadpoles to test stages 51 and 54 will remain at 50 mL/minute.

Reason for Change:

Change recommended by U.S. EPA ORD/MED (Duluth, MN) via telephone conversation with Study Director and Study Team Leader.

Approval:

Study Director:	[signature]	Date:	2/17/04
WAL:	[signature]	Date:	3/1/04
Program Management:	[signature]	Date:	3/3/04
QAU:	[signature]	Date:	2/17/04

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Frog Metamorphosis Assay Demonstration	
Amendment Number: 1	**Document ID Number**: WA 3-11 (Protocol # BATT02-1)
Submitted By: John Young Flowers	**Date**: 2/17/04
Amendment Relating To:	
[X] Protocol [ ] Study Plan [ ] QAPP [ ] QMP [ ] SOP	
[ ] Other (describe):	

Original Specifications:

Page 5, Paragraph 2, Line 6. "The flow rate to each tank will be 50 mL/minute."

Planned Change:

Change flow rate for assay to 25 mL/minute. The flow rate for rearing the pre-test tadpoles to test stages 51 and 54 will remain at 50 mL/minute.

Reason for Change:

Change recommended by U.S. EPA ORD/MED (Duluth, MN) via telephone conversation with Study Director and Study Team Leader.

Approval:

Study Director:		Date:	2/17/04
WAL:		Date:	3/1/04
Program Management:		Date:	3/3/04
QAU:		Date:	2/17/04

PROTOCOL AMENDMENT FORM
Fort Environmental Laboratories

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: BATT02		DATE: March 05, 2004			
AMEDMENT NUMBER: 02		Document ID Number: WA 3-11 (Protocol # BATT02-1)			
TITLE OF STUDY: Frog Metamorphosis Assay Demonstration					
AMENDMENT RELATING TO: In-Life Sample Testing					
[ ]	QAPP	[ ]	QMP	[X]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

Protocol did not include in-life testing details

AMENDMENT: Add the following information to "Chemical Analysis" section.

1. MSL will serve as the chemical analysis laboratory for demonstration study WA 3-11 and provide sample containers and shipping kits for collection and transport of in-life samples.
Contact at MSL:
Michael E. Cobb
Chemical Repository Study Director
Battelle Marine Sciences Laboratory
1529 West Sequim Bay Road
Sequim, WA 98382
PH: 360-681-4580
FAX: 360-681-3699
Email: michael.cobb@pnl.gov

2. Samples will be collected at test initiation and weekly thereafter through the test including termination. Since the testing requires 21-d exposure (start stage 51) for one set of organisms and 21-d exposure (start stage 54), it is anticipated that four sets of samples collected at d 0, ca. d 7, d 14, and d 21 (termination). The d 7 sample time frame may vary slightly based on the set up of the second set of organisms. A sampling schedule will be provided to MSL by FEL prior to the initiation of each chemical exposure. Each set of samples will consist of: 1 dilution water sample, 1 stock solution sample, 1 sample from each of the 4 test concentrations from the diluter system, and 1 arbitrarily chosen duplicate sample for QA analysis at MSL, if needed. Thus, a total of 7 samples will be collected and shipped to MSL per event.

3. Twenty-five (25) mL samples will be collected in amber glass bottles and stored at approximately 4 C until packaged and shipped to MSL. Samples will be collected and shipped on blue ice (or wet ice) in a timely fashion (based on MSL stability testing) via overnight courier service under chain-of-custody. Samples will be stored refrigerated (2-6°C) prior to analysis unless contraindicated by MSL stability testing, samples should be extracted within 7-d (if required by method) and analyzed within the specified stability dating demonstrated in the Study Protocol:
Purity and Stability of Test Substances for Work Assignment 3-11 - EDSP Study Number: EDSP.311-01.

4. Since analytical methods will vary from test substance to test substance, and stability testing has not been performed with each material yet, the methods will be as defined by the MSL's Study Protocol for EDSP.311-01 on stability which must be on file at FEL prior to in-life testing.

REASON FOR CHANGE:

Section was not defined or included in original protocol.

Approval:

Fort Environmental Labs Principal Investigator	[signature]	Date 3/5/04
Work Assignment Leader	[signature]	Date 3/19/04
Battelle Program Manager	[signature]	Date 3/29/04
Fort Environmental Labs Study Team Leader	[signature]	Date 3/5/04
Fort Environmental Labs QAU	[signature]	Date 3/5/04
~~EPA Work Assignment Manager~~		Date
EPA Project Officer		Date

TLP not necessary 3-29-04

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

FEL

DOCUMENT AMENDMENT FORM
Endocrine Disruptor Screening Program

Document or Study Title: Amphibian Metamorphosis Assay Demonstration	
Amendment Number: 03	**Document ID Number:** WA 3-11
Submitted By: Douglas J. Fort	**Date:** 11/4/2004

Amendment Relating To:

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

[ ] Other (describe):

Original Specifications:

1) Study guidance documents (Exposure System, Chemical Analysis, and Test Methods sections, and Protocol Amendment 02).

2) Study guidance documents (Test Termination section) state that at test termination, all surviving specimens will be euthanized and preserved in Bouin's Solution for possible thyroid histology.

3) Study guidance documents (Thyroid Histology section).

4) Protocol (Study Schedule) states that the final report will be submitted to EPA no later than 8/31/2004.

Planned Change:

1) Due to the wide range of physical and chemical properties (including solubility and stability) of T4, dexamethasone, phenobarbital, and PCN, the test conditions as outlined in the Protocol, Study Plan, and QAPP may need to be changed, depending on the chemical being tested. In lieu of generating a new amendment for each change, EPA will issue a technical directive for each remaining chemical, outlining any changes specific to the chemical being tested; such as, flow through vs. static renewal system, analytical sampling schedule, number of test concentrations, test concentration dilution factors, test solution volumes, etc. The EPA directive will also address any items listed as "TBD" (to be determined) in the original study guidance documents.

2) All surviving specimens will be euthanized and preserved in Bouin's Solution for 72 to 120 hours (3 to 5 d). The specimens will then be triple rinsed in 70% v/v reagent alcohol and preserved in 10% (w/v) neutral buffered formalin acetate for possible thyroid histology.

3) Thyroid gland histopathology will be handled as a separate work assignment (WA 5-8).

4) EPA has extended the final report deadline to 12/31/2004.

Reason for Change:

1) The use of EPA technical directives specific for each test chemical is the most direct and simplistic way to handle the unforeseen changes in test set up and conditions that may occur. These changes will not compromise the study, but should have a positive impact on facilitating each test.
2) Prolonged exposure to Bouin's solution can adversely affect the preservation process in tissue.
3) As a critical endpoint for the WA 3-11 assay, EPA has decided that WA 5-8 is needed to provide additional histopathological examination of tissues collected under WA 3-11.
4) EPA extended the deadline due to delays on getting the go-ahead for the laboratory to start testing.

Approval:

Study Director: _____ Date: 11/4/04

WAL: _____ Date: 1/13/05

Program Management: _____ Date: 1/13/05

QAU: _____ Date: 11/4/2004

Study Guidance Documents:
WA 3-11 QAPP with Amendments

1.0 TITLE AND APPROVAL

Quality Assurance Project Plan

For Work Assignment 3-11

Frog Metamorphosis Assay Demonstration

for

EPA CONTRACT NUMBER 68-W-01-023

Project No.: Battelle's Pacific Northwest Division. Project 43496

Work Assignment Leader: Michael Blanton

Principal Investigator: Douglas J. Fort

Institution: Fort Environmental Laboratories, Inc.
Stillwater, Oklahoma

SIGNATURE PAGE
Quality Assurance Project Plan for WA 3-11
Frog Metamorphosis Assay Demonstration
EPA Contract Number 68-W-01-023
Concurrences and Approvals

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3.0 DISTRIBUTION LIST

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4.0 PROJECT/TASK ORGANIZATION

4.1 PROJECT MANAGEMENT

The U.S. Environmental Protection Agency (EPA) is implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. To support this program, EPA has contracted with Battelle to provide comprehensive toxicological and ecotoxicological testing services, including chemical, analytical, statistical, and quality assurance (QA)/quality control (QC) support to assist the EPA in developing, standardizing, and validating a suite of *in vitro*, mammalian, and ecotoxicological screens and tests for identifying and characterizing endocrine effects through exposure to pesticides, industrial chemicals, and environmental contaminants. The studies conducted will be used to develop, standardize, and validate methods; prepare appropriate guidance documents for peer review of the methods; and develop technical guidance and test guidelines in support of the Office of Prevention, Pesticides and Toxic Substances regulatory programs. The validation studies will be conducted under the EDSP Quality Management Plan (QMP) (Battelle, 2003), the study protocol, applicable Quality Assurance Project Plans (QAPPs), relevant program and facility standard operating procedures (SOPs), and guidance documents.

One of the Tier 1 assays under development is an amphibian metamorphosis screening assay designed to detect substances that interact with the thyroid systems of vertebrates. It is thought that the inclusion of the amphibian metamorphosis assay in Tier 1 is important because amphibian metamorphosis provides a well studied thyroid dependent process on which to build an assay. Additionally, the amphibian assay is considered essential in the overall screening battery to detect substances which may disrupt the vertebrate thyroid axis.

The Food Quality Protection Act of 1996 requires the EPA to develop and implement a screening program using valid tests for determining the potential in humans for estrogenic effects from pesticides. EPA proposed a two-tiered screening program in a Federal Register notice in 1998 (63 FR 71542-71568, Dec. 28, 1998) that covered not only pesticides but also commercial chemicals subject to regulation under the Toxic Substances Control Act (TSCA; 15 USC 2601) and environmental and drinking water contaminants. A previous work assignment in this contract (WA 2-20) was initiated to provide a detailed review of amphibian metamorphosis assays suitable for use in the EDSP. One of the candidate protocols described was a Prometamorphosis Assay with *Xenopus*. The recommended protocol further outlined a method beginning with Nieuwkoop and Faber (1994) stage 51 or 54 and continuing for 14-21 days with key endpoints based on changes in developmental stage and thyroid histology. This approach has already been utilized for several compounds at the U.S. EPA, Office of Research and Development/Mid-Continent Ecology Division (ORD/MED) laboratory. The purpose of this work assignment is to perform a multiple chemical demonstration and evaluation of the amphibian metamorphosis assay as developed by the U.S. EPA, ORD/MED.

4.2 GOALS

A number of man-made environmental pollutants have the potential to interfere with endocrine function (Tulner, 1961; Welch *et. al.*, 1969; Jobling and Sumpter, 1993; Palmiter and Mulvihill, 1978; Gray *et. al.*, 1994; and Kelce *et. al.*, 1995), and there is evidence of reproductive or endocrine dysfunction in wildlife species that have been exposed environmentally to such endocrine-disrupting contaminants (Guillette *et. al.*, 1994; Guillette *et. al.*, 1996; Crain *et. al.*, 1997; Crain *et. al.*, 1998; Folmar *et. al.*, 1996; Purdom *et. al.*, 1994; and Van der Kraak *et. al.*, 1992). While there have been recent reports of alterations of the stress hormone axis in amphibians (Gendron *et. al.*, 1997; and Hayes *et. al.*, 1997) there is little or no published evidence of reproductive dysfunction or developmental effects in this group as a result of exposure to endocrine disrupting chemicals (EDCs). A recent special report on environmental endocrine disruption by the U.S. EPA cited no reports of such effects in amphibians, although it concluded that "this class of vertebrate represents a unique sentinel animal model for laboratory and field exposure studies" (U.S. EPA, 1997).

The primary experimental objective is to determine the differential sensitivity of beginning exposure with stage 51 and 54 larvae and to establish the necessary duration of exposure. The primary endpoints will be growth, mortality and developmental stage. To achieve this objective, two concurrent studies (one cohort exposed from stage 51; another cohort exposed from stage 54) will be conducted for each chemical with individuals from the same cohort of organisms. One will be initiated with stage 51 larvae and conducted for 21 days and the other with stage 54 larvae conducted for 14 days.

The completion of Work Assignment (WA) 3-11 will provide data to address the following:

1. Demonstrate and further evaluate the feasibility of an amphibian metamorphosis assay developed by the U.S. EPA ORD/MED for screening thyroid axis disrupting chemicals;
2. Determine which exposure regime is the most sensitive in the proposed assay design; and
3. Provide additional data regarding the thyroid disrupting capacity of several test methods.

Refer to Section 19.2, Statistical Analysis, of this document for a more detailed discussion.

There are four administrative and two research tasks required under WA 3-11.

Task 1. Prepare Work Plan and Overall Study Management
Task 2. Consultation with Frog Experts(s)
Task 3. Develop a Study Plan
Task 4. Prepare a Quality Assurance Project Plan (QAPP) and Quality Assurance Oversight
Task 5. Conduct the Studies
Task 6. Analyze Data and Prepare Final Report

Task 1: Prepare Work Plan and Overall Study Management

Battelle and Fort Environmental Laboratories have prepared a Work Plan providing a detailed description of the technical approach that will be followed to accomplish the tasks, a detailed staffing plan, a time line for completion of tasks and due dates for deliverables, and a detailed cost estimate. All aspects of study preparation, conduct, and oversight will be managed by Battelle.

Task 2: Consultation with Frog Expert(s)

Battelle and Fort Environmental Laboratories will consult with the EPA Work Assignment Manager (WAM), Les Touart, and staff from U.S. EPA, ORD/MED, Duluth, MN regarding development of the Study Plan in Task 3. Assistance of EPA staff is especially important in designing the Protocol and to obtain additional unpublished information. Battelle's Work Assignment Leader (WAL) and Fort Environmental Laboratories' Principal Investigator will consult with the U.S. EPA, ORD/MED key staff to allow in depth discussion of the study objectives with the technical experts. If deemed necessary, a visit by the Principal Investigator to ORD/MED Duluth Lab to further review methods and laboratory procedures may occur but is not a requirement. In addition, Fort Environmental Laboratories will periodically hold telephone consultations with Battelle, the U.S. EPA WAM, and U.S. EPA, ORD/MED during the execution of all phases of this Work Assignment.

Task 3: Develop a Study Plan

In conjunction with Michael Blanton, the WAL, Fort Environmental Laboratories have developed a Study Plan (Battelle, 2004) to conduct a *Xenopus laevis* metamorphosis assay with six known or suspected thyroid EDCs. The Study Plan contains the Study Protocol BATT02-1, Frog Metamorphosis Assay Demonstration. Concurrent trials using the same cohort of frog larvae will be initiated comparing initial stage of exposure for NF stage 51 and NF stage 54 (Nieuwkoop and Faber, 1994). The following description of the method was used in preparing the Study Plan:

Experimental Design

Objective

The primary experimental objective is to determine the differential sensitivity of beginning exposure with stage 51 and 54 larvae and to establish the necessary duration of exposure. The primary endpoints will be growth, mortality and developmental stage. To achieve this objective, two concurrent studies (one cohort exposed from stage 51; another cohort exposed from stage 54) will be conducted for each of the specified six test chemicals of interest (Table 4-1) with individuals from the same cohort of organisms. One will be initiated with stage 51 larvae and conducted for 21 days and the other with stage 54 larvae conducted for 14 days.

Table 4-1. Test Substance Requirements

Test Substance[1]	CAS #	Stock Solution Conc.	Exposure Conc.[3]	Estimated Neat Chemical Required[2]	Analytical Chemistry Method[3]
6-n-Propyl-thiouracil (PTU) ($C_7H_{10}N_2OS$)	51-52-5	700 mg/L	2.5, 5.0, 10.0, 20.0 mg/L	800 g	HPLC with UV/Vis Detector
Phenobarbital ($C_{12}H_{12}N_2O_3$)	50-06-6	TBD[4]	TBD[4]	TBD	HPLC with UV/Vis Detector
Pregnenolone-16-alpha-carbonitrile ($C_{22}H_{31}NO_2$)	1434-54-4	TBD[4]	TBD[4]	TBD	TBD
L-thyroxine (T4) ($C_{15}H_{10}I_4NO_4Na$)	51-48-9	4.04 mg/L	0.5, 1.0, 2.0, 4.0 µg/L	4.0 g	Stability: HPLC with UV/Vis Detector In-Life: HPLC with ICP-MS
Dexamethasone ($C_{22}H_{29}FO_5$)	50-02-2	TBD[4]	TBD[4]	TBD	TBD
Methimazole ($C_4H_6N_2S$)	60-56-0	2,500 mg/L	6.25, 12.5, 25, 50 mg/L	2,000 g	TBD
Dilution Water (Dechlorinated [aged] tap water)	-	0.0 mg/L	0.0 mg/L	-	Per chemical

[1] The Battelle Chemical Repository will provide test chemicals at 98% or greater purity, as confirmed by analyses conducted by the Repository.

[2] This amount allows for 2 replicates per test and an additional amount for contingencies to insure all tests use the same chemical lot number.

[3] The Battelle Chemical Repository will perform chemical analysis on exposure concentrations on samples collected at test initiation, at least weekly thereafter, and at test termination. Analytical test methods will be provided by U.S. EPA, ORD/MED, if available. Those methods not provided by U.S. EPA, ORD/MED will be provided by the contractor. Addendums to the QAPP will be provided, as methods are determined through literature searches.

[4] Stock solution preparation to be determined (TBD) in consultation with EPA technical experts. Addendums to the QAPP will be provided, as concentrations are determined through literature searches and range finding tests.

It is recognized that Table 4-1 is not complete, and that analysis information listed as "To Be Determined" or "TBD" will be addressed by preparing addendums to this QAPP as information becomes available. It is likely that range finding tests and literature information will be needed (per U.S. EPA ORD/MED). Three test chemicals may require range-finding studies (dexamethasone, phenobarbital, and pregnenolone-16-alpha-carbonitrile). The approach will be to expose stage 51 larvae for 4 d in the diluter system using a limit test approach starting at the limit of water solubility and using log or augmented log concentration series.

Animals

The *Xenopus laevis* larvae used for these studies will be obtained from an in-house culture where adults are injected with human chorionic gonadotropin (HCG) to induce reproduction. The genus and species will be verified by a taxonomic identification referencing the appropriate key(s) used. The embryos are held at approximately 21°C for four days to allow for hatching, at which time they are divided into groups of 250/50 L (5 larvae/L, which is the same density as will be used in the actual test) and maintained in a constant flow (50 mL/minute) of dechlorinated tap water at approximately 21°C until they reach the desired developmental stages used in the studies. Larvae are to be fed a blended mixture of TetraFin® (Tetra Sales, Blacksburg, VA, USA), *Spirulina* algae discs (The Wardley Corporation, Secaucus, N.J., USA), Silver Cup Trout Starter (Nelson & Sons Inc., Murray Utah, USA) along with live brine shrimp (Bio-Marine® Brand, Bio-Marine Inc., Hawthorne CA, USA) twice/day on weekdays and once/day on the weekends regardless of treatment.

Test Initiation

At 14-17 days post-hatch, larvae will be anesthetized using 100 mg/L of MS-222 (tricaine methane sulfonate) buffered with 200 mg/L of sodium bicarbonate, sorted by stage (Nieuwkoop and Faber, 1994), and allowed to recover from anesthesia in dechlorinated tap water. After recovery, 200 stage 51 tadpoles will be counted and randomly divided into tanks (20 tadpoles/tank). Remaining tadpoles will be placed back in dechlorinated tap water until they reach stage 54 (24-26 days post-hatch) when they will be used to start (using similar methods used for the stage 51 tadpoles) the stage 54 portion of each study. Stage 51 and 54 larvae will be exposed to 4 test concentrations and a control. Each test concentration and control will be replicated with 20 organisms per replicate.

Test concentrations are scheduled to be 2.5, 5.0, 10.0, and 20.0 mg/L for PTU; 0.5, 1.0, 2.0, and 4.0 µg/L for T4; and 6.25, 12.5, 25, and 50 mg/L for methimazole (Table 4-1). Concentrations for the other three chemicals will be determined in consultation with the technical experts from U.S. EPA, ORD/MED and/or range-finding studies, and documented as an addendum to this QAPP.

Test Termination

Once in the test system, mortality observations will be made daily and any dead larvae will be removed. On exposure days 21 and 14 for the stage 51 and stage 54 experiments respectively, final mortality observations will be made, and all organisms in the study will be anesthetized in MS-222, evaluated for developmental stage, weighed, and preserved in Bouin's solution for possible histological analysis of the thyroid glands.

Toxicant solutions

The six test chemicals listed in Table 4-1 will be utilized in this work assignment: PTU, phenobarbital, pregnenolone-16-alpha-carbonitrile, T4, dexamethasone, and methimazole. Primary stock solutions for PTU (700 mg/L) will be made in glass or Teflon™ coated containers by dissolving the chemical in dechlorinated tap water heated to 40°C and mixed using a high-speed magnetic stirrer. The working stock solution (20.0 mg/L) will be made by diluting the primary stock solution with dechlorinated tap water. Primary stock solutions for T4 (4.04 mg/L) will be made in glass or °™ coated containers by dissolving chemical in 200 mL of 50 millimolar sodium hydroxide (NaOH) solution and diluting to 18 L with dechlorinated tap water. This solution will then be added to dechlorinated tap water to make the working stock solution (4.0 µg/L). Primary stock solutions for methimazole (2,500 mg/L) will be made in glass or Teflon™ coated containers by dissolving the chemical in dechlorinated tap water and mixed using a high-speed magnetic stirrer. The working solution (50 mg/L) will be made by diluting the primary stock solution with dechlorinated tap water. Stock solution preparation for the remaining three chemicals will be determined in consultation with the technical experts from U.S. EPA, ORD/MED. Addendums to this QAPP will be added for each TBD in Table 4-1 based on literature searches and range finding tests. Range finding tests will be based on a log or augmented log based concentration scale using a short-term exposure method to establish lethal concentrations.

Approach:

1. Literature Search to possibly identify effective concentrations.
2. Short-term exposure with stage 51 larvae for 4 days in a flow-through diluter system to determine sub-lethal concentrations and define test concentrations for metamorphosis assay.

Exposure system

A continuous flow mini-diluter exposure system will be used for all studies (Benoit et al., 1982). The diluter system consists of four major components or cells; the toxicant and water cell, the dilution cell, the flow booster cell, and the flow splitter cell. The system components coming in contact with test solutions will be limited to glass and silicone, stainless steel, and/or Teflon™, will be capable of generating five replicated exposure concentrations for each chemical with a dilution factor of 0.5 for replicated test compounds, as well as replicated controls. The chemical stock solution is first pumped to

the toxicant cell using a metering pump with stainless steel head and Teflon™ tubing. From there, the system relies on gravity to feed the test solution and dilution water to a series of headboxes, dilution cells, booster cells, and splitter cells where stock solution is mixed, diluted, and split to obtain the appropriate treatment concentrations. Adjustable Teflon™ tips will be used to maintain the proper flow rate of 50 mL/minute. Exposure tanks will be glass aquaria (approx. 22.5 x 14.0 x 16.5 cm deep = 5.2 L) equipped with 13.0 cm standpipes, which should provide an actual working tank volume of at least 4.0 L. The flow rate to each tank will be 50 mL/minute. Fluorescent lamps will provide a photoperiod of 12 hr light: 12 hr dark at an intensity that will range between 61 to 139 lumens at the water surface.

Chemical Analysis

Diluter system performance will be monitored daily by measuring the flow rates to each exposure chamber and adjusting, as needed, to maintain the target flows of 50 mL/minute. For QC purposes, the dilution water (dechlorinated tap water) will be analyzed for each of the six test substances prior to starting the study. Exposure concentrations will be analyzed at test initiation, at least weekly thereafter, and at test termination. Analytical procedures will be obtained from U.S. EPA, ORD/MED and are summarized in Table 4-1. Those methods not provided by U.S. EPA, ORD/MED will be provided by the contractor. The Battelle Chemical Repository (Sequim, WA) will perform the required analyses to determine actual concentrations tested.

Histological procedures

The histology and pathology interpretation will be performed by Experimental Pathology Laboratories (Herndon, VA).

The thyroid gland will be cut in three step sections and mounted on one slide if possible and stained with hematoxylin and eosin. After processing, a board-certified veterinary pathologist will evaluate the sections microscopically and prepare a summary with appropriate tables of the findings. Digital photographs will be taken to illustrate changes, if any, in the thyroid glands of exposed frogs compared to thyroid glands of control frogs. A photograph log describing what is depicted will accompany digital photographs.

Statistics

Developmental stage will analyzed based on the distribution of stages after 14 and 21 days of exposure for both tests conducted with stage 51 and 54 larvae. The non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) will be conducted on the entire data set for each stage. Pair wise comparisons between treated and control treatments will be conducted using the Mann-Whitney U test, with a significance level of $p \leq 0.05$. Wet weight will be analyzed by analysis of variance followed by pair wise comparisons between treated and control using the Bonferroni method, with a significance level of $p \leq 0.05$.

Other Study Plan Issues

As discussed in Task 6, the Study Plan will form the first section of the Final Report. The Study Plan addresses:

1. Purpose of the study,
2. Endpoints of interest,
3. Dose selection for each of the six test chemicals, and
4. Detailed study protocol.

All chemicals will be at least 98% pure, as confirmed by analyses conducted by the Contractor under the EDSP Chemical Repository Work Assignment (i.e., WA 4-2 or its follow-on work assignment in Option Year 3). At least four test chemical concentrations and a control will be tested in each assay, with a minimum of two replicates per treatment. At a minimum, exposure concentrations will be verified at test initiation, at least weekly thereafter, and at test termination.

Task 4: Prepare a Quality Assurance Project Plan and Quality Assurance Oversight

In conjunction with the WAL and QA Unit Specialist for Battelle, Sequim, the Principal Investigator and QA Unit Manager at Fort Environmental Laboratories have prepared a QAPP in accordance with all applicable EPA QA guidance - in particular, the "EPA Requirements for Quality Assurance Project Plans" (EPA QA/R-5, March 2001, http://www.epa.gov/quality1/qs_docs/r5_final.pdf). The study will be audited by the Fort Environmental Laboratories QA Unit Manager in accordance with this QAPP at a level commensurate with level of QA for a demonstration study. No laboratory work will begin until EPA's QA Manager has approved the QAPP.

Task 5: Conduct the Studies

Fort Environmental Laboratories will conduct the chemical demonstration studies in accordance with the EPA-approved Study Plan developed in Task 3 using aliquots of chemical obtained from the Chemical Repository. The studies shall be coordinated with purity and storage stability testing performed by the Battelle Chemical Repository, so that purity and stability of the test chemicals is assured during the course of the studies. Test exposure concentrations will be analyzed by the EDSP Chemical Repository at test initiation, and at least weekly through test termination.

Task 6: Analyze Data and Prepare Final Report

The Study Plan developed in Task 3 will form the basis for the first section of the Final Report. Statistical analysis of the data collected shall be performed by Fort Environmental Laboratories with review by Valerie Cullinan of Battelle, Sequim as described in the approved Study Plan. It is anticipated that EPA will perform an independent analysis of the raw data comparing the alternative approaches in the context of life stages and endpoints to detecting

EDCs with known modes of action for the Final Report. This analysis may also be published in the scientific literature by U.S. EPA, ORD/MED.

The report will also contain a section on the QA program activities and QC data indicating

- Deviations, if any, from the QAPP or the Study Plan that were encountered and how they were documented;
- Problems, if any, concerning study quality or data quality that are encountered, the corrective actions employed and documentation of the deviations and problems and the impact on the data.

Fort Environmental Laboratories' Principal Investigator will prepare a Microsoft PowerPoint presentation of the results for delivery by him to the Endocrine Disruptor Methods Validation Subcommittee (EDMVS).

4.3 ORGANIZATION

Fort Environmental Laboratories, under the direction and oversight of Battelle, will conduct the assays specified under WA 3-11 as outlined according to the specifications and subsequent modifications by the EPA. A summary of the work assignment organization is shown in Figure 4-1.

The overall work assignment will be managed by **Mr. Michael Blanton,** the Work Assignment Leader (WAL). Michael Blanton is responsible for preparing the technical work assignment, assigning appropriate staff to complete specified tasks within this work assignment, and monitoring the progress of both technical and fiscal milestones, as outlined in the technical work plan. Mr. Blanton will report progress of Fort Environmental Laboratories on the work assignment to **Dr. David Houchens** at Battelle through a series of planned conference calls and through the use of written monthly reports.

General scientific direction and supervision of the work performed under this work assignment is provided by **Dr. Douglas Fort**. Dr. Fort is the Principal Investigator (PI) for the Frog Metamorphosis Assay Demonstration component of this contract; he is responsible for preparing the study protocol for submission and approval by the EPA. Dr. Fort will ensure that the study is being conducted according to the appropriate study protocol and animal care and use guidelines. **Mr. John Thomas**, the Study Team Leader will report to Dr. Fort and will oversee the laboratory components of the study.

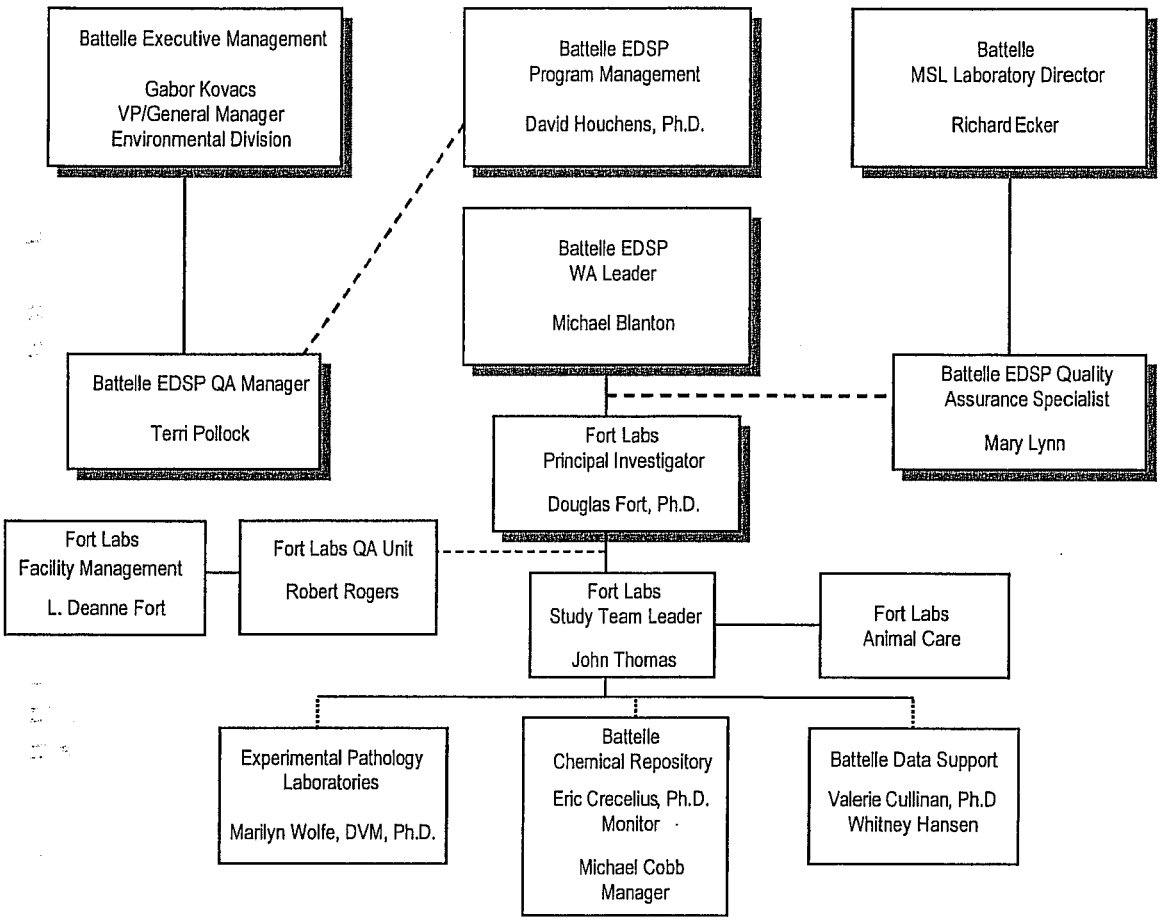


Figure 4-1. WA 3-11 Project Organization Overview

Xenopus laevis larvae used for these studies will be obtained from an in-house culture at Fort Environmental Laboratories where adults are injected with human chorionic gonadotropin (HCG) to induce reproduction. The study team leader, John Thomas, a Fort Environmental Laboratories employee, will provide support for embryo and larvae development over the course of the study to include ambient and water temperature, light intensity, and photoperiod monitoring, under the direction of Dr. Fort. Study team members Andra Noll and Brody Buzzard will assist Mr. Thomas, as needed, with the study to include daily feeding and tank cleaning activities.

Dr. Marilyn J. Wolfe, an independent contractor of her own firm, Experimental Pathology Laboratories, (located in Herndon, VA) will be responsible for histological analyses of thyroid glands. Mr. Thomas, Fort Environmental Laboratories, will prepare samples and embed organs to create slides for histological analysis. Dr. Wolfe will perform histological analyses and provide digital photo-documentation to illustrate changes, if any, in the thyroid glands of exposed frogs compared to thyroid glands of control frogs.

Dr. Valerie Cullinan is the lead statistician for the project and is responsible for overseeing the statistical design, analysis, and interpretation of the data. Dr. Cullinan has over 15 years of experience in the design, analysis, and interpretation of multidisciplinary experimental research. Dr. Cullinan will work with Dr. Doug Fort and report to Mr. Michael Blanton.

The Battelle, EDSP Quality Assurance Unit (QAU) is established as an objective, independent monitor of the work performed at Battelle. Thus, the Battelle, EDSP QAU, **Ms. Mary Lynn,** operationally informs Mr. Michael Blanton, who has overall responsibility for the Battelle ESDP QA program, of QA Program status and implementation. Ms. Mary Lynn will provide oversight and assist when requested in meeting QA objectives.

Mr. Robert Rogers of Fort Environmental Laboratories is the designated lead QA Unit and he is responsible for approving the QAPP and he will be the designated facility QAU for this work assignment. In this capacity, he will administer the QAPP, and will perform surveillances and assessments, review data, and provide project support during external assessments and facility inspections. The specific responsibilities of Mr. Rogers include:

1. Assist the Battelle, EDSP QAU and the Principal Investigator in preparing the individual QAPP for the WA by defining appropriate QA requirements according to EPA/QA-R5, EPA Requirements for Quality Assurance Project Plans for Environmental Data Operations.

2. Interact with the Principal Investigator to ensure that WA personnel understand QA and QC procedures.

3. Conduct technical systems audits (TSAs) and audits of data quality (ADQs) to evaluate the implementation of the program WAs with respect to the EDSP QMP, the WA QAPP, the Study Plan, and applicable program and facility SOPs.

4. Prepare and track reports of deficiencies and submit them to both line and program management.

5. Consult with the Principal Investigator and, as necessary, the EDSP Battelle QA Manager and Program Manager on actions required to correct deficiencies noted during the conduct of the WA.

6. Ensure that all data produced as part of the EDSP WA are maintained in a secure, environmentally protected archive.

7. Ensure, during the conduct of TSAs, that all staff participating on the EDSP are adequately trained.

8. Maintain complete facility-specific QA records related to the program.

9. Submit copies of resolved audits to the EDSP Battelle QA Manager.

10. Submit a QA Statement to the EDSP Battelle QA Manager and Program Manager with each written deliverable that describes the audit and review activities completed and any outstanding issues that could affect data quality or interpretation of the results discussed in the report.

11. Maintain effective communication with the Battelle QAU and the EDSP QA Manager.

12. Act as the facility's EDSP SOP Custodian for all SOPs received from the SOP Administrator.

Dr. Eric Crecelius of Battelle, Sequim will provide technical oversight for the team of professional chemists and laboratory technicians supporting the EDSP Chemical Repository and who will prepare, deliver, and store the chemical test samples to be used by all the participating laboratories on the program. Dr. Crecelius is the manager of the Environmental Chemistry group at the Battelle, Sequim facility and will be the monitor of the Chemical Repository.

Mr. Michael Cobb of Battelle, Sequim will have ultimate responsibility for the quality of all chemical test samples used on the program and for their timely delivery to the participating laboratories. Mr. Cobb is the manager of the EDSP Chemical Repository, in the Environmental Chemistry group at the Battelle, Sequim facility. He is responsible for ensuring that appropriate and comparable technical procedures are used for sample analysis, ensuring that performance evaluation samples, standard reference materials, and certified standards are a routine part of the laboratory quality control program, and for providing technical expertise to the analytical laboratories. He is also responsible for ensuring that analyses are scheduled and completed by their due dates, that holding times are met, and that reporting schedules are not compromised. He will also ensure that corrective actions are assigned and completed. Mr. Cobb reports the status of laboratory analyses and potential problems to the MSL Laboratory Director, Mr. Richard Ecker, to the WAL, Mr. Michael Blanton, and to EDSP Program and QA Managers, Dr. David Houchens and Ms. Terri Pollock respectively. Mr. Cobb reports directly to the MSL Laboratory Director, Mr. Richard Ecker.

As EDSP Manager, **Dr. David Houchens** will have ultimate responsibility for quality, timeliness, and budget adherence for all activities on the contract. He will also serve as the principal interface with the EPA's Project Officer on all contract-level administrative and technical issues. Because of the high level of subcontracting and purchases required by the program, such as test laboratory subcontracts and purchases of chemical supplies, Dr. Houchens will be assisted by an administrative deputy manager, **Mr. James Easley**. Mr. Easley will manage the procurement of all subcontracts, consultants, and purchased materials and services, and will facilitate schedule and cost control. He has played a similar role on ten other large, multi-year, level-of-effort task-order contracts for EPA. Thus, he will be able to assure that all purchases are compliant with government regulations and that EPA is provided timely, accurate accounting of these substantial costs in our monthly progress reports.

Ms. Terri Pollock, the EDSP QA Manager at Battelle, will direct a team of QA specialists, including **Ms. Mary Lynn** at Battelle, Sequim, who will monitor the technical activities on the chemical repository program and provide oversight to all associated QA

functions. Ms. Pollock will be responsible for reporting her findings and any quality concerns to Dr. Houchens. Ms. Pollock reports, for the purposes of this program, to **Mr. Gabor Kovacs**, Vice President and General Manager of Battelle's Environmental Division. This reporting relationship assures that the QA function is independent of the technical activities on the program.

5.0 PROBLEM DEFINITION/BACKGROUND

5.1 PROBLEM DEFINITION

In spite of increasing alarm over global population declines in amphibian species over the last 20 years, only recently has evidence of reproductive toxicity due to xenobiotic exposure been provided. Habitat destruction, fragmentation, or disturbance may be sufficient to explain some declines, but not all. Other potential causes include habitat acidification, predation, competition by introduced species (Wake, 1991), increased ultraviolet (UV) radiation resulting from atmospheric ozone depletion (Blaustein *et. al.*, 1994), and exposure to toxic environmental contaminants (Hall and Henry, 1992; Carey and Bryant, 1995). Amphibians may be particularly vulnerable to waterborne environmental contaminants due to their largely aquatic life histories and their highly permeable skin (Nieuwkoop and Faber, 1994).

Metamorphosis is a period of substantial morphological change in which an organism alters its mode of living and occurs in all major chordate groups with the exception of amniotes. In fact, metamorphosis is developmentally comparable to post-embryonic organogenesis in mammals. Three primary characteristics define metamorphosis, 1) change in non-reproductive structures between a post-hatch or larval state and sexual maturity, 2) form of the larvae enable it to occupy a unique ecological niche different from that used by the adult life stage, and 3) the morphological changes that occur at the conclusion of larval development depend on some environmental stimulus, either external (i.e., temperature or food supply), or internal (hormonal changes). Each of the three classes of amphibians, anurans, urodeles, and caecilians undergo metamorphosis, although not all species within each class metamorphose. For example, obligatory neotenic urodeles do not metamorphose, and reproduce as an aquatic "adult larvae". Three primary morphological changes occur during metamorphosis, 1) resorption or regression of tissue or organ systems that have primary function only in the larval life stage, 2) the remodeling of larval organ systems to their adult form, which are suitable only for the adult, and 3) *de novo* development of tissues in the adult that are not required by the larvae. These changes are most marked in anuran species, and less obvious in urodeles and caecilians. In each of the three classes of amphibians, metamorphosis is controlled by thyroid hormone (TH), although less is currently known about the role of TH in the metamorphosis of caecilian species. Amphibian metamorphosis has been most widely studied in anurans, primarily due to the dramatic nature of metamorphosis and the ease in use of anuran species in research. However, within the anurans, metamorphosis has only been reasonably well studied in three species, *Xenopus laevis* (South African clawed frog), *Rana catesbeiana* (bull frog), and *R. pipiens* (Northern Leopard frog). Anuran metamorphosis is separated into three distinct periods, premetamorphosis, prometamorphosis, and metamorphic climax. Premetamorphosis refers to a period of embryonic and early larvae development that takes place without thyroid hormone. Some advanced

morphological developments occur during this stage including hind limb bud development. More specific morphogenesis, such as differentiation of the toes and rapid growth (elongation) of the hind limbs, occurs during prometamorphosis. Biochemically, prometamorphosis is characterized by rising concentrations of endogenous TH. The final period is metamorphic climax in which a surge of TH triggers the final processes associated with metamorphosis, including forelimb development and resorption of the tail. Drastic internal transformations at the organ system, tissue, and biochemical levels are also taking place during prometamorphosis and metamorphic climax.

From an evolutionary standpoint, amphibians are distinctively separated phylogenetically from other vertebrates. However, specific aspects of the thyroid axis are conserved amongst most chordates at both the morphological and molecular levels. Thus, the conserved nature of the thyroid axis enhances the ability to use an amphibian, particularly an anuran, as a general model for evaluating thyroid disruption that can be extrapolated to other vertebrate species.

The thyroid axis represents one potential target for environmental chemicals. Environmental agents, toxicants, natural products, and complex mixtures can alter metamorphosis by interacting with the thyroid axis. Further, the complexity of the thyroid axis yields many different possible mechanisms of inhibiting metamorphic processes in amphibians at differing biochemical and molecular levels. Thus, from this end, use of amphibians to screen for thyroid disrupting chemicals or chemical mixtures as a representative chordate is not unreasonable.

To date, the debate on endocrine disruptors has mostly revolved around gonadal steroids including estrogens and androgens, because of controversy regarding their possible link to infertility, breast cancer, and lower sperm counts. Thus, the thyroid has received little attention comparatively. The effects of synthetic chemicals in the environment on thyroid function have been surveyed recently. This review confirms the hypothesis of thyroid disruption by environmental chemicals in wildlife and supports the need for human population and laboratory animal studies on compounds already identified as thyroid disruptors. The effects of over 40 pesticides and 45 industrial chemicals on the thyroid axis are described.

The amphibian metamorphosis screening assay is designed to detect substances that interact with the thyroid systems of vertebrates. It is thought that the inclusion of the amphibian metamorphosis assay in Tier 1 EDSP studies is important because amphibian metamorphosis provides a well studied thyroid dependent process on which to build an assay. Additionally, the amphibian assay is considered essential in the overall screening battery to detect substances, which may disrupt the vertebrate thyroid axis.

The primary experimental objective is to determine the differential sensitivity of beginning exposure with stage 51 and 54 larvae and to establish the necessary duration of exposure. The primary endpoints will be growth, mortality and developmental stage. To achieve this objective, two concurrent studies (one cohort exposed from stage 51; another cohort exposed from stage 54) will be conducted for each chemical with individuals from the same cohort of organisms. One will be initiated with stage 51 larvae and conducted for 21 days and the other with stage 54 larvae conducted for 14 days.

5.2 BACKGROUND

A previous work assignment in this contract (WA 2-20) was initiated to provide a detailed review of amphibian metamorphosis assays suitable for use in the EDSP. One of the candidate protocols described was a Prometamorphosis Assay with *Xenopus*. The recommended protocol outlined a method beginning with Nieuwkoop and Faber (1994) stage 51 or 54 and continuing for 14-21 days with key endpoints based on changes in developmental stage and thyroid histology.

The process of selection of the test chemicals is based on the selection of known compounds already identified as thyroid disruptors. Stock solution preparation for the remaining four chemicals will be determined in consultation with the technical experts from U.S. EPA, ORD/MED.

6.0 PROJECT/TASK DESCRIPTION

6.1 PROJECT OVERVIEW

The purpose of this work assignment is to perform a multiple chemical demonstration and evaluation of the amphibian metamorphosis assay as developed by the U.S. EPA, ORD/MED.

6.1.1 Exposure Regime

The embryos are held at approximately 21°C for four days to allow for hatching, at which time they are divided into groups of 250/50 L (5 larvae/L, which is the same density as will be used in the actual test) and maintained in a constant flow (50 mL/minute) of dilution water at approximately 21°C until they reach the desired developmental stages used in the studies.

A summary of the experimental conditions to be used in the present study are provided in Table 6-1. At 14-17 days post-hatch, larvae will be anesthetized using 100 mg/L of MS-222 (tricaine methane sulfonate) buffered with 200 mg/L of sodium bicarbonate, sorted by stage (Nieuwkoop and Faber, 1994), and allowed to recover from anesthesia in dechlorinated tap water. After recovery, 200 stage 51 tadpoles will be counted and randomly divided into tanks containing 4 L of test solution (20 tadpoles/tank, at a density of 5 tadpoles/L). Remaining tadpoles will be placed back in dechlorinated tap water until they reach stage 54 (24-26 days post-hatch) when they will be used to start (using similar methods used for the stage 51 tadpoles) the stage 54 portion of each study. Stage 51 and 54 larvae will be exposed to 4 test concentrations and a control. Each test concentration and control will be replicated with 20 organisms per replicate. Concentrations for PTU are scheduled to be 2.5, 5.0, 10.0, and 20.0 mg/L and for T4 should be 0.5, 1.0, 2.0, and 4.0 ug/L. Proposed concentrations for methimazole are 6.25, 12.5, 25, and 50 mg/L. Concentrations for the other three chemicals will be determined in consultation with the technical experts from U.S. EPA, ORD/MED and documented as an addendum to this QAPP.

Table 6-1. Experimental Conditions

Test Type	Metamorphosis Assay
Test Animal	*Xenopus laevis* Larvae
Initial Larval Stages	Stage 51 and Stage 54
Test Duration:	21 Days for Stage 51 and 14 Days for Stage 54
Number of Test Substances:	6
Dilution Media:	Dechlorinated (aged) Tap Water
Test Solution Volume/Tank	4.0 L
Larvae/Tank	20
Photoperiod	12 hr Dark and 12 hr Light
Light Intensity at Water Surface	61 to 139 lumens
Water Temperature	21°C ± 1°C
Number of Test Concentrations	4
Number of Controls	1
Replicates/Test Concentration and Control	2

6.1.2 Test Termination

Once in the test system, mortality observations will be made daily and any dead larvae will be removed. On exposure day 21 and 14 for the stage 51 and stage 54 experiments, respectively, all organisms in the study will be anesthetized in MS-222, evaluated for developmental stage, weighed, and preserved in Bouin's solution for possible histological analysis of the thyroid glands.

6.1.3 Endpoints

The primary endpoints, which will be evaluated in the present study, will be mortality, developmental stage, growth, and thyroid histology. These endpoints including the measurement format and the frequency of measurement during the proposed studies are described in Table 6-2. Apical morphology will be measured as a secondary endpoint.

Table 6-2. Primary and Secondary Endpoints

Primary Endpoint	Frequency of Measurement	Measurement Format
Mortality	Daily	% Mortality at Test Conclusion
Developmental Stage	At Test Conclusion	Individual Stage Distribution
Growth	At Test Conclusion	Weight
Thyroid Histology	At Test Conclusion	Pathology Summary
Secondary Endpoint		
Gross Morphology	At Test Conclusion	% External Abnormality

6.1.4 Test Materials

Six test chemicals, which are summarized in Table 4-1, will be evaluated in the present study including: PTU, phenobarbital, pregnenolone-16-a-carbonitrile, T4, dexamethasone, and methimazole. Primary stock solutions for PTU (700 mg/L) will be made in glass or Teflon™ coated containers by dissolving the chemical in dechlorinated tap water heated to 40°C and mixed using a high-speed magnetic stirrer. The working stock solution (20.0 mg/L) will be made by diluting the primary stock solution with dechlorinated tap water. Primary stock solutions for T4 (4.04 mg/L) will be made in glass or Teflon™ coated containers by dissolving chemical in 200 mL of 50 millimolar sodium hydroxide (NaOH) solution and diluting to 18 L with dechlorinated tap water. This solution will then be added to dechlorinated tap water to make the working stock solution (4.0 µg/L). Primary stock solutions for methimazole (2,500 mg/L) will be made in glass or Teflon™ coated containers by dissolving the chemical in dechlorinated tap water and mixed using a high-speed magnetic stirrer. The working solution (50 mg/L) will be made by diluting the primary stock solution with dechlorinated tap water. Stock solution preparation for the remaining three chemicals will be determined in consultation with the technical experts from U.S. EPA, ORD/MED. All chemicals should be confirmed to be at least 98% pure. At least four test chemical concentrations and a control will be tested in each assay, with two replicates per treatment.

6.2 SPECIFIC STUDY OBJECTIVES

The primary experimental objective is to determine the differential sensitivity of beginning exposure with stage 51 and 54 larvae and to establish the necessary duration of exposure. The primary endpoints will be growth, mortality, developmental stage, and thyroid histology. To achieve this objective, two concurrent studies (one cohort exposed from stage 51; another cohort exposed from stage 54) will be conducted for each chemical with individuals from the same cohort of organisms. One will be initiated with stage 51 larvae and conducted for 21 days and the other with stage 54 larvae conducted for 14 days.

7.0 QUALITY OBJECTIVES AND CRITERIA FOR MEASUREMENT DATA

A number of the specified tasks incorporate quality objectives relative to the accuracy of the measures to be made (refer to Table 7-1). Standard applications of data quality objectives do not readily apply to this work. Measurement systems and equipment will be calibrated before use. Much of the data to be collected are counts, observations, weights, and measures, which are discussed in detail in Section 9.4. Developmental stage and external abnormality assessments depend on the level of training of staff. Fort Environmental Laboratories' SOP 10.1, Developmental Staging and Scoring of Test Specimens, will provide a strong reference for staff training and completing this task. Pathology experts will perform histology.

Table 7-1. Quality Objectives and Criteria for Measurement Data

Parameter	Units	Comments
Organism Care and Breeding		
Temperature	°C	Thermometer
pH	su	Meter/Electrode
DO	mg/L	Meter/Probe
Flow	Target 50 mL/min	Stopwatch and Graduated Cylinder
Feeding	Twice/day weekdays Once/day weekends	Record feeding
Test Initiation		
Sorting by Stage 20	NA	Microscopic Evaluation
Sorting by Stage 51	NA	Microscopic Evaluation
Sorting by Stage 54	NA	Microscopic Evaluation
Test Concentration Sampling	mL	
Test Conditions		**Monitored Daily**
Photoperiod	12 hr light: 12 hr dark	24 hr Timer Switch
Light Intensity at Water Surface	61-139 Lumens	Dual Range Light Meter (traceability to NIST)
Water Temperature	21°C ±1°C	Thermometer
Daily Observations		
Mortality	Counts	Observations Removal of dead larvae
Diluter System Performance	Cycles	Read meter counts
Weekly Observations		
Exposure concentrations	mg/L or ug/L	Exposure concentrations will be analyzed at test initiation, at least weekly thereafter, and at test termination.
Test Termination		
Mortality	Counts	Observations
Developmental Stage	NA	Microscopic Evaluation
Organism Weight	Individual body weights (g)	Analytical Balance
Thyroid Histology	Visual and histological assessment	
Gross Morphology	% external abnormality	Microscopic Evaluation

8.0 SPECIAL TRAINING/CERTIFICATION

Chemicals PTU and T4 may be potentially harmful if swallowed, inhaled, or absorbed through skin. Therefore, staff handling the preparation and distribution of these chemicals will be trained relative to the hazards and safe handling of PTU and T4 and will wear appropriate personal protective equipment as determined by an Environmental Safety and Health subject area expert. Material Safety Data Sheets (MSDS) will be provided prior to handling any of the six test chemicals. Also refer to Section 10.1, Test Substance, below.

The only requirements for specialized training for the exposures to be performed under this WA are related to the use of phenobarbital, a controlled substance. Fort Environmental Laboratories will obtain the appropriate research permit to use this chemical, adopt controlled substance inventory, custody, handling, and disposal procedures. Fort Environmental Laboratories will appoint a Controlled Substance Coordinator to direct these tasks. In addition, Fort Environmental Laboratories will demonstrate to EPA's satisfaction its ability to do any of the tasks listed in the protocols by offering to have each technician who will be doing these tasks demonstrate proficiency (i.e., during an audit).

9.0 DOCUMENTS AND RECORDS

9.1 RETENTION OF SPECIMENS AND RECORDS

All specimens and records that remain the responsibility of Fort Environmental Laboratories will be retained in the project data files for the length of time stipulated in the contract. The files will remain with the Principal Investigator until the study is complete and then be placed in archived files. Data files, correspondence, QA records, and reports will be archived indefinitely. Storage of archived files will be maintained in a two-tier manner. Archived files will first be kept in locked, fire-proof file cabinets, located in the study facility laboratory, for a period of 1 year or until final report has been reviewed and accepted by the client. After which time, the files will be transferred to storage file boxes and archived off premises at a secured commercial storage facility. The preserved test specimens will be labeled and archived in the laboratory until such time as specimen degradation renders the samples useless. Records are maintained according to a policy of limited access. The archivist is responsible for archiving and retrieving WA materials. An archive inventory will be maintained and storage capability will provide for the expedient retrieval of materials. Specimens and samples will be disposed of only after assessing that they no longer afford evaluation.

9.2 QUALITY ASSURANCE PROJECT PLAN

This QAPP will be distributed to Fort Environmental Laboratories and Battelle project participants, initially, and whenever revised. Previous versions either will be marked as "obsolete" when newer versions are distributed, or will be collected and destroyed so that there is no confusion regarding the version in effect. The right-justified document control header example shown here

Version 1
Month Year
Page x of y

is used to ensure that revision numbers and dates are obvious to document users. The QAPP will be reviewed annually if the project extends beyond one year, and a determination made to either modify the document, based on new or modified project requirements, or leave as is. Copies of the QAPP are maintained, tracked, and managed by Battelle.

9.3 STANDARD OPERATING PROCEDURES

Applicable procedures for conducting work exist within the Fort Environmental Laboratories QA Program. The following existing SOPs will be followed:

LABORATORY EQUIPMENT

3.1. Analytical Balance (Ohaus E10640)
3.2. Top Loading Balance (Ohaus CS200)
3.3. Conductivity Meter (Corning Model 441)
3.4. Dissolved Oxygen (D.O.) Meter (YSI Model 52)
3.5. pH Meter (Denver Basic)
3.6. Microscope (Fisher Stereomaster Zoom)
3.7. Spectrophotometer (Hach DR/2010)
3.8. Water Treatment Systems
3.10. Light Meter

ADMINISTRATIVE / DOCUMENTATION

4.1. Standard Operating Procedure
4.2. Study Protocol
4.3. Documentation of Error Corrections
4.4. Personnel Qualifications and Training
4.5. Study Personnel Responsibilities
4.6. QAU Study Protocol Review
4.7. QAU Study Inspections
4.8. QAU Study File and Report Audits
4.9. QAU Inspection Reports, Audit Reports, and Report Routing
4.10. Study File Document Collection and Organization
4.11. Maintenance of Study Archives
4.12. Documentation and Tracking of Controlled Substance

ANALYTICAL CHEMISTRY TEST METHODS

5.1. Temperature
5.2. pH
5.3. Dissolved Oxygen (D.O.)
5.4. Total Hardness
5.5. Conductivity
5.6. Total Alkalinity

5.7. Ammonia-Nitrogen
5.8. Total Chlorine

SAMPLE HANDLING AND PREPARATION
6.1. Sample Tracking and Handling
6.2. Waste Collection, Storage, and Disposal

ANIMAL CULTURE HUSBANDRY
8.1. *Xenopus* Tadpole and Froglet Care and Maintenance
8.2. *Xenopus* Adult Frog Care and Maintenance
8.3. *Xenopus* Breeding
8.4. *Xenopus* Egg Collection

BIOASSAY METHODS
9.1. Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX)

TEST CULTURE SCORING FOR ABNORMALITIES
10.1. Scoring of Test Specimens

TEST CULTURE PRESERVATION / CATALOGUING
12.1. Archiving of Test Specimens

This project requires requesting test chemicals from the EDSP Chemical Repository. The EDSP documents directing this work will be followed.

Routine animal care procedures will also be followed. Data reporting will be in conformance with:

EDSP.D-003-01	Transmission of Information to the EDSP Data Coordination Center (DCC)
EDSP.D-013-01	Information to be Delivered for QA Documentation to the EDSP Data Coordination Center (DCC)

A Training Assignment matrix will be used to define the required training for each project participant based on the required work activities.

Deviations from SOPs will be documented using the EDSP Deviation Documentation Form from the EDSP QMP. The form provides tracking and documentation of both miscellaneous issues and deviations from SOPs. All issues documented using this form must be assigned a deviation number by the Fort Environmental Laboratories' QA Unit Manager.

9.4 DATA REPORTING PACKAGE

Records for the data report package are listed below. Except for the animal room environmental conditions and the statistical analysis records, the data are recorded manually on

data forms. All data forms include a title identifying the type of data to be recorded, the unique study name, and the initials and date of the data recorder(s) to authenticate the records:

- Study Protocol and any Amendments,
- List of any Protocol Deviations,
- List of applicable Standard Operating Procedures,
- Study Journal to document daily observations and comments,
- Culture Maintenance Log,
- Culture Breeding Log,
- Study Tracking Sheet,
- Test Information Sheet,
- Culture Mortality/Stage Data Sheet,
- Specimen Weight Sheets,
- Chain of Custody Forms for samples received or shipped,
- Sample Tracking Log for tracking work order and sample numbers assigned,
- General Water Chemistry Sheet,
- Statistical Analysis Records (see Section 19.3 for software information), and
- Correspondence.

9.4.1 Draft and Final Reports

The Principal Investigator will generate draft reports within three months of the completion of the various phases of the studies. Project staff and Fort Environmental Laboratories QA Unit Manager will review draft reports. The Work Assignment Leader and EDSP Program Management will review final reports before reports are forwarded to U.S. EPA.

General format requirements include:

1. ***"Draft or Final Report" and "Page 1 of _n", where n is the total number of report pages on the title page***

2. ***Contract number in the header, right justified***

3. ***Text, which includes the following sections:***

- Abstract
- Objectives
- Materials and Methods
- Results

- Discussion
- Conclusions
- References
- Protocol Deviations, if any
- Summary in-life and endpoint data with statistical analyses when applicable.

4. Appendices containing the individual animal data (in-life and necropsy), the protocol and any amendments, and historical control dataset. A histopathology report will also be provided.

9.4.2 Data Package Report Contents

Data package report contents will include the following in addition to the data package contents specified in Section 19.1:

9.4.2.1 Test Substance. The report will include a detailed description of the test substances, including information on the CAS numbers, sources, lot numbers, and purity. However, it needs to be stated that test substance will be provided by the EDSP Chemical Repository, which is responsible for purchasing, testing, and preparing the test substance at the request of the WA 3-11 PI.

9.4.2.2 Protocol and Any Amendments. The data package will document the methods (protocol) used, list of any protocol deviations, provide a data summary, and the results of any statistical analysis performed, The recommended test protocol is intended to be a product of WA 3-11; this is the basis of this WA. However, there may be unforeseen circumstances that might lead to a protocol deviation.

9.4.3 QA Assessment Reports

QA assessment reports (see Section 20) are maintained as confidential files by the QA Unit Manager.

9.4.4 Status Reports

Monthly status reports will be provided to the EDSP Program Manager and WAL.

9.5 ENVIRONMENTAL CONDITIONS

Fort Environmental Laboratories' toxicological testing facilities are state of the art with over 3,800 square feet of space. This is the only commercial amphibian and reptilian research center in the U.S. Additional facilities available include minimum exposure incubators, and over 2,500 square feet of analytical chemistry laboratory space. There are specific requirements for environmental conditions for *Xenopus laevis* larvae to be used in this study. Refer to Table 6-1 above.

Housing and Environmental Conditions

Light will be provided by fluorescent lights that emit a spectrum simulating that of daylight (61-139 lumens). The photoperiod in the room housing the test animals will be 12 hours dark and 12 hours light, maintained by a 24 hour dial timer switch. The water temperature requirement ranges from about 20 to 22°C.

10.0 EXPERIMENTAL DESIGN

10.1 TEST SUBSTANCE

All test substances will be analytically pure (at least 98%), and the purity will be reported, along with the percentage of each impurity.

Chemical	Description
6-n-Propyl-thiouracil (PTU)	PTU is being tested because it has been established as a known thyroid axis inhibitor. PTU may be potentially harmful if swallowed, inhaled, or absorbed through skin. There appear to be no significant health effects from the use of this chemical at the specified concentrations when used with appropriate personal protection equipment (PPE). Avoid mixing with metals and oxidizing materials.
Phenobarbital	Phenobarbital is being tested because it has the potential to disrupt thyroid axis homeostasis. Phenobarbital is classified as a Schedule IV controlled substance by the U.S. Drug Enforcement Administration (DEA). It will be inventoried and dispersed for use by the Controlled Substance Coordinator. There appear to be no significant health effects from the use of this chemical at the specified concentrations when used with appropriate PPE.
Pregnenolone-16-alpha-carbonitrile	Pregnenolone-16-alpha-carbonitrile is being tested because it has the potential to disrupt thyroid axis homeostasis. Chemical may be potentially harmful if swallowed, inhaled, or absorbed through skin. There appear to be no significant health effects from the use of this chemical at the specified concentrations when used with appropriate PPE.

Chemical	Description
Dexamethasone	Dexamethasone is being tested because it is a possible thyroid axis stimulator. There appear to be no significant health effects from the use of this chemical at the specified concentrations when used with appropriate PPE. Avoid mixing with oxidizing materials.
Methimazole	Methimazole is being tested because it has been established as a known thyroid axis inhibitor. There appear to be no significant health effects from the use of this chemical at the specified concentrations when used with appropriate PPE.
Dilution Water	Dechlorinated (aged) tap water will be used as the diluent for this study. Tap water will be passed through a 5 μm pre-filter for solids removal, a 1.2 cf carbon canister for chlorine removal, and a 5 μm post-filter.

10.2 TEST ANIMALS

The *Xenopus laevis* larvae used for these studies will be obtained from an in-house culture where adults are injected with human chorionic gonadotropin (HCG) to induce reproduction. The genus and species will be verified by a taxonomic identification referencing the appropriate key(s) used. The embryos are held at approximately 21°C for four days to allow for hatching, at which time they are divided into groups of 250/50 L (5 larvae/L, which is the same density as will be used in the actual test) and maintained in a constant flow (50 mL/minute) of dechlorinated tap water at 21°C ± 1°C until they reach the desired developmental stages used in the studies. Larvae are to be fed a blended mixture of TetraFin® (Tetra Sales, Blacksburg, VA, USA), *Spirulina* algae discs (The Wardley Corporation, Secaucus, N.J., USA), Silver Cup Trout Starter (Nelson & Sons Inc., Murray Utah, USA) along with live brine shrimp (Bio-Marine® Brand, Bio-Marine Inc., Hawthorne CA, USA) twice/day on weekdays and once/day on the weekends regardless of treatment.

10.3 TOTAL NUMBER AND AGE OF TEST ORGANISMS

The total number and age of *Xenopus laevis* to be used in testing is 20 organisms/tank X 2 replicates X 5 test concentrations (including controls) X 6 chemicals X 2 stages (51 and 54) = 2,400 larvae.

10.4 DURATION OF TEST

At 14-17 days post-hatch, larvae are anesthetized and 200 stage 51 larvae will be counted and randomly divided into tanks (20 larvae/tank). Remaining larvae will be placed back in dechlorinated tap water until they reach stage 54 (24-26 days post-hatch) when they will be used to start (using similar methods used for the stage 51 tadpoles) the stage 54 portion of each study. Stage 51 and 54 larvae will be exposed to 4 test concentrations and a dilution water control

(dechlorinated tap water). On exposure day 21 and 14 for the stage 51 and stage 54 experiments, respectively, all organisms in the study will be terminated for endpoint assessment. Therefore, test duration is estimated to be 21 days and 24 days for the stage 51 and stage 54 experiments, respectively.

10.5 OBSERVATIONS OF RECORD

Disposition of Test Organisms: At test termination, all surviving organisms will be euthanized using MS-222 and preserved in Bouin's Solution. All euthanasia methods will be documented in the raw data. Fifty animals (10 from each chemical plus control) from each test will be set aside for potential thyroid histology. All remaining organisms will be archived for further study reference, if needed.

10.6 QUARANTINE

Quarantine procedures do not apply because all test organisms are from an in-house culture *of Xenopus laevis* larvae.

10.7 ANIMAL IDENTIFICATION

Individual animal identification is not required. Exposure tanks will be externally labeled as to test chemical, concentration and replicate.

10.8 LIMITATION OF DISCOMFORT

Although not anticipated, some toxicity may occur with exposure to the test chemicals. Discomfort will be limited, in that if any animal becomes severely debilitated or moribund at any time during the study, it will be humanely terminated by use of MS-222.

10.9 BREEDING

The *Xenopus laevis* larvae used in this study are as randomly bred as possible within the confines of the population available. All animals are monitored daily for health and any possible abnormalities. Genetic vigor of test population is maintained by introducing new lines of animals on a routine basis. Since breeding is controlled by the pairs selected, records of breeding success and fecundity can be maintained.

11.0 SAMPLING METHODS

Once in the test system, mortality observations will be made daily and any dead larvae will be removed. On exposure days 21 and 14 for the stage 51 and stage 54 experiments respectively, all organisms in the study will be anesthetized in MS-222, evaluated for developmental stage, weighed, and preserved in Bouin's solution for possible histological analysis of the thyroid glands.

One L samples of test concentrations will be collected in one L glass amber bottles provided by Battelle, Sequim and shipped to the EDSP Chemical Repository under Chain of Custody using cold preservation to maintain the cooler temperature between 2-6°C.

12.0 SAMPLE HANDLING AND CUSTODY

Sample handling is specified in the study protocol. Samples that may be shipped to Experimental Pathology Laboratories (Herndon, VA) for histological analysis of the thyroid glands will be shipped overnight via Fed Ex. Custody of samples for verification of exposure concentrations is addressed above and in the SOP 6.1, Sample Tracking and Handling.

12.1 DIET SAMPLING AND ANALYSIS

The diet is blended mixture of TetraFin®, *Spirulina* algae discs, and Silver Cup Trout Starter along with commercially available live brine shrimp. The diets are not expected to contain the six test chemicals. Manufacturer's diet analyses will be documented. A determination will be made among Battelle, the EPA, and the WAM as to the efficacy of collecting and analyzing the blended feed to evaluate the presence of the test substances in the frog diet. If samples are deemed necessary, containers and recommended diet sample weights will be obtained from the Chemical Repository at Battelle, Sequim.

12.2 HISTOLOGY SAMPLES

At termination, organisms in the study will be anesthetized in MS-222, evaluated for developmental stage, weighed, and preserved. Those specimens chosen for possible histological analysis of the thyroid glands by Experimental Pathology Laboratories (Herndon, VA) will be preserved in Bouin's solution.

Bouin's solution is a picric acid- acetic acid –formaldehyde fixative and preserving fluid. Samples preserved in Bouin's solution will be shipped by Federal Express. The samples will either be shipped as a non-hazard or a corrosive liquid, depending on volume of preservatives and final pH (if the pH is 2 or less, it is classified as a corrosive). It is recommended that samples be fixed, and shipped with a Ziploc bag with a paper towel moistened with fixative. Excess fixative can be drained off and the bag vacuum-packed. This will meet requirements for shipping as a non-hazard. Hazardous samples may require additional packaging and labeling.

13.0 ANALYTICAL METHODS

The assays will be performed as specified in the study protocol presented in the approved study plan. No deviations from the protocols are expected, but if deviations occur, they will be documented and reported to the EDSP and EPA managers.

The analytical methods known to date to test for the six exposure chemicals are listed in Table 4-1. The methods will be documented when developed and become part of the data

documentation. Test chemicals need to be 98% pure, soluble in water, and stable for the duration of the test.

All test substance calculations will be based on the purity of the test substance as received or will be corrected for purity of the active ingredient in the test substance.

Staff handling the preparation and distribution of the exposure chemicals will be trained relative to the hazards and safe handling of each chemical and will wear appropriate personal protective equipment as determined by an Environmental Safety and Health subject area expert. Area access is restricted by posting appropriately labeled signs, identifying potential hazards if any, on doors leading into the study area.

To minimize cross-contamination among the exposures, each exposure will be conducted separately in time from the others. The aquaria will be washed in concentrated cleaning solution designed for laboratory use, rinsed with tap water to remove all soap, and triple rinsed with deionized water between tests. All mixing equipment will be cleaned with ethanol and rinsed using deionized water between each test and dose. Where possible, dedicated mixing vessels will be used per dose.

14.0 QUALITY CONTROL MEASURES

The experimental design was determined to provide a statistically-valid sample size for all phases of the study. Refer to Section 19.2, Statistical Analyses.

15.0 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

The following equipment is needed to perform the work at Fort Environmental Laboratories:

Aquaria
Diluter System
Chemical Metering System
Thermometer
pH Meter and Electrode
DO Meter and Probe
Timer Clock for Photoperiod
Light Meter
Balances – body, tissue weights, feed
Calibration Verification Weight Set (Class 1)
Refrigerators for storing feed
Refrigerators/freezers for storing samples.

All equipment will be evaluated to ensure that it is functional and operable before use. Equipment requiring calibration will be calibrated as specified below.

16.0 INSTRUMENT/EQUIPMENT CALIBRATION AND FREQUENCY

The necessary project equipment is summarized in Table 16-1. Each time a calibration or calibration verification is made it needs to be recorded – either in the instrument calibration book, on an appropriate data sheet. The record will note the date, initials of who made the calibration, the result, the expected result, and whether or not the calibration was within the acceptable limit. If the result is not within the acceptable limit, the corrective action must be documented.

Table 16-1. Project Equipment Calibration and Frequency Requirements

Measure/ Use	Measuring or Test Equipment	Manufacturer/ Model	Serial Number or Unique ID	FEL Location	Primary Calibration	Secondary Calibration
Weight (mg/g)	Analytical Balance	Ohaus E10640	F0241119500 150	Chem Lab Room	Aldinger Co., Dallas TX, annual	Daily with use, Std. Class 1 Weight Set
Weight (mg/g)	Top –loading Balance	Ohaus CS200		Chem Lab Room	Aldinger Co., Dallas TX, annual	Daily with use, Std. Class 1 Weight Set
Weight (mg/g)	Calibration Verification Weight Set	Denver Instrument Company Class 1 NIST-traceable	01-J54903-42	Chem Lab Room	Does an external group calibrate your weight set at some frequency? NIST-traceable	Check with use
Diluter System	Proportional Diluter	Environmental Consulting & Testing		Exposure Room #3		Daily monitoring
Chemical Metering System	Metering Pumps			Exposure Room #3		Daily monitoring
Animal care	Aquaria: Temperature			Exposure Room #3, Chem Lab Room, Chem Lab Room	NIST traceable, general monitoring use; Calibrated daily using standards; Internal calibration daily	Daily monitoring
	pH	Denver Meter/Basic	14071			Daily monitoring
	DO	YSI Meter/Model 52	00E0085			Daily monitoring
Animal care	Photoperiod	24 hour Dial Timer Switch	T103	Exposure Room #3		Daily monitoring
Animal care	Light Meter	Fisher Scientific	0666263	Exposure Room #3	NIST traceable	Daily monitoring
Animal care	Flow Rate	Manual Measurement		Exposure Room #3		Daily monitoring
Sample/ Reagent Storage	Laboratory Refrigerator@ 4°C			Chem Lab Room	NIST traceable thermometer	Weekly monitoring
Feed Storage	Freezer if applicable. Temp -20 °C			Exposure Room #2	NIST traceable thermometer	Weekly monitoring
Feed Storage	Refrigerator if applicable @ 4°C			Exposure Room #2	NIST traceable thermometer	Weekly monitoring

16.1 BALANCES

Fort Environmental Laboratories' balances are calibrated annually by an external contractor (Aldinger Company, Dallas, TX), and verified daily as specified in SOP, 3.1, Analytical Balance (Ohaus E10640) and SOP 3.2, Top Loading Balance (Ohaus CS200).

Balances will be calibrated daily and verified with certified Class 1 weights. Calibration verification weights will be recorded in the appropriate balance logbook and the % standard weight recovery plotted in the QC Performance Chart. If weight readings are not within the specified tolerance, two more measures will be made, and an average weight computed. If this average is not within the specified tolerance, then corrective actions will be implemented. The first action will be to ensure that the balance is level and that the certified weight is clean and free from degradation. Another weight from a different weight set might also be measured. If the calibration cannot be verified, then the balance will be placed on a service call list. All maintenance, both routine and major, will be recorded in the balance logbook, which will be kept with the balance.

16.2 pH METER

The pH meter (Denver Instruments Basic Meter) will be calibrated daily, prior to initial use, using standardized buffer solutions (pH 7, pH 4, and/or pH 10), as specified in SOP 3.5. Calibration verification measurements will be recorded in the pH meter logbook and plotted in the QC Performance Chart located in the logbook. If pH readings are not within the specified tolerance, the meter will be recalibrated and measurements taken again. If pH readings are still outside tolerance specifications, corrective actions will be implemented (to include replacing standard buffer with fresh solution and electrode maintenance as outlined in the manufacturer's manual). If the problem can not be resolved, then the meter and electrode will be placed on a service call list. All maintenance, both routine and major, will be recorded in the pH meter logbook, which will be kept with the meter.

16.3 DO METER

A YSI Model 52 DO Meter will be used to measure the dissolved oxygen. The meter will be calibrated daily, prior to initial use, or more often if necessary. The meter will be calibrated, based on altitude, by placing the probe in air at 100% relative humidity and following the manufacturer's calibration procedures as outlined in SOP 3.4. Readings will be recorded in the DO Meter logbook. All maintenance performed on the DO meter and probe, both routine and major, will be recorded in the pH meter logbook, which will be kept with the meter.

16.4 LIGHT METER

A Fisher Scientific dual range light meter with certificate of traceability to NIST will be used to measure light intensity (SOP 3.10). Light intensity will be measured at the water surface in aquaria daily and recorded in study journal. Adjustments will be made as needed by additional or less lighting.

16.5 PHOTOPERIOD FLOW RATE

Photoperiod is controlled using a 24 hour dial time switch. The light and dark cycles are verified daily by checking clock setting in timer and adjusting as needed.

16.6 FLOW RATE

Flow rates to the aquaria will be measured daily using a stopwatch and graduated cylinder and recorded in the study journal. Flow rates will be adjusted as needed to maintain target flow rate using adjustable Teflon™ tips.

16.7 REFRIGERATORS AND FREEZERS

Refrigerators and freezers will be subjected to weekly temperature monitoring (more often if circumstances deem it necessary) and documented in the Refrigerator / Freezer Temperature Record / Maintenance Logbook in accordance with SOP 5.1. If for any reason recorded temperatures fall outside established tolerance limits, corrective action will be taken and documented in the logbook. After corrective action is taken, temperature will be monitored until it is within acceptable limits. If corrective action does not rectify the problem, then the appliance will be placed on a service call list. All maintenance, both routine and major, will be recorded in the logbook.

17.0 INSPECTION / ACCEPTANCE OF SUPPLIES AND CONSUMABLES

Upon receipt, purchased items are inspected for conformance to quality requirements prior to use. All expiration dates are expected to be within the expected use of the product, when applicable.

18.0 NONDIRECT MEASUREMENTS

No collection of any samples or sample data is obtained from non-direct measures such as computer databases or programs.

19.0 DATA MANAGEMENT

19.1 DATA MANAGEMENT OVERVIEW

Data will be collected on data sheets according to the end points listed above. In general, data are entered into Excel spreadsheets and analyzed using SigmaStat® or SysStat® (SPSS, Corte Madera, CA), SAS, Statview, or Minitab using the statistical and data presentation abilities of the applications.

19.2 STATISTICAL ANALYSES

Developmental stage will analyzed based on the distribution of stages after 14 and 21 days of exposure for both tests conducted with stage 51 and 54 larvae. The non-parametric Kruskal-Wallis one way analysis of variance will be conducted on the entire data set for each stage. Pair wise comparisons between treated and control treatments will be conducted using the Mann-Whitney U test, with a significance level of $p \leq 0.05$. Wet weight will be analyzed by analysis of variance followed by pair wise comparisons between treated and control using the Bonferroni method, with a significance level of $p \leq 0.05$.

19.3 DATA TRANSFER

Data will primarily be collected by hand-entering data on data sheets. Data will be transferred to Excel files and verified. 100% verification is required when transferring hand entered data to an electronic database. Excel data can then be analyzed using the applications available.

20.0 ASSESSMENTS AND RESPONSE ACTIONS

The QA Unit Manager may perform assessments on WA 3-11 activities and operations affecting data quality, but will at a minimum review the raw data and final report. Assessments of important phase activities and operations will be identified and scheduled by the PI and the QA Unit. Any findings will be reported to the PI and the Work Assignment Leader and other line management as necessary to ensure that the requirements in relevant SOPs, WA protocols and QAPP, and the QMP are met. The assessments for this WA include Technical Systems Audits (TSA) and Audits of Data Quality (ADQ). Performance Evaluations do not apply to this QAPP.

20.1 TECHNICAL SYSTEMS AUDITS

A TSA is a process by which the quality of a WA is assessed through evaluating a WA activity's conformance with the protocols, applicable facility or program SOPs, the WA QAPP, and the EDSP QMP. The acceptance criteria are that WA activities and operations must meet the requirements of these planning documents or be explained and evaluated in a deviation report by the Principal Investigator.

20.2 TYPE, SCHEDULING, AND PERFORMANCE OF TECHNICAL SYSTEMS AUDITS

The QA Unit Manager, with the concurrence of the Principal Investigator, will determine the critical phases of WA 3-11 if any, based on the activities specified in the protocols. Whenever possible, TSAs should be done at the commencement of the WA critical phase to ensure WA integrity based on compliance with the protocols and QAPP. Critical phases targeted for TSAs may include:

- QAPP and/or procedure review,

- Personnel training files for documentation that EDSP SOPs and WA QAPP have been read and understood by WA personnel before any activities begin, and
- Calibration and data records.

During the TSA, EDSP QA team members record observations to be used later in preparing the audit report. EDSP QA team members observe the procedure, data recording, and any equipment maintenance and calibration procedures and/or documentation, noting whether or not the activities adhered to the WA protocols and QAPP, applicable SOPs, and the EDSP QMP. Any findings will be communicated to the technical personnel at the completion of the WA activity unless an error could compromise the WA (e.g., lost or broken samples). EDSP QA team members immediately notify the Principal Investigator in person or by telephone and/or e-mail of any adverse findings that could impact the conduct of the WA. This direct communication is also documented in the audit report.

20.3 AUDITS OF DATA QUALITY

An ADQ is a process by which the accuracy of data calculations and reporting is assessed to ensure that the reported results are of high quality and accurately reflect the raw data and that the executive summary accurately describes the materials used in the WA. The acceptance criteria for the ADQ are that data collection, analysis, and reporting must meet the requirements of the applicable facility and program SOPs, the WA protocols and QAPP, and the EDSP QMP or be explained and evaluated in a deviation report.

20.4 SCHEDULING AND PERFORMANCE OF AUDITS OF DATA QUALITY

Direct and frequent communication between the Principal Investigator and the QA Unit Manager will provide for sufficient time to perform an ADQ, so that the submission date of the audited executive summary meets that specified in the WA 3-11 protocols. The scheduling process should also allow for a reasonable amount of time for corrections and subsequent verification of the corrections by QA.

WA personnel submit all data and records for review at the time of the data and executive summary audit. An EDSP QA team member reviews the data package for completeness and, if incomplete, requests that the additional records needed for review be submitted. EDSP QA team members review a minimum of 10% of the raw data, the tabulated data, and WA records of performance and methods to ensure compliance with the planning documents mentioned previously. At least 10% of the raw data are compared to the tabulated individual data, and a minimum 10% of the summary data tables are checked. EDSP QA team members will also check all tabulated data designated as statistically significant. Findings are reported, and corrective actions undertaken as described earlier. EDSP QA team members review the executive summary using the audited data and corrected tables to ensure that the reported results are of high quality and accurately reflect the raw data, and that the summary accurately describes the materials used in the WA. Findings are then reported and corrective actions undertaken, as described earlier.

20.5 AUDIT REPORT FORMAT

The audit report consists of a cover page for WA information and additional page(s) with the audit findings. All pages have header information containing the WA protocol number, audit report date, and audit code. The audit report date is the date on which the EDSP QA Unit Manager signs the audit report and sends it to the Principal Investigator and EDSP management. The audit code is an abbreviation of the audit type (e.g., D = dosing, DA/RA = data audit/report audit).

The cover page contains the WA protocol title, number, and code; contract number; EPA Sponsor; PI; audit type; audit date(s); EDSP QA Unit; distribution list; the dated signature of the auditor; the date that the Work Assignment Leader received the audit report; and the dated signatures of the PI and management. The distribution list may include additional names for individuals who have findings pertaining to their area of responsibility and is used to ensure that the report is sent by e-mail attachment to all who will receive an electronic copy of the report. The official audit report is the hard copy containing corrective actions and dated signatures of the EDSP QA Unit, PI, and management. Subsequent page(s) contain the audit finding(s), any recommended remedial actions, and space for the PI to respond to the findings and document remedial actions taken or to be taken.

20.6 RESPONSE ACTIONS AND RESOLUTION OF ISSUES

The PI shall respond to a TSA report within five working days of receipt of the report. There is no deadline for the PI's response to an ADQ report except for the time constraint deriving from the submission date of the final WA 3-11 report. The PI forwards the audit report to management for review. Management adds comments as necessary, signs and dates the report, and returns it to the EDSP QA Team Member. The EDSP QA Team Member assesses the responses and verifies the corrective actions. If a disagreement between the PI and EDSP QA team member arises over a finding, it will be discussed among the other EDSP QA team members. The EDSP QA team member will then present the majority opinion to the PI for further consideration. If the disagreement remains, the issue is reported to the PI's management and, if necessary, the Fort Environmental Laboratories' Director. The action decided on by management will be documented in the QA files.

During an assessment, if the auditor determines that adverse health effects could result or WA 3-11 objectives of acceptable quality cannot be achieved, the auditor follows the Stop Work Procedure specified in the EDSP QMP (Section 3.3).

20.7 INDEPENDENT ASSESSMENTS

The EDSP Battelle QAM or designee may conduct an independent TSA and ADQ during the conduct of this work assignment. Typically, one independent audit will be conducted during the work assignment. If major deficiencies are uncovered, additional independent audits may be scheduled. The conduct and reporting of the audits will be consistent with the procedures described in the EDSP QMP (Section 3.3). In addition, the EDSP EPA QAM or designee has the option of conducting external TSAs/ADQs.

21.0 REPORTS TO MANAGEMENT

The Fort Environmental Laboratories QA Unit Manager routinely prepares and distributes quarterly reports. If this frequency is not considered adequate to convey EDSP concerns, the EDSP Ecotoxicology Project Leader will receive project-specific briefings so that project-specific reports can be made on a monthly basis. The QA Unit Manager will send copies of resolved assessment reports containing findings, responses, and corrective actions taken to the EDSP Battelle QA Manager as soon as they are completed.

22.0 DATA REVIEW, VERIFICATION, AND VALIDATION

The data produced under this work assignment will be reviewed by the technical personnel for the validation process and by EDSP QA team members for the verification process (see Section 23.3). The criteria used for validation depend on the type of data. Compromised samples are not analyzed and any deviations resulting from such circumstances will be addressed.

23.0 VERIFICATION AND VALIDATION METHODS

23.1 CHAIN OF CUSTODY FOR TEST SOLUTIONS

Documentation for the preparation, purity, and stability of test solutions is evaluated for accuracy and completeness as part of the EDSP Chemical Repository operations. Test solution data maintained to support WA 3-11 must be traceable to the Chain of Custody documentation to provide the link to Chemical Repository data.

23.2 DATA VALIDATION

Data validation is a process by which the Principal Investigator and/or other technical personnel ensure that the processes for data entry, reduction, and reporting provide the desired product and meet project objectives. WA 3-11 personnel are responsible for reviewing the data, evaluating any technical deviations or non-conformances, and then determining the degree to which the data meet the quality criteria stated Section 7.0.

23.3 DATA VERIFICATION

Data verification constitutes part of the ADQ process performed by EDSP QA team members and described earlier. Verification ensures that 1) the data are of high quality and were collected according to the planning documents' requirements, and 2) the reported results accurately reflect the raw data. Each data type is evaluated against its collection and reduction requirements, using the data calculations required by the protocol as specified in the planning documents. Errors discovered during the data evaluation are corrected. The reported

conclusions drawn from the data are verified by EDSP QA team members during the report audit to confirm that they are true and accurate. The procedure for resolving issues of data verification has been detailed in prior sections of this document.

24.0 RECONCILIATION AND USER REQUIREMENTS

Proposed methods for data analysis, which will include a test for statistical outliers, are specified above, in Section 19.2.

25.0 REFERENCES

Battelle, 2003. Endocrine Disruptor Screening Program, Quality Management Plan, Version 2, Battelle, Columbus, Ohio. 12 May 2003.

Battelle, 2004. Final Study Plan on Amphibian Metamorphosis Assay Demonstration Study. Battelle, Columbus, Ohio. 7 January 2004.

Benoit, D.A., Mattson, V.R., and Olson, D.L. A continuous-flow mini-diluter system for toxicity testing. Water Res. 16:457-464, 1982.

Blaustein, A.R., Hoffman, P.D., Hokit, K.G., Kiesecker, J.M., Walls, S.C. and Hays, J.B. UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines. Proc. Natl. Acad. Sci. USA 91:1791-1795, 1994.

Carey, C. and Bryant, C.J. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environ. Health Perspect. 103(suppl 4):13-17, 1995.

Crain, D.A., Guillette, L.J. Jr., Rooney, A.A. and Pickford, D.B. Alterations in steroidogenesis in alligators (Alligator mississippiensis) exposed naturally and experimentally to environmental contaminants. Environ. Health Perspect. 105:528-533, 1997.

Crain, D.A., Guillette, L.J. Jr., Pickford, D.B., Percival, H.F. and Woodward, A.R. Sex steroid and thyroid hormone concentrations in juvenile alligators (Alligator mississippiensis) from contaminated and reference lakes in Florida, USA. Environ. Toxicol. Chem. 17:446-452, 1998.

Folmar, L.C., Denslow, N.D., Rao, V., Chow, M., Crain, D.A., Enblom, J., Marcino, J. and Guillette, L.J. Jr. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (Cyprinus carpio) captured near a major metropolitan sewage treatment plant. Environ. Health Perspect. 104:1096-1101, 1996.

Gendron, A.D., Bishop, C.A., Fortin, R. and Hontela, A. In vivo testing of the functional integrity of the corticosterone-producing axis in mudpuppy (Amphibia) exposed to chlorinated hydrocarbons in the wild. Environ. Toxicol. Chem. 16:1694-1706, 1997.

Gray, L.E.J., Ostby, J.S. and Kelce, W.R. Developmental effects of an environmental antiandrogen: the fungicide vinclosolin alters sex differentiation of the male rat. Toxicol. Appl. Pharmacol. 129:46-52, 1994.

Guillette, L.J. Jr., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F. and Woodward, A.R. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect. 102:680-688, 1994.

Guillette, L.J. Jr., Pickford, D.B., Crain, D.A., Rooney, A.A. and Percival, H.F. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. Gen. Comp. Endocrinol. 101:32-42, 1996.

Hall, R.J. and Henry, P.F.P. Assessing effects of pesticides on amphibians and reptiles: status and needs. Herpetol. J. 2:65-71, 1992.

Hayes, T.B., Wu, T.H. and Gill, T.N. DDT-like effects as a result of corticosterone treatment in an anuran amphibian: is DDT a corticoid mimic or a stressor? Environ. Toxicol. Chem. 16:1948-1953, 1997.

Jobling, S. and Sumpter, J.P. Detergent components in sewage effluent are weakly oestrogenic to fish: an in vitro study using rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquat. Toxicol. 27:361-372, 1993.

Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Kemppainen J.A. and Wilson, E.M. Persistent DDT metabolite p,p'-DEE is a potent androgen receptor antagonist. Nature 375:581-585, 1995.

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

Palmiter, R.D. and Mulvihill, E.R. Estrogenic activity of the insecticide kepone on the chicken oviduct. Science 201:356-358, 1978.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Taylor, C.R. and Sumpter, J.P. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 8:275-285, 1994.

Tulner, W.W. Uterotrophic action of the insecticide methoxychlor. Science 133:647-648, 1961.

U.S. EPA. Special report on environmental endocrine disruption: an effects assessment and analysis. EPA/630/R-96/012. Washington, DC: U.S. Environmental Protection Agency, 1997.

Van der Kraak, G.J., Munkittrick, K.R., McMaster, M.E., Portt, C.B. and Chang, J.P. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. Toxicol. Appl. Pharmacol. 115:224-233, 1992.

Wake, D.B. Declining amphibian populations. Science 253:860, 1991.

Welch, R.M., Levin, W. and Conney, A.H. Estrogenic action of DDT and its analogues. Toxicol. Appl. Parmacol. 14:358-367, 1969.

DOCUMENT AMENDMENT FORM
Fort Environmental Laboratories

Document or Study Title: Frog Metamorphosis Assay Demonstration	
Amendment Number: 1	**Document ID Number:** WA 3-11
Submitted By: John Denny Flowers	**Date:** 2/17/04
Amendment Relating To:	
[ ] Protocol [ ] Study Plan [X] QAPP [ ] QMP [ ] SOP	
[ ] Other (describe):	

Original Specifications:

1) Page 12, Paragraph 1, Lines 6, 9 and Paragraph 2, Line 3: "50 mL/minute" assay flow rate.
   Page 23, Table 7-1, Row 6: "Target 50 mL/min".
2) Page 25, Laboratory Equipment, 3.4 and 3.5: "D.O. Meter (YSI Model 52)" and "pH Meter (Denver Basic).
   Page 34, Table 16-1, Row 7: pH and DO meters.
   Page 35, Paragraphs 3 and 4: pH Meter and DO Meter.
3) Page 15, Paragraph 1, Line 6: "Study team members Andra Noll and Brody Buzzard.....".

Planned Change:

1) Change flow rate for assay to 25 mL/minute. The flow rate for rearing the pre-test tadpoles to test stages 51 and 54 will remain at 50 mL/minute.
2) Add portable pH meter (manufacturer WTW, model pH315i, SN 03450006) and portable DO meter (manufacturer YSI, model 550A, Fisher catalog no. 13298119) to study equipment list.
3) Specific study team members may change during the study. However, QAU will ensure that any new staff has been adequately trained prior to participating in the study.

Reason for Change:

1) Change recommended by U.S. EPA ORD/MED (Duluth, MN) via telephone conversation with Study Director and Study Team Leader.
2) Portable pH and DO meters will increase efficiency, while the existing non-portable meters will serve as back-ups, in case of meter failure.
3) Because of study length and unforeseen circumstances, Fort Environmental Laboratories cannot guarantee that the list study team members will not change before study completion.

Approval:

Study Director:		Date:	2/17/04
WAL:		Date:	3/1/04
Program Management:		Date:	3/3/04
QAU:	Robert Rogers	Date:	2/17/04

FEL

DOCUMENT AMENDMENT FORM
Endocrine Disruptor Screening Program

Document or Study Title: Amphibian Metamorphosis Assay Demonstration	
Amendment Number: 03	**Document ID Number**: WA 3-11
Submitted By: Douglas J. Fort	**Date**: 11/4/2004

Amendment Relating To:

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

[ ] Other (describe):

Original Specifications:

1) Study guidance documents (Exposure System, Chemical Analysis, and Test Methods sections, and Protocol Amendment 02).

2) Study guidance documents (Test Termination section) state that at test termination, all surviving specimens will be euthanized and preserved in Bouin's Solution for possible thyroid histology.

3) Study guidance documents (Thyroid Histology section).

4) Protocol (Study Schedule) states that the final report will be submitted to EPA no later than 8/31/2004.

Planned Change:

1) Due to the wide range of physical and chemical properties (including solubility and stability) of T4, dexamethasone, phenobarbital, and PCN, the test conditions as outlined in the Protocol, Study Plan, and QAPP may need to be changed, depending on the chemical being tested. In lieu of generating a new amendment for each change, EPA will issue a technical directive for each remaining chemical, outlining any changes specific to the chemical being tested; such as, flow through vs. static renewal system, analytical sampling schedule, number of test concentrations, test concentration dilution factors, test solution volumes, etc. The EPA directive will also address any items listed as "TBD" (to be determined) in the original study guidance documents.

2) All surviving specimens will be euthanized and preserved in Bouin's Solution for 72 to 120 hours (3 to 5 d). The specimens will then be triple rinsed in 70% v/v reagent alcohol and preserved in 10% (w/v) neutral buffered formalin acetate for possible thyroid histology.

3) Thyroid gland histopathology will be handled as a separate work assignment (WA 5-8).

4) EPA has extended the final report deadline to 12/31/2004.

Reason for Change:

1) The use of EPA technical directives specific for each test chemical is the most direct and simplistic way to handle the unforeseen changes in test set up and conditions that may occur. These changes will not compromise the study, but should have a positive impact on facilitating each test.
2) Prolonged exposure to Bouin's solution can adversely affect the preservation process in tissue.
3) As a critical endpoint for the WA 3-11 assay, EPA has decided that WA 5-8 is needed to provide additional histopathological examination of tissues collected under WA 3-11.
4) EPA extended the deadline due to delays on getting the go-ahead for the laboratory to start testing.

Approval:

Study Director:	[signature]	Date:	11/4/04
WAL:	[signature]	Date:	1/13/05
Program Management:	[signature]	Date:	1/13/05
QAU:	Robert Rogers	Date:	11/4/2004

Study Guidance Documents:

WA 5-8 Study Plan

For Review and Approval
s:\houch\oscp\g6687\Task 8\WA 5-8 Draft Study Plan ltr

Project No. G668708-01
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Internal Distribution

Lee/ OSCP files
D. Houchens
M. Blanton
D. Ford
RMO

	Name	Initials	Date
Originator	M. Blanton		11-9-04
	T. Pollock		11-9-04
Approved	D. Houchens		11/9/04

Sent Via: FedX + email

November 9, 2004

Dr. Les Touart
U.S. Environmental Protection Agency
EPA East Building
Room 4106-F, Mail Code 7201M
1201 Constitution Avenue, N.W.
Washington, DC 20004

Dear Les:

Contract No. 68-W-01-023
Work Assignment WA 5-8
Frog Metamorphosis Assay Histopathology
Draft Study Plan

Enclosed is the Draft Study Plan for Work Assignment 5-8, "Frog Metamorphosis Assay Histopathology".

If you have any questions regarding this work plan, please contact Mike Blanton at (360) 681-4568.

We look forward to performing this work assignment for EPA.

Sincerely,

David P. Houchens, Ph.D.
Program Manager

DPH:lnl
Enclosure

cc: Dr. Linda Phillips, EPA Project Officer (letter only)

DRAFT STUDY PLAN

on

AMPHIBIAN METAMORPHOSIS ASSAY HISTOPATHOLOGY

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 5-8

November 9, 2004

Prepared for

LES TOUART, PH.D.
WORK ASSIGNMENT MANAGER
U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

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1. INTRODUCTION

A number of man-made environmental pollutants have the potential to interfere with endocrine function (1-6), and there is evidence of reproductive or endocrine dysfunction in wildlife species that have been exposed environmentally to such endocrine-disrupting contaminants (EDCs) (7-13). While there have been recent reports of alterations of the stress hormone axis in amphibians (14,15), there is little or no published evidence of reproductive dysfunction or developmental effects in this group as a result of exposure to EDCs. A recent special report on environmental endocrine disruption by the U.S. Environmental Protection Agency cited no reports of such effects in amphibians, although it concluded that "this class of vertebrate represents a unique sentinel animal model for laboratory and field exposure studies" (16).

The U.S. Environmental Protection Agency is implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is an amphibian metamorphosis screening assay designed to detect substances that interact with the thyroid systems of vertebrates. It is thought that the inclusion of the amphibian metamorphosis assay in Tier 1 is important because amphibian metamorphosis provides a well-studied thyroid dependent process on which to build an assay. Additionally, the amphibian assay is considered essential in the overall screening battery to detect substances that may disrupt the vertebrate thyroid axis.

A previous work assignment in this contract (WA 3-11) was initiated to perform a multiple chemical demonstration and evaluation of the amphibian prometamorphosis assay as developed by the US-EPA, ORD/MED. A critical endpoint for this assay is the histopathology of the thyroid gland. Under WA 3-11, thyroid tissues collected did not receive a fully sufficient histopathological examination. The purpose of this work assignment is to perform additional histopathological examination of tissues collected as part of WA 3-11 and to prepare a complete and integrated study report from WA 3-11 and the analyses performed under this work assignment. This work assignment will not duplicate any of the work conducted under WA 3-11.

2. HISTOPATHOLOGICAL EVALUATION

Tissue Preservation

All surviving specimens from each amphibian metamorphosis assay performed by Fort Environmental Laboratories, Inc. (FEL) will be euthanized and preserved in Bouin's Solution for 72 to 120 hours (3 to 5 d). The specimens will then be triple rinsed in 70% v/v reagent alcohol and preserved in 10% (w/v) neutral buffered formalin acetate for possible thyroid histology.

Selection of Specimens

FEL will randomly select 5 preserved larval specimens per replicate per test concentration per test substance and the control, and will ship to Experimental Pathology Laboratories, Inc. (EPL) for histopathological evaluation of the thyroid gland, under the direction of pathologist Dr. Marilyn Wolfe.

Test Substances

Amphibian metamorphosis assays were completed on 6 test chemicals and preserved specimens from 1 of the experimental trials, 6-n-propylthiouracil (PTU), were histologically evaluated by EPL, under WA 3-11. Preserved larval specimens from the remaining 5 assays (phenobarbital, pregnelone-16-a-carbonitrile [PCN], L-thyroxine [T4], dexamethasone, and methimazole) will receive histopathological evaluation under WA 5-8.

Histopathological Procedures

Tissue sampling will be by transverse section of the head taken caudal to the eyes. Sectioning should be in 5 um sections, 2 serial sections at 5 steps 30 um apart for a total of 10 sections, or otherwise as appropriate. Both right and left lobes should be evaluated if possible. It is expected that Richard and Allen Hematoxylin and Eosin Y staining will be used, but other stains (e.g., Periodic Acid Schiff) may also be considered for use to better delineate colloid content. Qualitative analysis should include overall size of the thyroid gland, follicle size, follicle shape, colloid content, colloid density, follicular cell shape, follicular cell height, structure of the epithelium, and follicular cell hyperplasia. Quantitative measures may also be optionally considered and would include follicular cell height, colloid density (colorimetric), thyroid gland cross section area, and follicle cross section area.

3. REPORTS

Progress, results, and other information will be provided by EPL in monthly reports to FEL and Battelle. A Pathology Summary, which will include the materials, methods, and results comparing the thyroid tissue from control frogs with exposed frogs, will be written for each amphibian metamorphosis assay performed. Appropriate Individual Animal Tables and Summary Incidence Tables will accompany the Pathology Summary. Digital photographs will be taken to illustrate changes, if any, in the thyroid glands of exposed frogs as compared to thyroid glands of control frogs. The final results of the pathological examinations under WA 5-8 will be combined by FEL with the data and observations collected under WA 3-11, and integrated into a comprehensive final report.

Quality Assurance

The final report shall also contain a section on Quality Assurance indicating: 1) deviations, if any, from the Quality Assurance Project Plan (QAPP) completed for WA 3-11 and for this work assignment (WA 5-8) that were encountered and how they were dealt with; and 2) problems, if any, concerning study quality or data quality that were encountered and how they were handled.

4. REFERENCES

1. Tulner, W.W. Uterotrophic action of the insecticide methoxychlor. Science 133:647-648, 1961.

2. Welch, R.M., Levin, W. and Conney, A.H. Estrogenic action of DDT and its analogues. Toxicol. Appl. Parmacol. 14:358-367, 1969.

3. Jobling, S. and Sumpter, J.P. Detergent components in sewage effluent are weakly oestrogenic to fish: an *in vitro* study using rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquat. Toxicol. 27:361-372, 1993.

4. Palmiter, R.D. and Mulvihill, E.R. Estrogenic activity of the insecticide kepone on the chicken oviduct. Science 201:356-358, 1978.

5. Gray, L.E.J., Ostby, J.S. and Kelce, W.R. Developmental effects of an environmental antiandrogen: the fungicide vinclosolin alters sex differentiation of the male rat. Toxicol. Appl. Pharmacol. 129:46-52, 1994.

6. Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Kemppainen J.A. and Wilson, E.M. Persistent DDT metabolite p,p'-DEE is a potent androgen receptor antagonist. Nature 375:581-585, 1995.

7. Guillette, L.J. Jr., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F. and Woodward, A.R. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect. 102:680-688, 1994.

8. Guillette, L.J. Jr., Pickford, D.B., Crain, D.A., Rooney, A.A. and Percival, H.F. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. Gen. Comp. Endocrinol. 101:32-42, 1996.

9. Crain, D.A., Guillette, L.J. Jr., Rooney, A.A. and Pickford, D.B. Alterations in steroidogenesis in alligators (Alligator mississippiensis) exposed naturally and experimentally to environmental contaminants. Environ. Health Perspect. 105:528-533, 1997.

10. Crain, D.A., Guillette, L.J. Jr., Pickford, D.B., Percival, H.F. and Woodward, A.R. Sex steroid and thyroid hormone concentrations in juvenile alligators (Alligator mississippiensis) from contaminated and reference lakes in Florida, USA. Environ. Toxicol. Chem. 17:446-452, 1998.

11. Folmar, L.C., Denslow, N.D., Rao, V., Chow, M., Crain, D.A., Enblom, J., Marcino, J. and Guillette, L.J. Jr. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environ. Health Perspect. 104:1096-1101, 1996.

12. Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Taylor, C.R. and Sumpter, J.P. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 8:275-285, 1994.

13. Van der Kraak, G.J., Munkittrick, K.R., McMaster, M.E., Portt, C.B. and Chang, J.P. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. Toxicol. Appl. Pharmacol. 115:224-233, 1992.

14. Gendron, A.D., Bishop, C.A., Fortin, R. and Hontela, A. *In vivo* testing of the functional integrity of the corticosterone-producing axis in mudpuppy (Amphibia) exposed to chlorinated hydrocarbons in the wild. Environ. Toxicol. Chem. 16:1694-1706, 1997.

15. Hayes, T.B., Wu, T.H. and Gill, T.N. DDT-like effects as a result of corticosterone treatment in an anuran amphibian: is DDT a corticoid mimic or a stressor? Environ. Toxicol. Chem. 16:1948-1953, 1997.

16. U.S. EPA. Special report on environmental endocrine disruption: an effects assessment and analysis. EPA/630/R-96/012. Washington, DC: U.S. Environmental Protection Agency, 1997.

Study Guidance Documents:
WA 5-8 QAPP

1.0 TITLE AND APPROVAL

Quality Assurance Project Plan

for

Work Assignment 5-8

Amphibian Metamorphosis Assay Histopathology

EPA Contract Number 68-W-01-023

Project No.: Battelle's Pacific Northwest Division. Project 43496

Work Assignment Leader: Michael L. Blanton

Principal Investigator: Douglas J. Fort

Institution: Fort Environmental Laboratories, Inc.
Stillwater, Oklahoma

SIGNATURE PAGE
Quality Assurance Project Plan for WA 5-8

Amphibian Metamorphosis Assay Histopathology
EPA Contract Number 68-W-01-023
Concurrences and Approvals

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4.0 PROJECT ORGANIZATION

4.1 PROJECT MANAGEMENT

The U.S. Environmental Protection Agency (EPA) is implementing the Endocrine Disruptor Screening Program (EDSP). To support this program, EPA has contracted with Battelle to provide comprehensive toxicological and ecotoxicological testing services, including chemical, analytical, statistical, and quality assurance (QA)/quality control (QC) support to assist the EPA in developing, standardizing, and validating a suite of *in vitro*, mammalian, and ecotoxicological screens and tests for identifying and characterizing endocrine effects through exposure to pesticides, industrial chemicals, and environmental contaminants. The studies conducted will be used to develop, standardize, and validate methods; prepare appropriate guidance documents for peer review of the methods; and develop technical guidance and test guidelines in support of the Office of Prevention, Pesticides and Toxic Substances regulatory programs. The validation studies will be conducted under the EDSP Quality Management Plan (QMP), study protocol, applicable Quality Assurance Project Plans (QAPPs), relevant program and facility standard operating procedures (SOPs), and guidance documents.

The Food Quality Protection Act of 1996 requires the EPA to develop and implement a screening program using valid tests for determining the potential in humans for estrogenic effects from pesticides. EPA proposed a two-tiered screening program in a Federal Register notice in 1998 (63 CFR 1542-71568, Dec. 28, 1998) that covered not only pesticides but also commercial chemicals subject to regulation under the Toxic Substances Control Act (TSCA; 15 USC 2601) and environmental and drinking water contaminants.

This QAPP addresses the need for experimental data regarding:

1. Histopathology of a Frog Metamorphosis Assay.

4.2 GOALS

Under Work Assignment (WA) 3-11, work was conducted to determine the differential sensitivity of beginning exposure with stage 51 and 54 larvae and to establish the necessary duration of exposure for model compounds. The primary endpoints were growth, mortality, developmental stage, and thyroid gland histopathology. To achieve this objective, two concurrent studies (one cohort exposed from stage 51; another cohort exposed from stage 54) were conducted for each chemical with individuals from the same cohort of organisms. One was initiated with stage 51 larvae and conducted for 21 days and the other with stage 54 larvae conducted for 14 days. The laboratory testing and analysis for WA 3-11 has been completed (or is in the final stages of completion). The completion of WA 5-8 will provide additional data regarding the thyroid disrupting capacity of several test chemicals. There are 3 administrative and 2 research tasks required under WA 5-8.

Goal 1. Conduct histopathological analyses, and
Goal 2. Analyze Data and Prepare Final Report.

4.3 ORGANIZATION

Battelle, in conjunction with Fort Environmental and EPL Inc., will conduct the histopathology investigation of frog tissues specified under WA 5-8. A summary of the work assignment organization is shown in Figure 4-1.

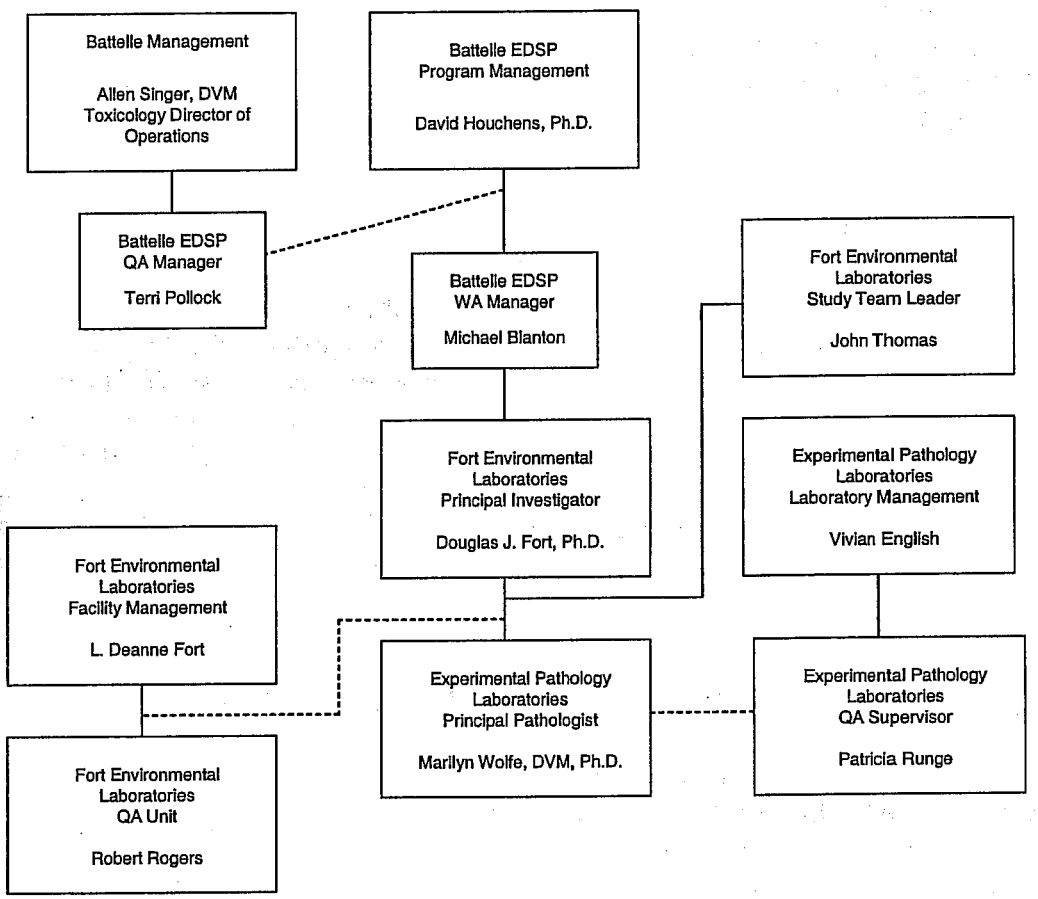


Figure 4-1. WA 5-8 Project Organization Overview

As EDSP Manager, **Dr. David Houchens** will have ultimate responsibility for quality, timeliness, and budget adherence for all activities on the contract. He will also serve as the principal interface with the U.S. EPA's Project Officer on all contract-level administrative and technical issues. Because of the high level of subcontracting and purchases required by the program, such as test laboratory subcontracts and purchases of chemical supplies, Dr. Houchens will be assisted by an administrative deputy manager, **Mr. James Easley**. Mr. Easley will manage the procurement of all subcontracts, consultants, and purchased materials and services, and will facilitate schedule and cost control.

Michael L. Blanton will serve as the Work Assignment leader and **Dr. Douglas Fort** will provide general scientific direction of the work performed under this work assignment. Dr.

Fort is the Principal Investigator (PI) for the Amphibian Metamorphosis Assay Demonstration WA 3-11 and will be the primary technical contact for Environmental Pathology Laboratories (EPL). Dr. Fort is also responsible for preparing the Study Plan for submission and approval by the U.S. EPA. **Mr. John Thomas**, the Study Team Leader will report to Dr. Fort and will oversee specimen preservation, selection, tracking, and shipment to EPL.

Dr. Marilyn J. Wolfe of EPL (located in Herndon, VA) is the principal pathologist and is responsible for the thyroid gland histopathological analyses. Dr. Wolfe will provide to Dr. Fort monthly progress reports and pathology summaries for each assayed chemical. **Ms. Patricia Runge** is the QA Supervisor for the Virginia laboratory and is responsible for auditing the histopathology data generated for this work assignment

Mr. Robert Rogers is the Fort Environmental Laboratories, Inc (FEL) QAU Manager and is responsible for approving the QAPP for this work assignment. In this capacity, he will aid in the preparation of the WA 5-8 QAPP and assist the Principal Investigator and EPL QAU personnel with QA issues. Mr. Rogers will also review EPL's final data summaries, prior to inclusion into the final study report as part of the final report audit, as defined in WA 3-11.

Ms. Terri Pollock, the EDSP QA Manager at Battelle, will provide oversight to all associated QA functions. Ms. Pollock will be responsible for reporting her findings and any quality concerns to Dr. Houchens. Ms. Pollock reports to **Dr. Allen Singer,** Director of Operations, Toxicology Division. This reporting relationship assures that the QA function is independent of the technical activities on the program.

5.0 PROBLEM DEFINITION/BACKGROUND

The U.S EPA is implementing an EDSP comprised of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is an amphibian metamorphosis screening assay designed to detect substances that interact with the thyroid systems of vertebrates. It is thought that the inclusion of the amphibian metamorphosis assay in Tier 1 is important because amphibian metamorphosis provides a well-studied thyroid dependent process on which to build an assay. Additionally, the amphibian assay is considered essential in the overall screening battery to detect substances that may disrupt the vertebrate thyroid axis.

A previous work assignment in this contract (WA 3-11) was initiated to perform a multiple chemical demonstration and evaluation of the amphibian prometamorphosis assay as developed by the US-EPA, ORD/MED. A critical endpoint for this assay is the histopathology of the thyroid gland. Under WA 3-11, thyroid tissues collected did not receive a fully sufficient histopathological examination. The purpose of this work assignment is to perform additional histopathological examination of tissues collected as part of WA 3-11 and to prepare a complete and integrated study report from WA 3-11 and the analyses performed under this Work Assignment.

6.0 PROJECT/TASK DESCRIPTION

6.1 PROJECT OVERVIEW

The purpose of this work assignment is to perform additional histopathological examination of tissues collected as part of WA 3-11 and to prepare a complete and integrated study report from WA 3-11 and the analyses performed under this work assignment.

6.2 SPECIFIC STUDY OBJECTIVES

The specific objectives of the study are to:

1. Conduct histopathology evaluation of frog tissues obtained from WA 3-11.

6.3 SUMMARY

HISTOPATHOLOGICAL EVALUATION

Tissue Preservation. Under WA 3-11, all surviving specimens from each amphibian metamorphosis assay performed by FEL were euthanized and preserved in Bouin's Solution for 72 to 120 hours (3 to 5 d). The specimens will then be triple rinsed in 70% v/v reagent alcohol and preserved in 10% (w/v) neutral buffered formalin acetate for possible thyroid histology.

Selection of Speciments. FEL will randomly select 5 preserved larval specimens per replicate per test concentration per test substance and the control, and will ship to EPL for histopathological evaluation of the thyroid gland, under the direction of pathologist Dr. Marilyn Wolfe.

Test Substances. Amphibian metamorphosis assays were completed on 6 test chemicals and preserved specimens from 1 of the experimental trials, 6-n-propylthiouracil (PTU), were histologically evaluated by EPL, under WA 3-11. Preserved larval specimens from the remaining 5 assays (phenobarbital, pregnelone-16-a-carbonitrile [PCN], L-thyroxine [T4], dexamethasone, and methimazole) will receive histopathological evaluation under WA 5-8.

Histopathological Procedures. Tissue sampling will be by transverse section of the head taken caudal to the eyes. Sectioning will be in 5 um sections, 2 serial sections at 5 steps 30 um apart for a total of 10 sections, or otherwise as appropriate. Both right and left lobes will be evaluated if possible. It is expected that Richard and Allen hematoxylin and eosin staining will be used and Periodic Acid Schiff will additionally be used for phenobarbital specimens to better delineate colloid content. Qualitative analysis will include overall size of the thyroid gland, follicle size, follicle shape, colloid content, colloid density, follicular cell shape, follicular cell height, structure of the epithelium, and follicular cell hyperplasia. Quantitative measures may also be optionally considered and would include follicular cell height, colloid density (colorimetric), thyroid gland cross section area, and follicle cross section area.

7.0 QUALITY OBJECTIVES AND CRITERIA

Standard applications of data quality objectives do not readily apply to the work to be conducted for WA 5-8. The specified tasks incorporate quality objectives relative to the accuracy of the measures to be made. The large numbers of frogs used in WA 3-11 were needed to provide a statistically-valid sample to detect effects from the test chemical. Measurement systems and equipment will be calibrated before use. Histology will be performed by experts. Quality control procedures used during the analysis are described in Section 14.0.

8.0 SPECIAL TRAINING/CERTIFICATION

Training for personnel at the subcontracted laboratory is documented by the facility and those records are retained at the facility. Histopathology assessments will be conducted by a qualified pathologist.

9.0 DOCUMENTS AND RECORDS

9.1 RETENTION OF SPECIMENS AND RECORDS

All specimens and records generated at FEL, EPL and Battelle will remain the responsibility of testing facility that generated the data and will be retained in the project data files for the length of time stipulated in the contract. Each contracted facility will retain the raw data and specimens generated at their respective facility. The period of time for retention is specific to each facility. No records will be disposed of at any given facility without the authorization of Battelle. Records may be returned to Battelle for retention at their request.

9.2 QUALITY ASSURANCE PROJECT PLAN

This QAPP will be distributed to project participants, initially, and whenever revised. Previous versions either will be marked as "obsolete" when newer versions are distributed, or will be collected and destroyed so that there is no confusion regarding the version in effect. The right-justified document control header example shown here

Version 1
Month Year
Page 1 of 1

is used to ensure that revision numbers and dates are obvious to document users. The QAPP will be reviewed annually if the project extends beyond one year, and a determination made to either modify the document, based on new or modified project requirements, or leave as is. Controlled copies of the QAPP are maintained, tracked, and managed by the QA Unit Manager through the use of a master distribution list.

9.3 STANDARD OPERATING PROCEDURES

Applicable procedures for conducting work exist within the SOP at each subcontracted facility. Deviations from SOPs will be documented as required by each facility.

9.4 DATA REPORTING PACKAGE

Each subcontracted facility will generate and maintain the data necessary to reconstruct the activities performed at that facility.

Records generated for the data report package are specified in the Study Plan for WA 3-11 and 5-8 are listed below for reference. Except for the animal room environmental conditions and the statistical analysis records, the data are recorded manually on data forms. All data forms include a title identifying the type of data to be recorded, the unique study name, and the initials and date of the data recorder(s) to authenticate the records:

- Protocol and any Amendments
- QAPP and any Amendments
- List of any Protocol or QAPP Deviations
- List of applicable Standard Operating Procedures
- Temperature and Humidity Records for the Animal Rooms
- Feed Type, Source, Lot Number, Dates Used, Certification, Analytical Results
- Dose Formulation Receipt, Transfer, and Use Records
- Sample Collection Records, including coding of samples
- Weight Sheets (body weight, necropsy – organs collected)
- Specimen Sample Collection Records and Chain of Custody
- Statistical Analysis Records
- Correspondence.

9.4.1 Draft and Final Reports

Progress, results, and other information will be provided by EPL in monthly reports to FEL and Battelle. A Pathology Summary, which will include the materials, methods, and results comparing the thyroid tissue from control frogs with exposed frogs, will be written for each amphibian metamorphosis assay performed. Appropriate Individual Animal Tables and Summary Incidence Tables will accompany the Pathology Summary. Digital photographs will be taken to illustrate changes, if any, in the thyroid glands of exposed frogs as compared to thyroid glands of control frogs. The final results of the pathological examinations under WA 5-8 will be combined by FEL with the data and observations collected under WA 3-11, and integrated into a comprehensive final report.

General format requirements include:

1. **"Draft or Final Report" and "Page 1 of _n", where n is the total number of report pages on the title page**

2. **Text, which includes the following sections:**
   - Abstract
   - Objectives
   - Materials and Methods
   - Results
   - Discussion
   - Conclusions
   - References
   - Protocol Deviations, if any
   - Summary in-life and endpoint data with statistical analyses when applicable.

The report will also contain a section on deviations listing any differences from the Quality Assurance Project Plan completed for WA 3-11 and for this work assignment that were encountered and how they were dealt with. This section will also address problems, if any, concerning study quality or data quality that were encountered and how they were dealt with.

The EPL written report will be based on the histopathologic evaluation of the slides. The report will consist of a short summary of the protocol used by the laboratory conducting the experiment. The treatment effects will be based on comparing the results in tissues sampled from the test substance at various exposure concentrations with negative controls and positive controls (if available). The severity and frequency of observed treatment effects will be considered. A draft report will be provided to Battelle for review prior to completion of a final report. Comments from an EPA review will be incorporated into the final report.

All EPL reports will be submitted to Battelle electronically in the following formats:

- Pathology Summary - MS Word
- Pathology Data File - MS Excel
- Images - TIFF format.

9.4.2 QA Assessment Reports

QA assessment reports (see Section 20) are maintained as confidential files by the QA Unit.

9.4.3 Status Reports

Monthly status reports will be provided by EPL to FEL and the EDSP Program Manager and Work Assignment Leader.

10.0 SAMPLING PROCESS DESIGN (EXPERIMENTAL DESIGN)

This work assignment (WA 5-8) is focused on developing a final report using data generated from a separate work assignment (WA 3-11) that is governed by its own specific Study Plan and Quality Assurance Project Plan. Therefore, there is no specific experimental

design associated with this work assignment. Histological examination of tissues samples generated from WA 3-11 will be evaluated.

11.0 SAMPLING METHODS

Sampling methods were discussed in detail in the QAPP, Study Plan and Study Protocol for WA 3-11.

12.0 SAMPLE HANDLING AND CUSTODY

Sample handling is described below and in the study plan. Chain of custody of samples will be documented.

12.1 HISTOLOGY SAMPLES

TEST SPECIMENS FOR THYROID HISTOPATHOLOGY

During the Amphibian Metamorphosis Assay Demonstration Study, stage 51 and 54 *X. laevis* larvae were exposed to 6 test chemicals, using 4 to 5 treatment concentrations per chemical (Table 12-1). At the completion of each amphibian metamorphosis assay, surviving *X. laevis* larvae were preserved as outlined in the WA 5-8 Study Plan (Battelle, 2004). Five specimens from each replicate (with the exclusion of replicates from the PTU assay, which were histologically examined under WA 3-11) will be selected for thyroid gland histopathological evaluation (reference WA 5-8 Study Plan [Battelle, 2004]).

Table 12-1. Test Substance Requirements

Test Substance	CAS #	Stock Solution Concentration	Treatment Concentrations
6-n-propyl-thiouracil (PTU)	51-52-5	700 mg/L	2.5, 5.0, 10.0, 20.0 mg/L
Phenobarbital	50-06-6	8,000 mg/L	Stg 51 – 125, 250, 500, 1000 g/L Stg 54 – 250, 500, 1000, 1500 mg/L
Pregnelone-16-α-carbonitrile (PCN)	1434-54-4	200 µg/L	7.4, 22.2, 66.7, 200 µg/L
L-thyroxine (T4)	51-48-9	4.04 mg/L	0.5, 1.0, 2.0, 4.0 µg/L
Dexamethasone	50-02-2	20.0 mg/L	1.0, 3.9, 15.6, 62.5, 250 µg/L
Methimazole	60-56-0	2,500 mg/L	6.25, 12.5, 25.0, 50.0 mg/L
Dilution Water	-	0.0 mg/L	0.0 mg/L

Tissue sampling will be by transverse section of the head taken caudal to the eyes. Sectioning will be in 5 um sections, 2 serial sections at 5 steps 30 um apart for a total of 10 sections, or otherwise as appropriate. Both right and left lobes will be evaluated if possible. It is

expected that Harris' hematoxylin and eosin staining will be used and Periodic Acid Schiff will additionally be used for phenobarbital specimens to better delineate colloid content. Qualitative analysis will include overall size of the thyroid gland, follicle size, follicle shape, colloid content, colloid density, follicular cell shape, follicular cell height, structure of the epithelium, and follicular cell hyperplasia. Quantitative measures may also be optionally considered and would include follicular cell height, colloid density (colorimetric), thyroid gland cross section area, and follicle cross section area.

All of the histology work conducted by EPL will be performed according to the standards of Good Laboratory Practice (GLP) as standard business operations. However, WA 3-11 and 5-8 are not GLP activities.

Preparation of the specimens for histopathology will be conducted according to the appropriate Standard Operating Procedures of EPL. Glass slides will be prepared for each sample section utilizing the EPL standard blocking scheme.

Specified tissues will be dissected according to the study plan and will be handled as follows:

A. At embedding, tissues will be appropriately oriented.
B. One representative section will be cut from each embedded block.
C. All slides will be stained with hematoxylin and eosin and covered with glass coverslips.
D. All paraffin blocks with residual tissue will be sealed with paraffin.

13.0 ANALYTICAL METHODS

This activity does not involve any analytical measurements.

14.0 QUALITY CONTROL

The final report shall also contain a section on Quality Assurance indicating: 1) deviations, if any, from the Quality Assurance Project Plan (QAPP) completed for WA 3-11 and for this work assignment (WA 5-8) that were encountered and how they were dealt with; and 2) problems, if any, concerning study quality or data quality that were encountered and how they were handled. EPL QC is provided in their SOPs and is conducted to meet GLP.

15.0 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

Equipment used by Battelle is operated and tested according to approved SOPs. Each subcontracted laboratory is responsible to provide the equipment necessary to perform the procedures as defined by this WA and is responsible for testing, inspecting and maintaining the required equipment.

16.0 INSTRUMENT/EQUIPMENT CALIBRATION AND FREQUENCY

Each piece of equipment used to obtain a measurement or used in the generation of data (e.g., balances, pipettes, thermometers) is calibrated or tested on the schedule specified in the SOP for that piece of equipment or as recommended by the manufacturer (see Section 15.0 for additional information). In general, equipment is calibrated on a schedule as recommended by the manufacturer or more frequently.

Equipment is calibrated using NIST traceable or other certified standard(s). Calibration will be conducted either by qualified facility personnel or equipment service representatives. The procedure for calibration is specified in the facility SOPs, equipment manual or service representative procedures. Documentation of any calibration conducted will be retained. In general, this documentation will consist of the unique identification of the piece of equipment being calibration, the date of calibration, who conducted the calibration, the expected result, the actual result and if the calibration was within the acceptable limit. If the calibration is outside the acceptable limit, the corrective action will be documented.

17.0 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

Upon receipt, purchased items are inspected for conformance to quality requirements and again prior to use. All products are expected to be used prior to the expiration dates of the product, when applicable.

18.0 NONDIRECT MEASUREMENTS

No collection of any samples or sample data for this Task is obtained from nondirect measures.

19.0 DATA MANAGEMENT

19.1 DATA MANAGEMENT OVERVIEW

Data will be collected to data sheets according to the end points listed above. In general, data are entered into Excel spreadsheets and analyzed using SAS, Statview, or Minitab using the statistical and data presentation abilities of the applications.

19.2 STATISTICAL ANALYSES

Statistical analysis is described in the WA 3-11 Study Plan and QAPP.

19.3 DATA TRANSFER

Each subcontractor will provide a report to Battelle detailing their results and observations (see Section 9.0).

20.0 ASSESSMENTS AND RESPONSE ACTIONS

Each subcontracted facility is responsible to audit the report and data generated at their respective facility. The following sections (20.1 through 20.4) describe the audit procedure by the Battelle QAU when auditing the data and report generated as a result of WA 3-11 and this WA. FEL role in WA 5-8 is focused primarily on report writing and data interpretation, and EPL will be conducting histology analysis of samples from WA 3-11 under GLP.

20.1 AUDITS OF DATA QUALITY

An Audit of Data Quality (ADQ) is a process by which the accuracy of data calculations and reporting is assessed to ensure that the reported results are of high quality and accurately reflect the raw data and that the executive summary and final report accurately describes the materials used in the WA. The acceptance criteria for the ADQ are that data collection, analysis, and reporting must meet the requirements of the applicable facility and program SOPs, the WA study protocols and QAPP, and the EDSP QMP or be explained and evaluated in a deviation report.

Direct and frequent communication between the PI and the Battelle QA Unit will provide for sufficient time to perform an ADQ, so that the submission date of the audited data meets that specified in the WA protocols. The scheduling process will also allow for a reasonable amount of time for corrections and subsequent verification of the corrections by QA.

WA personnel submit all data and records for review at the time of the ADQ. An EDSP QA team member or delegate reviews the data package for completeness and, if incomplete, requests that the additional records needed for review be submitted. EDSP QA team members review a minimum of 10% of the raw data, the tabulated data, and WA records of performance and methods to ensure compliance with the planning documents mentioned previously. At least 10% of the raw data are compared to the tabulated individual data, and a minimum 10% of the summary data tables are checked. EDSP QA team members will also check all tabulated data designated as statistically significant. Findings are reported as described below. EDSP QA team members review any data summary using the audited data and corrected tables to ensure that the reported results are of high quality and accurately reflect the raw data, and that the summary accurately describes the materials used in the WA. Findings are reported as described below.

20.2 AUDIT REPORT FORMAT

The audit report consists of a cover page for WA information and additional page(s) with the audit findings. All pages have header information containing the WA study protocol number,

audit date(s), and auditor. The audit report date is the date on which the EDSP QA Unit signs the audit report and sends it to the PI and EDSP management.

The cover page contains the WA protocol title, number, and code; PI; audit type; audit date(s); EDSP QA Unit; distribution list; the dated signature of the auditor; the date that the WAL received the audit report; and the dated signatures of the PI and management. The distribution list may include additional names for individuals who have findings pertaining to their area of responsibility. The official audit report is the hard copy containing corrective actions and dated signatures of the EDSP QA Unit, PI, and management. Subsequent page(s) contain the audit finding(s), any recommended remedial actions, and space for the PI to respond to the findings and document remedial actions taken or to be taken.

20.3 RESPONSE ACTIONS

There is no deadline for the PI's response to an ADQ report except for the time constraint deriving from the submission date of the final WA report. The PI forwards the audit report to management for review. Management adds comments as necessary, signs and dates the report, and returns it to the EDSP QA Team Member. The EDSP QA Team Member assesses the responses and verifies the corrective actions. If a disagreement between the PI and EDSP QA Team Member arises over a finding, these individuals will attempt to resolve the issue. If necessary, the EDSP Quality Assurance Manager may be consulted and/or the issue brought to the attention of management for resolution. The final resolution will be documented in the QA files.

During an assessment, if the auditor determines that adverse health effects could result or WA objectives of acceptable quality cannot be achieved, the auditor follows the Stop Work Procedure specified in the EDSP QMP (Section 3.3).

20.4 INDEPENDENT ASSESSMENTS

The EDSP Battelle QAM or designee may conduct an independent TSA and ADQ during the conduct of this work assignment. Typically, one independent audit will be conducted during the work assignment. If major deficiencies are uncovered, additional independent audits may be scheduled. The conduct and reporting of the audits will be consistent with the procedures described in the EDSP QMP (Section 3.3).

21.0 REPORTS TO MANAGEMENT

Each audit report prepared at Battelle is distributed to the PI, Work Assignment Leader and management of the applicable facility (i.e., PI's manager). The QA Unit will send copies of resolved audit reports containing findings, responses, and corrective actions taken to the EDSP Battelle's QA Manager as they are completed. The EDSP QA Manager routinely prepares and distributes quarterly reports to EDSP program management.

Each subcontracted facility will report audit findings as specified in their respective SOPs.

22.0 DATA REVIEW, VERIFICATION, AND VALIDATION

The histological analysis of tissues by EPL will be conducted under GLP. The reporting of the WA 3-11 results and information supplied by EPL will be reviewed by the technical personnel as described in the WA 3-11 QAPP.

23.0 VERIFICATION AND VALIDATION METHODS

Based on the pre-established acceptance criteria defined in this document and the study protocol, the technical personnel will review the data produced to determine data validity and that data meet project objectives. Data will also reviewed to determine if any samples were compromised during the analysis. Any sample data determined to be invalid will be documented and reported as such and, generally, will not be used in the data analysis. The review for data validity will be conducted prior to the Audits of Data Quality (ADQ) (verification) conducted by the EDSP QA team members.

The EDSP QA verification review (i.e., ADQ) will be conducted to assure data were collected in accordance with the planning documents (e.g., study protocol, QAPP, facility and EDSP SOPs) and that the reported results accurately reflect the raw data. The ADQ(s) conducted by the EDSP QA team members will be conducted and reported as specified in approved facility QA SOPs and the QMP and as described previously in section 20.0.

24.0 RECONCILIATION AND USER REQUIREMENTS

The data requirements and proposed methods for data analysis for this Task have been specified in previous sections and will be included in the study plan. Results will be communicated to EDSP program management and EPA as indicated in the Work Plan for this Task.

25.0 REFERENCE

Battelle, 2004. Final Study Plan on Amphibian Metamorphosis Assay Histopathology. Battelle, Columbus, Ohio. November 2004.

Study Guidance Documents:
Teleconference Records

1414 South Sangre Road
Stillwater, OK 74074-1899
T—405-624-6771
F—405-377-3388
djfort@fortlabs.com

Fort Environmental Laboratories, Inc.

Memo

To: Dr. Dave Houchens; Manager, Endocrine Disruptor Program

From: Douglas J. Fort, PI

CC: Les Touart, WAM, USEPA; Michael Blanton, WAM, Battelle; Sig Degitz and Joe Tietge, ORD/MED

Date: 4/21/2005

Re: Conference Call Notes

Parties Involved:

D.J. Fort and J.H. Thomas, FEL; J. Tietge and S. Degitz, USEPA ORD/MED

Date & Time:

Thursday, February 5, 2004 at 1030 CST

Outline and Discussion:

1. Updated status of Demonstration Study, WA 3-11

    a. DJF indicated that a start date of 2/4/2004 was established with PTU tentatively scheduled to start during the week of 2/9. The QAPP is officially signed and in place. The system is calibrated and ready for the study (see comment regarding proposed change in flow rate used by the flow diluter system).

2. Reviewed Protocol

    a. DJF briefly outlined the protocol to facilitate discussion. DJF also described the flow of work between the Battelle Chemical Repository and FEL regarding sample chemistry analysis, distribution of the test substances, and the initiation of testing at FEL.

3. Test Materials

    a. Stock Preparation (PTU)

        i. SD indicated that PTU should be prepared using water diluent (water) and top stirring to increase wetability.

        ii. DJF indicated that the protocol for preparing T4 stock was described in detail in the Study Plan.

iii. SD/JT suggested that stock preparation for the other materials could be provided by them (dexamethasone) or the Chemical Repository (Battelle)

b. Range Finding Experiments

i. DJF-SD indicated that range testing would run up to the limit of water solubility in a log set of five concentrations. Range finding will be needed for phenobarbital and PCN.

c. Chemical Measurement

i. DF described the problems encountered by the Chemical Repository related by Michael Cobb regarding analysis of T4. Essentially, the methodologies used were not sensitive to measure the lower range of the proposed test concentration series. SD indicated that they typically measure the two greatest concentrations (not less than 1µg/L) and use the proportional dilution to determine the remaining test concentrations. SD and JT further indicated that the approach will only work if the system is properly calibrated.

4. Lighting and Feeding Regime

a. JT asked SD and JT about the units used to measure light intensity. FEL measures light intensity in lux (lumens/sq m), whereas EPA guidance suggests lumens in the Study Plan. It was suggested that the light intensity be measured consistent with normally performed at FEL and any unit conversions be performed following the study. We discussed the fact that both USEPA ORD/MED and FEL use a mini-Benoit flow through diluter system which minimizes differences in the exposure system.

5. Staging

a. SD and JT suggested that a peer-reviewed approach to determining stage in which the technical staff concurrently reviews and agrees upon stage designation (consensus stage) prior to specimen selection be used. The use of computer imaging to assist in this process was recommended. DJF indicated that this was s standard practice for FEL.

6. Flow Rates

a. SD and JT suggested that the flow rate in the diluter system be reduced from 50 mL./min. as indicated in the Study Plan, protocol, and QAPP to 25 mL/min. The flow rate for the cultures should be maintained at 50 mL/min. since the rate of organism growth was consistent with that routinely observed at ORD/MED. This will require re-calibration of the diluter system. An amendment to the Study Protocol/QAPP will be required.

1414 South Sangre Road
Stillwater, OK 74074-1899
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F—405-377-3388
djfort@fortlabs.com

Fort Environmental Laboratories, Inc.

Memo

To: Dr. Dave Houchens; Program Manager, Endocrine Disruptor Program

From: Douglas J. Fort, PI

CC: Les Touart, WAM, USEPA; Michael Blanton, WAL, Battelle;

Date: 4/21/2005

Re: Conference Call Notes

Parties Involved:

D.J. Fort, FEL; D. Houchens and M. Blanton, Battelle; L. Touart, USEPA

Date & Time:

Thursday, February 5, 2004 at 1300 CST

Outline and Discussion:

1. Updated status of Demonstration Study, WA 3-11

    a. DJF indicated that a start date of 2/4/2004 was established with PTU tentatively scheduled to start during the week of 2/9. The QAPP is officially signed and in place. The system is calibrated and ready for the study (see comment regarding proposed change in flow rate used by the flow diluter system).

2. Reviewed Protocol

    a. DJF briefly outlined the protocol to facilitate discussion. DJF also described the follow of work between the Battelle Chemical Repository and FEL regarding sample chemistry analysis, distribution of the test substances, and the initiation of testing at FEL. Additional range-finding tests with phenobarbital and PCN will need to be performed. ORD/MED will provide information regarding test concentrations for dexamethasone.

3. Change in Diluter System Flow Rates

    a. In the earlier conference call, ORD/MED suggested that the flow rate in the diluter system be reduced from 50 mL./min. as indicated in the Study Plan, protocol, and QAPP to 25 mL/min. The flow rate for the cultures should be maintained at 50 mL/min. since the rate of organism growth was consistent with that routinely observed at ORD/MED. This will require re-calibration of the diluter system. An amendment/deviation to the Study Protocol/QAPP will be required.

4. Discussed the use of Quick Response action to accommodate additional cost associated with range-finding studies and recalibration of diluter.

Robert Rogers

From: "Douglas Fort" <djfort@fortlabs.com>
To: "Robert Rogers" <rrogers@fortlabs.com>; "John Thomas" <jthomas@fortlabs.com>
Sent: Monday, June 07, 2004 12:18 PM
Attach: Sampling Plan - Dexamethasone and T4.doc
Subject: Fw:

----- Original Message -----
From: Douglas Fort
To: Michael Blanton
Cc: Dave Houchens ; Michael Cobb
Sent: Friday, June 04, 2004 5:28 PM

Les,

Thanks for taking time to discuss WA 3-11 with us today. This message contains our meeting notes from today's conference call summarized in the discussion below. Based on our discussion this afternoon, attached is the proposed sampling plans for T4 and dexamethasone. I have also provided information regarding the sampling used for PTU and methimazole. Please note that the testing format used for PTU and methimazole are consistent with information stated in the current protocol and QAPP. However, because of technical issues surrounding testing of dexamethasone and T4, we propose the attached amended sampling program.

The protocols requires sample collection of the stock and test dilutions every 7-d of the 21-d test (4 sets of samples). These samples were collected immediately prior to entry into the test aquaria. Since degradation was not an issue with these two introductory materials this approach appeared to be suitable. The analytical data appears to be quite robust and tracks well with the nominal concentrations.

For T4 and dexamethasone, stock solutions will be prepared every 5 d based on stability data. Each stock solution will be measured. For T4, 4, 2, and 1 ug/L will be measured; and for dexamethasone 250, 62.5, and 15.6 ug/L will be measured. Analysis intervals will be ca. every 7d. Samples will be collected from each respective replicate tank and composited such that on d 0 samples will be collected from both stage 51 replicates (and composited). On d 7 samples will be collected from the stage 54 replicate tanks, d 14 collections occurs from the stage 51 chambers, and on d 21 samples are collected from the stage 54 replicates. This provides a reasonable number of samples, but not exhaustive.

We need to get quick resolution of this proposed change to the protocol as we will have organisms ready for study by Wednesday of next week (6/9). Also, John Thomas and I have been asked by Sig/Joe to come for a site visit the second week of July to discuss and compare data etc. It would be good to have at least some T4 data to make the trip really worthwhile.

Let us know if you have questions. Thanks.

Doug

Douglas J. Fort, Ph.D.
President
Fort Environmental Laboratories, Inc.
1414 South Sangre Road
Stillwater, OK 74074

Voice - 405-624-6771
Fax - 405-377-3388

Client: Battelle
Internal Project No.: BATT03
WA: WA 3-11
Study: Frog Metamorphosis Assay Demonstration

Chemical Analysis Approach

PTU and Methimazole (complete)

Test Replicates: 2/stage (stage 51 and 54)
Stock Sample Interval: day 0, 7, 14, and 21
Stock Solution Preparation Interval: every 7 d
Dilution Series Tested: all dilutions tested and control
Dilution Series Test Interval: day 0, 7, 14, and 21
Total Samples Generated/Study: 24
Sampling Point: sample box prior to split into replicate tanks

T4

Test Replicates: 2/stage (stage 51 and 54)
Stock Sample Interval: day 0, 5, 10, 15, and 20
Stock Solution Preparation Interval: every 5 d
Dilution Series Tested: 4, 2, and 1 µg/L, and control
Dilution Series Test Interval: day 0 (stage 51 only), 7 (stage 54 only), 14 (stage 51 only), and 21 (stage 54 only)*
Total Samples Generated/Study: 21
Sampling Point: each replicate tank, but composited into one sample

*only two replicates alternating between 51 and 54 sets are analyzed per allotted time period for the dilutions specified.

Dexamethasone

Test Replicates: 2/stage (stage 51 and 54)
Stock Sample Interval: day 0, 5, 10, 15, and 20
Stock Solution Preparation Interval: every 5 d
Dilution Series Tested: 250, 62.5, 15.6 µg/L, and control
Dilution Series Test Interval: day 0 (stage 51 only), 7 (stage 54 only), 14 (stage 51 only), and 21 (stage 54 only)*
Total Samples Generated/Study: 21
Sampling Point: each replicate tank, but composited into one sample

*only two replicates alternating between 51 and 54 sets are analyzed per allotted time period for the dilutions specified.

Fort Environmental Laboratories, Inc.
Environmental Laboratory Services/Consulting

Re: Summary of Teleconference Meeting on July 22, 2004.

Teleconference Attendees:
D. Fort, FEL
S. Degitz, U.S. EPA-Duluth
J. Tietge, U.S. EPA-Duluth
L. Touart, U.S. EPA
M. Blanton, Battelle

Topics: WA 3-11
1. Phenobarbital Test Conditions
2. PCN Solubility
3. Time Extension

1. FEL observed lethality and sedation at 1,000 and 500 mg/L phenobarbital using stage 51 organisms in a 4-d static renewal (S/R) exposure.

   U.S. EPA (Duluth) ran a S/R test with stage 54 organisms for 14-d using 1,500; 1,000; 500; 250; 125; and 0 mg/L (control). Developmental effects were observed at 1,500 mg/L only. Histological effects on the thyroid were noted at 1,500; 1,000; and 500 mg/L phenobarbital. The effects were concentration-dependent.

   Sig/Joe recommended that phenobarbital be tested in a S/R format using the following conditions:
   - 20 organisms
   - 8 L test volume
   - 24-h renewal
   - Same feeding regime as for flow-through
   - Concentrations for stage 51: 1,000; 500; 250; 125; 0 mg/L
   - Concentrations for stage 54: 1,500; 1,000; 500; 250; 0 mg/L

   M. Blanton will check on phenobarbital measurement status.

   L. Touart will provide a technical directive on technique to be used for in-life test and the frequency and selection of concentrations to be measured.

2. D. Fort reported the solubility of PCN (aqueous) to be 0.2 – 0.5 mg/L. Range finding studies and stability work by the repository will commence.

3. L. Touart will request a no-cost extension until 12/31/2004.

Meeting concluded.

1414 South Sangre Road • Stillwater, Oklahoma 74074 • (405) 624-6771 • FAX (405) 377-3388

Study Guidance Documents:
EPA Technical Directives

Robert Rogers

From: "Douglas Fort" <djfort@fortlabs.com>
To: "Robert Rogers" <rrogers@fortlabs.com>; "John Thomas" <jthomas@fortlabs.com>
Sent: Tuesday, June 08, 2004 10:50 PM
Subject: Fw: WA 3-11

Here is the sampling protocol for at least T4 and dexamethasone. Be sure to ask me any questions if any of this information is unclear.

Doug

----- Original Message -----
From: <Touart.Les@epamail.epa.gov>
To: "Houchens, David P" <houchensd@BATTELLE.ORG>
Cc: "Crecelius, Eric A" <eric.crecelius@pnl.gov>; <Schweer.Greg@epamail.epa.gov>; <Kariya.Jim@epamail.epa.gov>; "Cobb, Michael E" <michael.cobb@pnl.gov>; "Blanton, Michael L" <ml.blanton@pnl.gov>; <hall.rosemary@epamail.epa.gov>; "Douglas Fort" <djfort@fortlabs.com>
Sent: Tuesday, June 08, 2004 4:12 PM
Subject: RE: WA 3-11

>
>
>
>
> Dave,
>
> I reviewed the proposed chemical analysis approach proposed by Dr. Fort
> for T4 and Dexamethasone and have the following direction to impose. My
> understanding is that in each chemical study the proportional diluter
> system will support four (4) replicates, two replicates for Stage 51
> tadpoles and two replicates for Stage 54 tadpoles. Also, for both
> chemicals, the stability data indicate that stock solutions need to be
> replaced on a 5 day cycle. Therefore, a sample for each new stock
> solution prepared will be collected and shipped for analysis for a total
> of 4 samples for each chemical trial (day 0, day 5, day 10, and day 15
> of the study). Additional twelve (12) samples will be collected from
> the replicate tanks, 3 from each of 4 sample dates (day 0, day 7, day
> 15, and day 21). Samples will be taken from alternate replicates from
> control, medium (2 ug/L T4, 62.5 ug/L dexamethasone), and high (4 ug/L
> T4, 250 ug/L dexamethasone) test levels. There will be no compositing
> of samples taken from the replicate tanks. On day 0, the samples will
> be taken from replicate 1 of the stage 51 tadpoles, on day 7 from
> replicate 1 of stage 54 tadpoles (day 0 for this stage), on day 15 from
> replicate 2 of stage 51 tadpoles, and on day 21 from replicate 2 of
> stage 54 tadpoles. This sampling regime will provide for six shipments
> and a total of 16 samples to the chemical repository for analysis with 1
> to 4 samples to be analyzed depending on shipment.
>

> If you need further clarification of the sampling schedule described,
> please let me know.
>
> Les
>
>
> Leslie W. Touart, Ph.D.
> U.S. Environmental Protection Agency
> OPPTS/Office of Science Coordination and Policy
> 1200 Pennsylvania Ave, NW (7203M)
> Washington, DC 20460
>
> ph: 202-564-8468
> fx: 202-564-8482
>

Robert Rogers

From: "Douglas Fort" <djfort@fortlabs.com>
To: "Robert Rogers" <rrogers@fortlabs.com>; "John Thomas" <jthomas@fortlabs.com>
Sent: Friday, August 13, 2004 2:28 PM
Subject: Fw: WA 3-11

Original Message -----
From: <Touart.Les@epamail.epa.gov>
To: "Houchens, David P" <houchensd@BATTELLE.ORG>
Cc: "Douglas Fort" <djfort@fortlabs.com>; <phillips.linda@epamail.epa.gov>; "Cobb, Michael E" <michael.cobb@pnl.gov>; "Blanton, Michael L" <ml.blanton@pnl.gov>
Sent: Friday, August 13, 2004 1:40 PM
Subject: Re: WA 3-11

>
>
>
>
> Dave,
>
> The following sampling and analysis schedule should be used for the
> phenobarbital experiment in WA 3-11.
>
> The stock solution should be analyzed on initial day 0 and at all
> subsequent replacements (old and new solutions).
> On initial day 0, one replicate from the NF 51 treatment levels 0, 125,
> 250, 500, and 1000mg/L should be analyzed.
> On day 7 (day 0 for NF 54) one replicate from NF 54 treatment levels 0,
> 250, 500, 1000, and 1500 mg/L should be analyzed.
> On day 14 (day 7 for NF 54) one replicate from NF 51 treatment levels 0
> and 250 mg/L and one replicate from NF54 treatment levels 0 and 500 mg/L
> should be analyzed.
> On day 21 (day 14 for NF 54) the alternate replicate (from day 0) from
> NF 51treatment levels 0, 125, 250, 500, and 1000 mg/L and the alternate
> replicate (from day 7) from NF 54 treatment levels 0, 250, 500, 1000,
> and 1500 mg/L should be analyzed.
> Note that only those treatment levels with surviving tadpoles would be
> analyzed.
>
> If you have any questions or need clarification for this sampling regime
> please let me know.
>
> Les
>
> Leslie W. Touart, Ph.D.
> U.S. Environmental Protection Agency
> OPPTS/Office of Science Coordination and Policy
> 1200 Pennsylvania Ave, NW (7203M)
> Washington, DC 20460
>

Environmental Protection Agency **Office of Science Coordination and Policy** **Exposure Assessment Coordination and Policy Division** **Technical Direction**	Contract No: 68-W-01-023 Contractor: Batelle WA No: 3-11 WA Task No: 5 TD No: 1 Page One of 1 Start date: 08/27/2003

WA Title: Amphibian Metamorphosis Assay Demonstration Study

Background:

Technical difficulties in performing the assay with Phenobarbital will necessitate some changes to the original study plan. The following technical direction addresses these changes to be made.

Technical Direction:

For phenobarbital, the experimental exposure condition should be changed from a flow-through condition to a static renewal condition. The specific conditions of the change are:

- 20 organisms/ replicate
- 8 L test volume
- 24-h renewal
- Same feeding regime as flow-through
- Concentrations for NF stage 51: 0, 125, 250, 500, and 1000 mg/L
- Concentrations for NF stage 54: 0, 250, 500, 1000, and 1500 mg/L

Working stocks should be prepared in consultation with Sigmund Degitz (US-EPA/MED) and the analytical sampling regime and number of samples will be determined based on the renewal period for the working stocks.

The change imposed by this technical direction is not anticipated to affect the total hours already budgeted for the WA.

Deliverables Schedule (if applicable):

The contact person for this TD is Les Touart (202) 564-8468. Please call with any questions.

Contracting Officer Representative's Signature: Les Touart
Telephone No. 202-564-8468 Date: 07/29/2004

Original Message -----
From: <Touart.Les@epamail.epa.gov>
To: "Houchens, David P" <houchensd@BATTELLE.ORG>
Cc: "Douglas Fort" <djfort@fortlabs.com>; <phillips.linda@epamail.epa.gov>;
"Blanton, Michael L" <ml.blanton@pnl.gov>
Sent: Wednesday, September 01, 2004 3:17 PM
Subject: RE: WA 3-11

> Dave,
>
> Based upon the information and suggested approach (static-renewal) provided, the following concentrations, sampling and analysis schedule should be used for the PCN experiment in WA 3-11.
>
> The stock solution should be analyzed on initial day 0 and at all subsequent replacements (old and new solutions) with replacements based
> on stability and/or volume usage.
>
> Test concentrations are recommended to be 0, 7.4, 22.2, 66.7, and 200 ug/L (if solubility under test conditions is less than 200 ug/L, then the test levels should be adjusted appropriately).
> .
> On initial day 0, one replicate from the NF 51 treatment levels 0, 7.4, 22.2, 66.7, and 200 ug/L should be analyzed.

> On day 7 (day 0 for NF 54) one replicate from NF 54 treatment levels 0, 7.4, 22.2, 66.7, and 200 ug/L should be analyzed.

> On day 14 (day 7 for NF 54) one replicate from NF 51 treatment levels 0 and 22.2 mg/L and one replicate from NF54 treatment levels 0 and 66.7 ug/L should be analyzed.

> On day 21 (day 14 for NF 54) the alternate replicate (from day 0) from NF 51 and NF 54 treatment levels 0, 7.4, 22.2, 66.7, and 200 ug/L should be analyzed.
>
> Note also that only those treatment levels/replicates with surviving tadpoles would be analyzed if mortality removes a level/replicate.
>
> If you have any questions or need clarification for this sampling regime> please let me know.
>
> Les
>
> Leslie W. Touart, Ph.D.
> U.S. Environmental Protection Agency
> OPPTS/Office of Science Coordination and Policy
> 1200 Pennsylvania Ave, NW (7203M)
> Washington, DC 20460
>

Study Guidance Documents:
Protocol Deviations

Protocol Deviations to WA 3-11 Frog Metamorphosis Assays Performed by Fort Environmental Laboratories

6-Propyl-2-Thiouracil (PTU):

No deviations noted in PTU study journal. No deviations found by QAU.

Methimazole:

1. Test vessel temperatures were outside the target temperature range of 21º ± 1ºC on study day 1 for stage 51 (21-d) assay. Temperatures ranged from 22º to 25.1ºC. All temperatures were within acceptable limits when rechecked. It was concluded that these deviations had no impact on the outcome of this assay.
2. One test vessel temperature (19.2ºC) was outside the target temperature range of 21º ± 1ºC on study day 2 for stage 51 (21-d) assay. Temperature was within acceptable limits when rechecked. It was concluded that the deviation had no impact on the outcome of this assay.
3. Two test vessel temperatures were outside the target temperature range of 21º ± 1ºC on study day 7 for stage 51 (21-d) assay. Both temperatures were 19.9ºC. Both temperatures were within acceptable limits when rechecked. It was concluded that these deviations had no impact on the outcome of this assay.
4. Two test vessel temperatures were outside the target temperature range of 21º ± 1ºC on study day 13 for stage 51 (21-d) assay. Temperatures were 22.1º and 22.3ºC. Both temperatures were within acceptable limits when rechecked. It was concluded that these deviations had no impact on the outcome of this assay.
5. One test vessel temperature (22.1ºC) was outside the target temperature range of 21º ± 1ºC on study day 5 for stage 54 (14-d) assay. Temperature was within acceptable limits when rechecked. It was concluded that the deviation had no impact on the outcome of this assay.

L-Thyroxine (T4):

1. Test vessel temperatures were outside the target range of 21º ± 1ºC on study day 6 for stage 51 (21-d) assay. Readings ranged from 22.1º to 22.3ºC. Temperatures were within acceptable limits when rechecked. It was concluded that this deviation had no impact on the outcome of this assay.

Dexamethasone:

1. Test vessel temperatures were outside the target range of 21º ± 1ºC on at test setup (day 0) for stage 51 (21-d) assay. Readings ranged from 22.6º to 22.9ºC. Chillers were installed in the water bath to help control higher temperatures experienced during summer months. The thermostat setting was also adjusted to lower ambient room temperature. Temperatures were within acceptable limits when rechecked. It was concluded that this deviation had no impact on the outcome of this assay.
2. Test vessel temperatures were outside the target range of 21º ± 1ºC on test day 6 for stage 51 (21-d) assay. Readings ranged from 22.1º to 22.4ºC. The thermostat setting was adjusted to

lower ambient room temperature. Temperatures were within acceptable limits when rechecked. It was concluded that this deviation had no impact on the outcome of this assay.

Phenobarbital:

1. Light intensity readings were not taken on test days 6 and 21. It was determined that this oversight had no impact on the outcome of this assay.
2. Based on EPA Technical Directive for phenobarbital, stock solutions were to be sampled for chemical analysis on the initial make-up day (new) and on replacement day (old). Six batches of stock solutions were prepared during the course of the 21-d assay. Batches 1,3,5, and 6 were sampled as new, but were completely spent when replaced, leaving nothing (old) to sample for analysis. New and old samples were collected for stock batches 2 and 4. Based on the stability report from Battelle Chemical Repository, phenobarbital is stable for 21 d, which would indicate that this deviation had no negative impact on the outcome of this assay.

Pregnenolone-16-α-Carbonitrile (PCN):

1. Due to an overnight cold front, test vessel temperatures were slightly below the target range of 21º ± 1ºC on test day 1 for stage 51 (21-d) assay. Readings ranged from 19.3º to 19.4ºC. The thermostat setting was adjusted to increase ambient room temperature. Temperatures were within acceptable limits when rechecked. It was concluded that this deviation had no impact on the outcome of this assay.
2. Analytical results for PCN in-life concentrations were consistent throughout the test with the exception of the last sampling event, which was reported as below detection limit. However, the stock concentration corresponding to the last sampling event, which was the same nominal concentration as the highest test concentration, was measurable and consistent with the results of the previous 3 sampling events. Because this difference could not be reconciled with the analytical laboratory, only results from the first three sampling events were used to calculate the average measured test concentrations reported in the study.

Appendix C
6-n-Propyl-Thiouracil (PTU)

Metamorphosis Assay – Raw Data:

Daily Operational Data
Test Termination Data – Stage and Growth
Statistical Data – Stage and Growth

Test Chemical Analytical Data from Battelle:

Purity / Stability Report
In-Life Test Chemical Analysis Report

Thyroid Histology Data from EPL:

Sample Identification Key
Thyroid Pathology Report

6-n-Propyl-Thiouracil (PTU) Metamorphosis Assay: Daily Operational Data

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00051 **Test No: 1**
Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 0.0 mg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc[1] (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	03/05/04			100.0	20.1	7.1	9.5	1690	24			100.0	20.1	7.2	9.6	1550	24	100.0	20.1	7.2	9.6	1620	24	0.009
1	03/06/04		20	100.0	21.9	7.3	9.2	1451	26		20	100.0	21.7	7.3	9.3	1454	26	100.0	21.8	7.3	9.3	1453	26	
2	03/07/04		20	100.0	21.8	7.2	9.1	1731	26		20	100.0	21.8	7.3	9.4	1614	26	100.0	21.8	7.3	9.3	1673	26	
3	03/08/04		20	100.0	21.6	7.2	8.8	1757	26	1	19	95.0	21.6	7.3	9.2	1553	26	97.5	21.6	7.3	9.0	1655	26	
4	03/09/04		20	100.0	21.4	7.4	8.7	1825	27		19	95.0	21.4	7.3	9.1	1560	27	97.5	21.4	7.4	8.9	1693	27	
5	03/10/04		20	100.0	21.0	7.3	8.4	1780	26		19	95.0	21.4	7.3	8.8	1580	26	97.5	21.2	7.3	8.6	1680	26	
6	03/11/04		20	100.0	21.0	7.1	8.6	1680	27		19	95.0	21.0	7.0	8.9	1539	27	97.5	21.0	7.1	8.8	1610	27	
7	03/12/04		20	100.0	21.0	7.2	8.1	1862	26		19	95.0	21.0	7.3	8.6	1522	26	97.5	21.0	7.3	8.4	1692	26	0.009
8	03/13/04		20	100.0	21.0	7.3	8.7	1762	26		19	95.0	21.0	7.3	8.9	1625	26	97.5	21.0	7.3	8.8	1694	26	
9	03/14/04		20	100.0	21.0	7.5	8.5	1752	26		19	95.0	21.0	7.1	8.7	1630	26	97.5	21.0	7.3	8.6	1691	26	
10	03/15/04		20	100.0	21.0	7.1	8.4	1870	26		19	95.0	21.0	7.3	8.8	1550	26	97.5	21.0	7.2	8.6	1710	26	
11	03/16/04		20	100.0	22.0	7.2	8.1	1841	26		19	95.0	21.0	7.2	8.5	1616	26	97.5	21.5	7.2	8.3	1729	26	
12	03/17/04		20	100.0	22.0	7.2	8.0	1976	27		19	95.0	22.0	7.2	8.3	1584	27	97.5	22.0	7.2	8.2	1780	27	
13	03/18/04		20	100.0	21.0	7.1	7.8	1325	27		19	95.0	21.0	7.2	8.3	1494	27	97.5	21.0	7.2	8.1	1410	27	
14	03/19/04		20	100.0	22.0	7.1	8.0	1780	27		19	95.0	22.0	7.2	8.2	1656	27	97.5	22.0	7.2	8.1	1718	27	0.009
15	03/20/04		20	100.0	22.0	7.0	8.1	1622	28		19	95.0	22.0	7.2	8.2	1488	28	97.5	22.0	7.1	8.2	1555	28	
16	03/21/04		20	100.0	22.0	7.2	7.8	1636	34		19	95.0	22.0	7.2	7.8	1626	34	97.5	22.0	7.2	7.8	1631	34	
17	03/22/04		20	100.0	22.0	7.2	7.7	1720	26		19	95.0	22.0	7.2	8.1	1601	26	97.5	22.0	7.2	7.9	1661	26	
18	03/23/04		20	100.0	22.0	7.1	7.1	1718	26		19	95.0	22.0	7.2	7.5	1622	26	97.5	22.0	7.2	7.3	1670	26	
19	03/24/04		20	100.0	21.0	7.2	7.5	1690	26		19	95.0	21.2	7.3	7.7	1370	26	97.5	21.1	7.3	7.6	1530	26	
20	03/25/04		20	100.0	21.9	7.3	7.0	1691	26		19	95.0	21.9	7.4	7.2	1450	26	97.5	21.9	7.4	7.1	1571	26	
21	03/26/04		20	100.0							19	95.0						97.5						0.009
	Median:				**21.6**	**7.2**	**8.1**	**1731**	**26**				**21.4**	**7.2**	**8.6**	**1560**	**26**		**21.5**	**7.2**	**8.4**	**1670**	**26**	
	Mode:				**21.0**	**7.2**	**8.1**	**1690**	**26**				**21.0**	**7.2**	**8.8**	**1550**	**26**		**21.0**	**7.3**	**9.3**	**#N/A**	**26**	
	Mean:			**100.0**	**21.5**	**7.2**	**8.2**	**1722**	**26.6**			**95.7**	**21.4**	**7.2**	**8.5**	**1556**	**26.6**	**97.8**	**21.4**	**7.2**	**8.4**	**1639**	**26.6**	**0.009**
	SEM:			**0.00**	**0.12**	**0.03**	**0.14**	**30.65**	**0.41**			**0.37**	**0.11**	**0.02**	**0.14**	**15.89**	**0.41**	**0.19**	**0.11**	**0.02**	**0.14**	**19.96**	**0.41**	**0.00**
	% CV:			**0.00**	**11.93**	**4.31**	**22.67**	**338.49**	**35.99**			**17.95**	**11.35**	**3.21**	**22.04**	**184.61**	**35.99**	**8.88**	**11.34**	**2.97**	**22.21**	**225.96**	**35.99**	**0.00**

[1]All measured test concentrations reported by Battelle as less than method detection limit (MDL) [0.0189 mg/L] or none detected (ND) are expressed as 0.009 mg/L (1/2 of MDL).

DAILY DATA

Workorder No: 00051 **Test No: 1**
Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 2.5 mg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	03/05/04			100.0	20.1	7.3	9.5	1590	24			100.0	20.1	7.3	9.6	1550	24	100.0	20.1	7.3	9.6	1570	24	3.18
1	03/06/04		20	100.0	21.6	7.4	9.3	1419	27		20	100.0	21.6	7.4	9.4	1460	27	100.0	21.6	7.4	9.4	1440	27	
2	03/07/04		20	100.0	21.6	7.3	9.3	1457	26		20	100.0	21.6	7.4	9.4	1620	26	100.0	21.6	7.4	9.4	1539	26	
3	03/08/04		20	100.0	21.6	7.4	9.2	1488	26		20	100.0	21.6	7.4	9.0	1509	26	100.0	21.6	7.4	9.1	1499	26	
4	03/09/04		20	100.0	21.4	7.4	9.1	1600	26		20	100.0	21.4	7.4	9.2	1925	26	100.0	21.4	7.4	9.2	1763	26	
5	03/10/04		20	100.0	21.3	7.3	8.8	1512	26		20	100.0	21.2	7.3	8.8	1827	26	100.0	21.3	7.3	8.8	1670	26	
6	03/11/04		20	100.0	21.0	7.2	8.7	1620	26		20	100.0	21.0	7.2	8.8	1707	26	100.0	21.0	7.2	8.8	1664	26	
7	03/12/04		20	100.0	21.0	7.3	8.5	1835	26		20	100.0	21.0	7.4	8.6	1493	26	100.0	21.0	7.4	8.6	1664	26	2.77
8	03/13/04		20	100.0	21.0	7.3	8.8	1494	26		20	100.0	21.0	7.4	8.9	1867	26	100.0	21.0	7.4	8.9	1681	26	
9	03/14/04		20	100.0	21.0	7.2	8.6	1434	26		20	100.0	21.0	7.3	8.7	1749	26	100.0	21.0	7.3	8.7	1592	26	
10	03/15/04		20	100.0	21.0	7.3	8.7	1465	25		20	100.0	21.0	7.3	8.7	1787	25	100.0	21.0	7.3	8.7	1626	25	
11	03/16/04		20	100.0	21.0	7.2	8.3	1525	25		20	100.0	21.0	7.3	8.4	1799	25	100.0	21.0	7.3	8.4	1662	25	
12	03/17/04		20	100.0	21.0	7.2	8.2	1418	25		20	100.0	22.0	7.2	8.3	1705	25	100.0	21.5	7.2	8.3	1562	25	
13	03/18/04		20	100.0	21.0	7.2	7.9	1420	24		20	100.0	21.0	7.2	7.9	1190	24	100.0	21.0	7.2	7.9	1305	24	
14	03/19/04		20	100.0	22.0	7.2	8.0	1475	24		20	100.0	22.0	7.2	7.9	1699	24	100.0	22.0	7.2	8.0	1587	24	2.45
15	03/20/04		20	100.0	22.0	7.2	8.4	1377	24		20	100.0	22.0	7.2	8.2	1318	24	100.0	22.0	7.2	8.3	1348	24	
16	03/21/04		20	100.0	22.0	7.3	7.8	1376	27		20	100.0	22.0	7.3	7.7	1660	27	100.0	22.0	7.3	7.8	1518	27	
17	03/22/04		20	100.0	22.0	7.2	7.9	1451	25		20	100.0	22.0	7.3	8.2	1682	25	100.0	22.0	7.3	8.1	1567	25	
18	03/23/04		20	100.0	22.0	7.2	7.3	1468	24		20	100.0	22.0	7.2	7.4	1751	24	100.0	22.0	7.2	7.4	1610	24	
19	03/24/04		20	100.0	21.2	7.3	7.6	1494	24		20	100.0	21.2	7.3	7.6	1649	24	100.0	21.2	7.3	7.6	1572	24	
20	03/25/04		20	100.0	21.8	7.4	7.0	1402	24		20	100.0	21.9	7.4	7.2	1651	24	100.0	21.9	7.4	7.1	1527	24	
21	03/26/04		20	100.0							20	100.0						100.0						2.15
	Median:				**21.3**	**7.3**	**8.5**	**1468**	**25**				**21.4**	**7.3**	**8.6**	**1682**	**25**		**21.4**	**7.3**	**8.6**	**1572**	**25**	
	Mode:				**21.0**	**7.2**	**9.3**	**1494**	**26**				**21.0**	**7.3**	**9.4**	**#N/A**	**26**		**21.0**	**7.2**	**9.4**	**#N/A**	**26**	
	Mean:			**100.0**	**21.4**	**7.3**	**8.4**	**1491**	**25.2**			**100.0**	**21.4**	**7.3**	**8.5**	**1648**	**25.2**	**100.0**	**21.4**	**7.3**	**8.4**	**1569**	**25.2**	**2.638**
	SEM:			**0.00**	**0.11**	**0.02**	**0.15**	**22.65**	**0.23**			**0.00**	**0.11**	**0.02**	**0.15**	**38.98**	**0.23**	**0.00**	**0.11**	**0.02**	**0.15**	**23.81**	**0.23**	**0.22**
	% CV:			**0.00**	**10.89**	**2.85**	**23.59**	**268.75**	**20.79**			**0.00**	**11.25**	**2.98**	**23.41**	**440.04**	**20.79**	**0.00**	**10.81**	**2.79**	**23.41**	**275.47**	**20.79**	**27.36**

DAILY DATA

Workorder No: 00051 **Test No: 1**
Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 5.0 mg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	03/05/04			100.0	20.1	7.3	9.5	1230	23			100.0	20.1	7.4	9.5	1560	23	100.0	20.1	7.4	9.5	1395	23	7.62
1	03/06/04		20	100.0	21.5	7.4	9.2	1415	21		20	100.0	21.6	7.4	9.3	1402	21	100.0	21.6	7.4	9.3	1409	21	
2	03/07/04		20	100.0	21.6	7.4	9.1	1489	25		20	100.0	21.5	7.4	9.3	1596	25	100.0	21.6	7.4	9.2	1543	25	
3	03/08/04		20	100.0	21.5	7.4	9.0	1509	26		20	100.0	21.4	7.4	9.3	1568	26	100.0	21.5	7.4	9.2	1539	26	
4	03/09/04		20	100.0	21.5	7.4	8.4	1540	27		20	100.0	21.2	7.4	8.6	1565	27	100.0	21.4	7.4	8.5	1553	27	
5	03/10/04		20	100.0	21.3	7.3	8.5	1485	23		20	100.0	21.0	7.3	8.8	1745	23	100.0	21.2	7.3	8.7	1615	23	
6	03/11/04		20	100.0	21.0	7.3	8.8	1870	22		20	100.0	21.0	7.3	8.9	1585	22	100.0	21.0	7.3	8.9	1728	22	
7	03/12/04		20	100.0	21.0	7.4	8.4	1440	22		20	100.0	21.0	7.4	8.7	1570	22	100.0	21.0	7.4	8.6	1505	22	4.87
8	03/13/04		20	100.0	21.0	7.3	8.7	1638	25		20	100.0	21.0	7.3	8.8	1605	25	100.0	21.0	7.3	8.8	1622	25	
9	03/14/04		20	100.0	21.0	7.3	8.2	1519	26		20	100.0	21.0	7.4	8.6	1673	26	100.0	21.0	7.4	8.4	1596	26	
10	03/15/04		20	100.0	21.0	7.3	8.7	1400	24		20	100.0	21.0	7.3	8.7	1680	24	100.0	21.0	7.3	8.7	1540	24	
11	03/16/04		20	100.0	21.0	7.2	8.4	1523	23		20	100.0	21.0	7.2	8.6	1689	23	100.0	21.0	7.2	8.5	1606	23	
12	03/17/04		20	100.0	22.0	7.2	8.2	1543	23		20	100.0	21.0	7.2	8.4	1820	23	100.0	21.5	7.2	8.3	1682	23	
13	03/18/04		20	100.0	22.0	7.2	7.9	1187	23		20	100.0	22.0	7.2	8.1	1191	23	100.0	22.0	7.2	8.0	1189	23	
14	03/19/04		20	100.0	22.0	7.2	8.0	1302	23		20	100.0	22.0	7.2	8.1	1588	23	100.0	22.0	7.2	8.1	1445	23	6.09
15	03/20/04		20	100.0	22.0	7.2	8.1	1360	24		20	100.0	22.0	7.3	8.5	1360	24	100.0	22.0	7.3	8.3	1360	24	
16	03/21/04		20	100.0	22.0	7.3	7.7	1472	23		20	100.0	22.0	7.3	7.7	1377	23	100.0	22.0	7.3	7.7	1425	23	
17	03/22/04		20	100.0	22.0	7.2	7.8	1490	22		20	100.0	22.0	7.2	8.1	1624	22	100.0	22.0	7.2	8.0	1557	22	
18	03/23/04		20	100.0	22.0	7.2	7.0	1276	21		20	100.0	21.8	7.2	7.5	1595	21	100.0	21.9	7.2	7.3	1436	21	
19	03/24/04		20	100.0	21.4	7.3	7.2	1208	21		20	100.0	21.4	7.3	7.5	1603	21	100.0	21.4	7.3	7.4	1406	21	
20	03/25/04		20	100.0	22.0	7.3	7.0	1401	23		20	100.0	21.8	7.4	7.0	1676	23	100.0	21.9	7.4	7.0	1539	23	
21	03/26/04		20	100.0							20	100.0						100.0						5.96
	Median:				**21.5**	**7.3**	**8.4**	**1472**	**23**				**21.4**	**7.3**	**8.6**	**1595**	**23**		**21.5**	**7.3**	**8.5**	**1539**	**23**	
	Mode:				**22.0**	**7.3**	**8.4**	**#N/A**	**23**				**21.0**	**7.4**	**9.3**	**#N/A**	**23**		**21.0**	**7.3**	**8.5**	**1539**	**23**	
	Mean:			**100.0**	**21.5**	**7.3**	**8.3**	**1443**	**23.3**			**100.0**	**21.4**	**7.3**	**8.5**	**1575**	**23.3**	**100.0**	**21.4**	**7.3**	**8.4**	**1509**	**23.3**	**6.134**
	SEM:			**0.00**	**0.11**	**0.02**	**0.15**	**34.10**	**0.37**			**0.00**	**0.11**	**0.02**	**0.14**	**30.91**	**0.37**	**0.00**	**0.11**	**0.02**	**0.15**	**26.79**	**0.37**	**0.56**
	% CV:			**0.00**	**11.32**	**2.85**	**23.98**	**411.43**	**34.85**			**0.00**	**10.99**	**3.07**	**22.70**	**356.88**	**34.85**	**0.00**	**10.87**	**2.87**	**23.21**	**316.07**	**34.85**	**45.60**

DAILY DATA

Workorder No: 00051 **Test No: 1**
Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 10.0 mg/L**

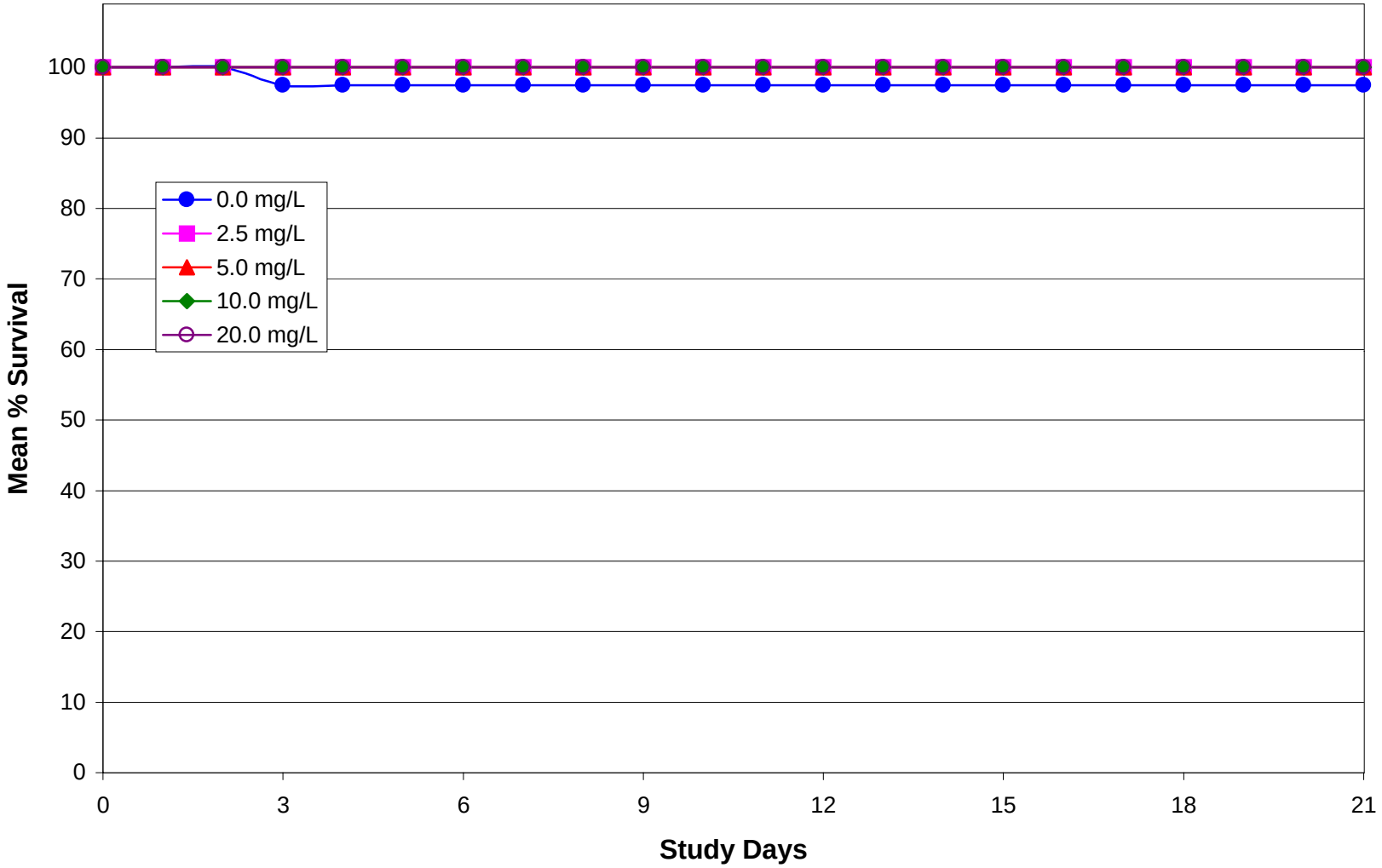
		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	03/05/04			100.0	20.1	7.4	9.6	1660	23			100.0	20.1	7.3	9.8	1570	23	100.0	20.1	7.4	9.7	1615	23	12.14
1	03/06/04		20	100.0	21.6	7.4	9.3	1435	20		20	100.0	21.5	7.4	9.4	1444	20	100.0	21.6	7.4	9.4	1440	20	
2	03/07/04		20	100.0	21.6	7.4	9.3	1792	24		20	100.0	21.4	7.4	9.5	1698	24	100.0	21.5	7.4	9.4	1745	24	
3	03/08/04		20	100.0	21.6	7.4	9.3	1772	25		20	100.0	21.5	7.3	9.5	1725	25	100.0	21.6	7.4	9.4	1749	25	
4	03/09/04		20	100.0	21.2	7.4	9.1	1895	24		20	100.0	21.3	7.3	9.5	1835	24	100.0	21.3	7.4	9.3	1865	24	
5	03/10/04		20	100.0	21.2	7.3	8.7	1801	24		20	100.0	21.0	7.3	9.0	1760	24	100.0	21.1	7.3	8.9	1781	24	
6	03/11/04		20	100.0	21.0	7.4	8.6	1755	20		20	100.0	21.0	7.3	8.8	1586	20	100.0	21.0	7.4	8.7	1671	20	
7	03/12/04		20	100.0	21.0	7.3	8.4	1805	19		20	100.0	21.0	7.3	8.6	1785	19	100.0	21.0	7.3	8.5	1795	19	11.48
8	03/13/04		20	100.0	21.0	7.2	8.6	1891	23		20	100.0	21.0	7.1	8.8	1841	23	100.0	21.0	7.2	8.7	1866	23	
9	03/14/04		20	100.0	21.0	7.5	9.1	1883	27		20	100.0	21.0	7.2	9.0	1858	27	100.0	21.0	7.4	9.1	1871	27	
10	03/15/04		20	100.0	21.0	7.3	8.7	1930	22		20	100.0	21.0	7.3	8.9	1813	22	100.0	21.0	7.3	8.8	1872	22	
11	03/16/04		20	100.0	21.0	7.2	8.4	1865	24		20	100.0	21.0	7.2	8.4	1875	24	100.0	21.0	7.2	8.4	1870	24	
12	03/17/04		20	100.0	21.0	7.2	8.3	1865	25		20	100.0	21.0	7.2	8.5	1837	25	100.0	21.0	7.2	8.4	1851	25	
13	03/18/04		20	100.0	22.0	7.2	8.1	1360	23		20	100.0	22.0	7.2	8.2	1367	23	100.0	22.0	7.2	8.2	1364	23	
14	03/19/04		20	100.0	22.0	7.2	8.1	1842	23		20	100.0	22.0	7.2	8.1	1766	23	100.0	22.0	7.2	8.1	1804	23	11.64
15	03/20/04		20	100.0	21.0	7.3	8.1	1573	23		20	100.0	22.0	7.3	8.2	1435	23	100.0	21.5	7.3	8.2	1504	23	
16	03/21/04		20	100.0	22.0	7.3	8.2	1526	25		20	100.0	22.0	7.3	8.2	1554	25	100.0	22.0	7.3	8.2	1540	25	
17	03/22/04		20	100.0	22.0	7.3	8.3	1781	24		20	100.0	22.0	7.2	8.4	1750	24	100.0	22.0	7.3	8.4	1766	24	
18	03/23/04		20	100.0	22.0	7.2	7.6	1796	25		20	100.0	21.8	7.2	7.7	1778	25	100.0	21.9	7.2	7.7	1787	25	
19	03/24/04		20	100.0	21.1	7.3	7.6	1544	25		20	100.0	21.1	7.3	7.8	1618	25	100.0	21.1	7.3	7.7	1581	25	
20	03/25/04		20	100.0	21.8	7.4	7.2	1721	25		20	100.0	21.7	7.4	7.4	1713	25	100.0	21.8	7.4	7.3	1717	25	
21	03/26/04		20	100.0							20	100.0						100.0						12.70
	Median:				**21.2**	**7.3**	**8.4**	**1792**	**24**				**21.3**	**7.3**	**8.6**	**1750**	**24**		**21.3**	**7.3**	**8.5**	**1766**	**24**	
	Mode:				**21.0**	**7.4**	**9.3**	**1865**	**25**				**21.0**	**7.3**	**9.5**	**#N/A**	**25**		**21.0**	**7.3**	**9.4**	**#N/A**	**25**	
	Mean:			**100.0**	**21.3**	**7.3**	**8.5**	**1738**	**23.5**			**100.0**	**21.4**	**7.3**	**8.7**	**1696**	**23.5**	**100.0**	**21.3**	**7.3**	**8.6**	**1717**	**23.5**	**11.990**
	SEM:			**0.00**	**0.11**	**0.02**	**0.14**	**35.12**	**0.42**			**0.00**	**0.11**	**0.02**	**0.14**	**32.88**	**0.42**	**0.00**	**0.11**	**0.02**	**0.14**	**33.43**	**0.42**	**0.28**
	% CV:			**0.00**	**10.99**	**3.37**	**21.59**	**386.04**	**40.03**			**0.00**	**10.92**	**2.91**	**22.36**	**365.88**	**40.03**	**0.00**	**10.64**	**2.83**	**21.90**	**369.73**	**40.03**	**15.90**

DAILY DATA

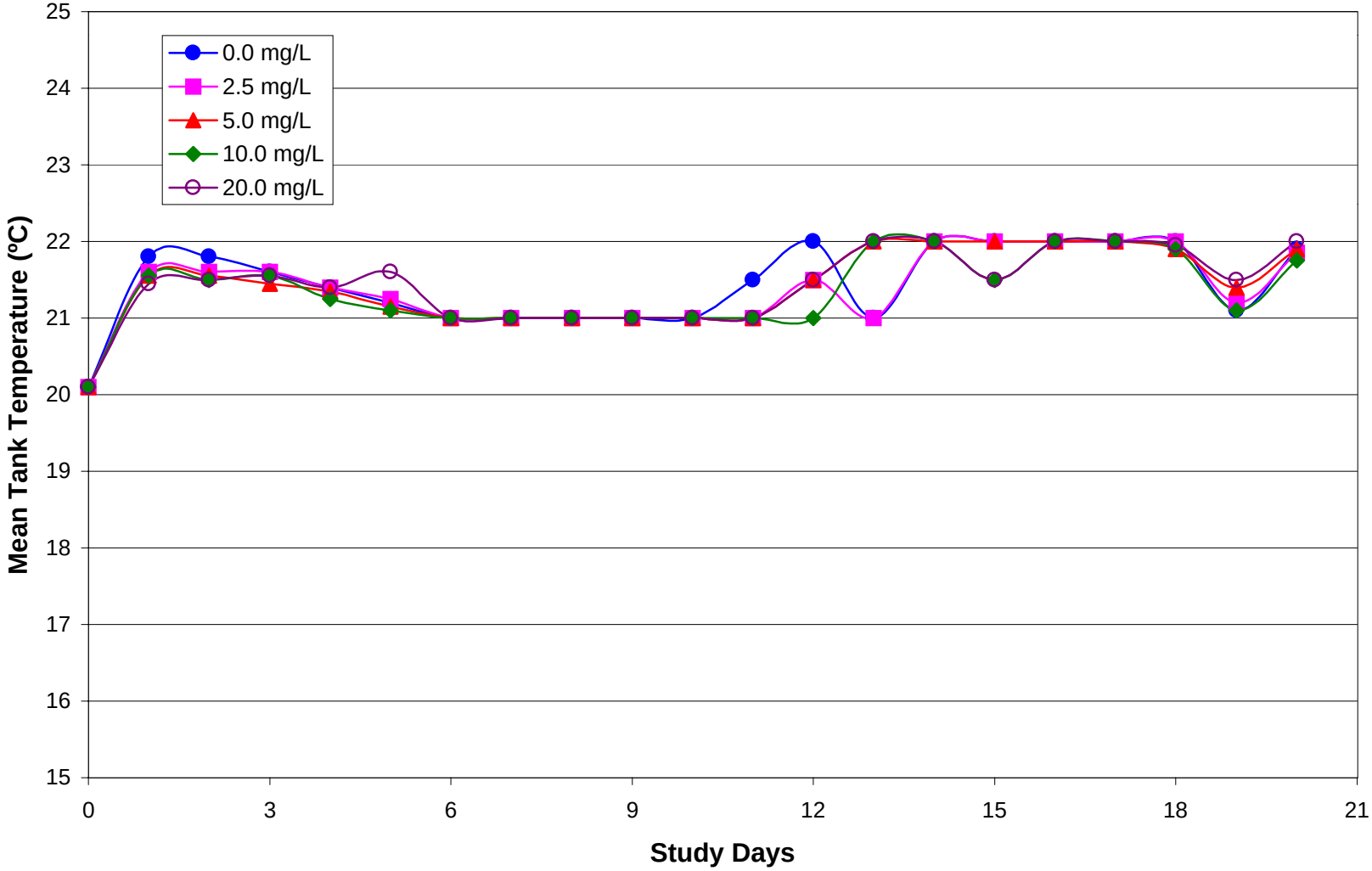
Workorder No: 00051 **Test No: 1**
Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 20.0 mg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	03/05/04			100.0	20.1	7.4	9.5	1740	19			100.0	20.1	7.3	9.8	1630	19	100.0	20.1	7.4	9.7	1685	19	20.51
1	03/06/04		20	100.0	21.4	7.4	9.4	1470	23		20	100.0	21.5	7.4	9.4	1391	23	100.0	21.5	7.4	9.4	1431	23	
2	03/07/04		20	100.0	21.5	7.4	9.4	1960	24		20	100.0	21.5	7.3	9.5	1704	24	100.0	21.5	7.4	9.5	1832	24	
3	03/08/04		20	100.0	21.6	7.4	9.2	1961	24		20	100.0	21.5	7.3	9.4	1775	24	100.0	21.6	7.4	9.3	1868	24	
4	03/09/04		20	100.0	21.4	7.4	9.1	1960	23		20	100.0	21.4	7.3	9.3	1790	23	100.0	21.4	7.4	9.2	1875	23	
5	03/10/04		20	100.0	21.8	7.3	7.0	1985	24		20	100.0	21.4	7.2	6.9	1880	24	100.0	21.6	7.3	7.0	1933	24	
6	03/11/04		20	100.0	21.0	7.3	8.8	1678	23		20	100.0	21.0	7.2	8.8	1788	23	100.0	21.0	7.3	8.8	1733	23	
7	03/12/04		20	100.0	21.0	7.5	8.6	1940	23		20	100.0	21.0	7.4	8.7	1941	23	100.0	21.0	7.5	8.7	1941	23	20.16
8	03/13/04		20	100.0	21.0	7.2	8.8	1938	23		20	100.0	21.0	7.2	8.7	1915	23	100.0	21.0	7.2	8.8	1927	23	
9	03/14/04		20	100.0	21.0	7.3	8.5	1967	25		20	100.0	21.0	7.4	8.7	1950	25	100.0	21.0	7.4	8.6	1959	25	
10	03/15/04		20	100.0	21.0	7.3	8.8	1910	22		20	100.0	21.0	7.3	8.6	1992	22	100.0	21.0	7.3	8.7	1951	22	
11	03/16/04		20	100.0	21.0	7.2	8.4	1929	22		20	100.0	21.0	7.2	8.2	1964	22	100.0	21.0	7.2	8.3	1947	22	
12	03/17/04		20	100.0	22.0	7.2	8.3	1875	27		20	100.0	21.0	7.2	8.1	1851	27	100.0	21.5	7.2	8.2	1863	27	
13	03/18/04		20	100.0	22.0	7.2	8.1	1487	20		20	100.0	22.0	7.2	7.9	1437	20	100.0	22.0	7.2	8.0	1462	20	
14	03/19/04		20	100.0	22.0	7.2	8.1	1799	21		20	100.0	22.0	7.2	8.0	1855	21	100.0	22.0	7.2	8.1	1827	21	20.43
15	03/20/04		20	100.0	22.0	7.2	8.3	1657	22		20	100.0	21.0	7.2	8.0	1638	22	100.0	21.5	7.2	8.2	1648	22	
16	03/21/04		20	100.0	22.0	7.3	8.2	1888	22		20	100.0	22.0	7.3	8.0	1553	22	100.0	22.0	7.3	8.1	1721	22	
17	03/22/04		20	100.0	22.0	7.2	8.4	1934	25		20	100.0	22.0	7.2	8.3	1876	25	100.0	22.0	7.2	8.4	1905	25	
18	03/23/04		20	100.0	22.0	7.2	7.4	1913	23		20	100.0	21.9	7.1	7.1	1830	23	100.0	22.0	7.2	7.3	1872	23	
19	03/24/04		20	100.0	21.5	7.3	7.1	1845	21		20	100.0	21.5	7.1	5.0	1712	21	100.0	21.5	7.2	6.1	1779	21	
20	03/25/04		20	100.0	22.0	7.4	7.2	1866	24		20	100.0	22.0	7.2	4.8	1902	24	100.0	22.0	7.3	6.0	1884	24	
21	03/26/04		20	100.0							20	100.0						100.0						20.71
	Median:				**21.5**	**7.3**	**8.4**	**1910**	**23**				**21.4**	**7.2**	**8.3**	**1830**	**23**		**21.5**	**7.3**	**8.4**	**1868**	**23**	
	Mode:				**22.0**	**7.2**	**8.8**	**1960**	**23**				**21.0**	**7.2**	**8.7**	**#N/A**	**23**		**21.0**	**7.2**	**#N/A**	**#N/A**	**23**	
	Mean:			**100.0**	**21.5**	**7.3**	**8.4**	**1843**	**22.9**			**100.0**	**21.4**	**7.2**	**8.2**	**1780**	**22.9**	**100.0**	**21.4**	**7.3**	**8.3**	**1811**	**22.9**	**20.454**
	SEM:			**0.00**	**0.12**	**0.02**	**0.16**	**33.24**	**0.39**			**0.00**	**0.11**	**0.02**	**0.29**	**36.93**	**0.39**	**0.00**	**0.11**	**0.02**	**0.22**	**33.01**	**0.39**	**0.11**
	% CV:			**0.00**	**11.39**	**3.51**	**25.80**	**354.86**	**37.58**			**0.00**	**10.86**	**3.24**	**46.02**	**401.18**	**37.58**	**0.00**	**10.62**	**3.08**	**35.19**	**355.44**	**37.58**	**5.01**

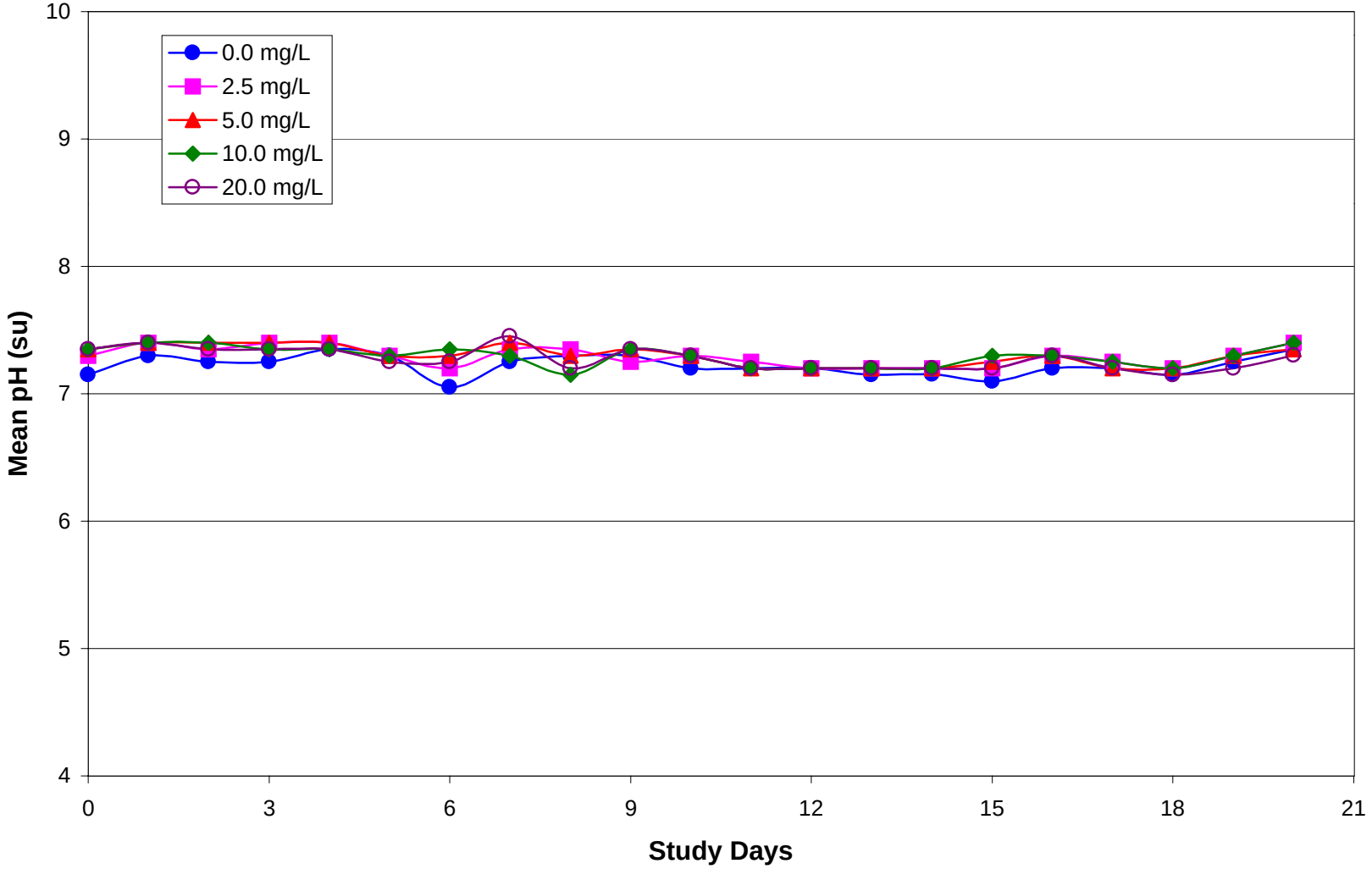
BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average of Larval Survival for Initial Stage 51 Test



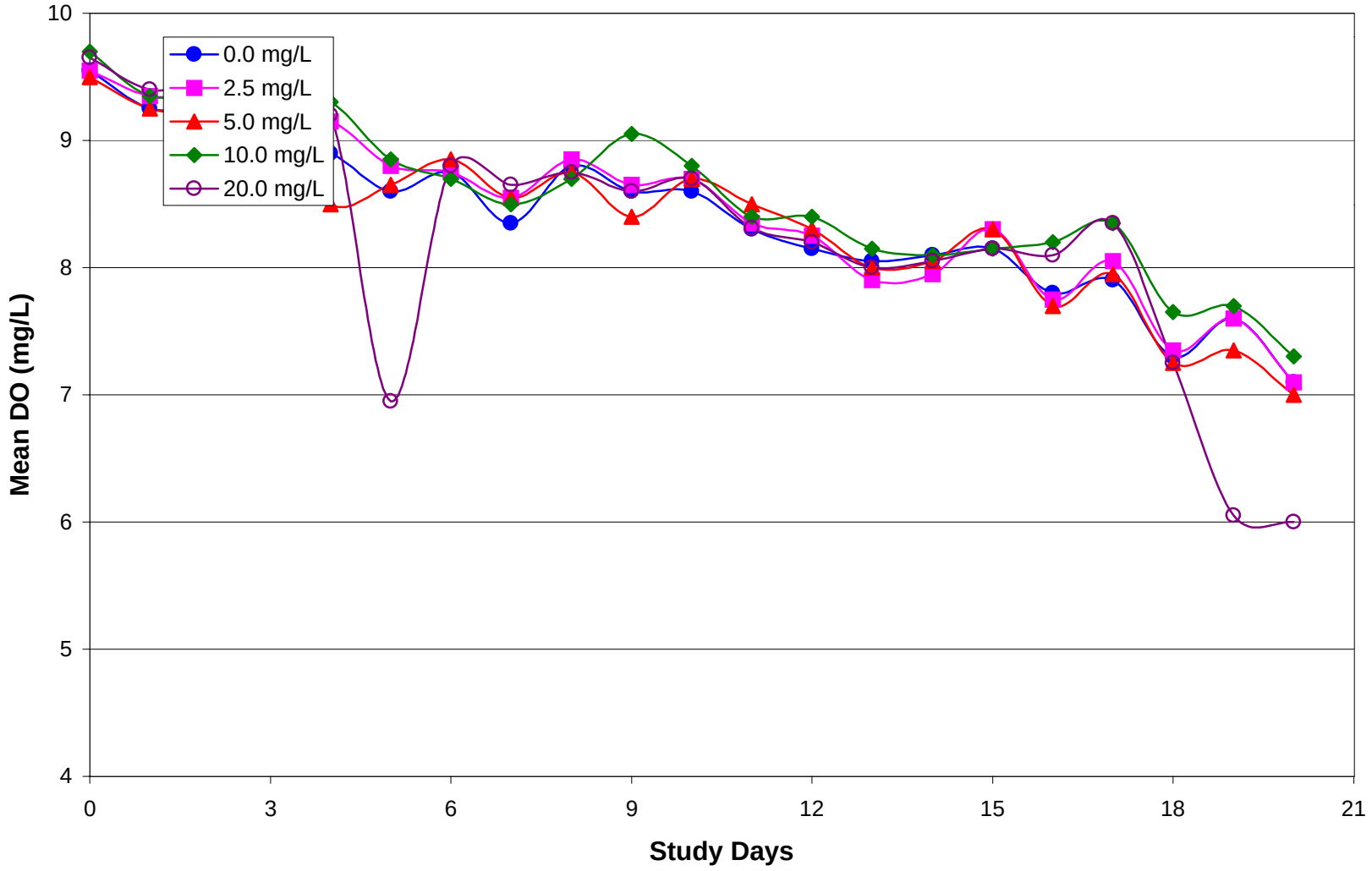
BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average Tank Temperatures for Initial Stage 51 Test



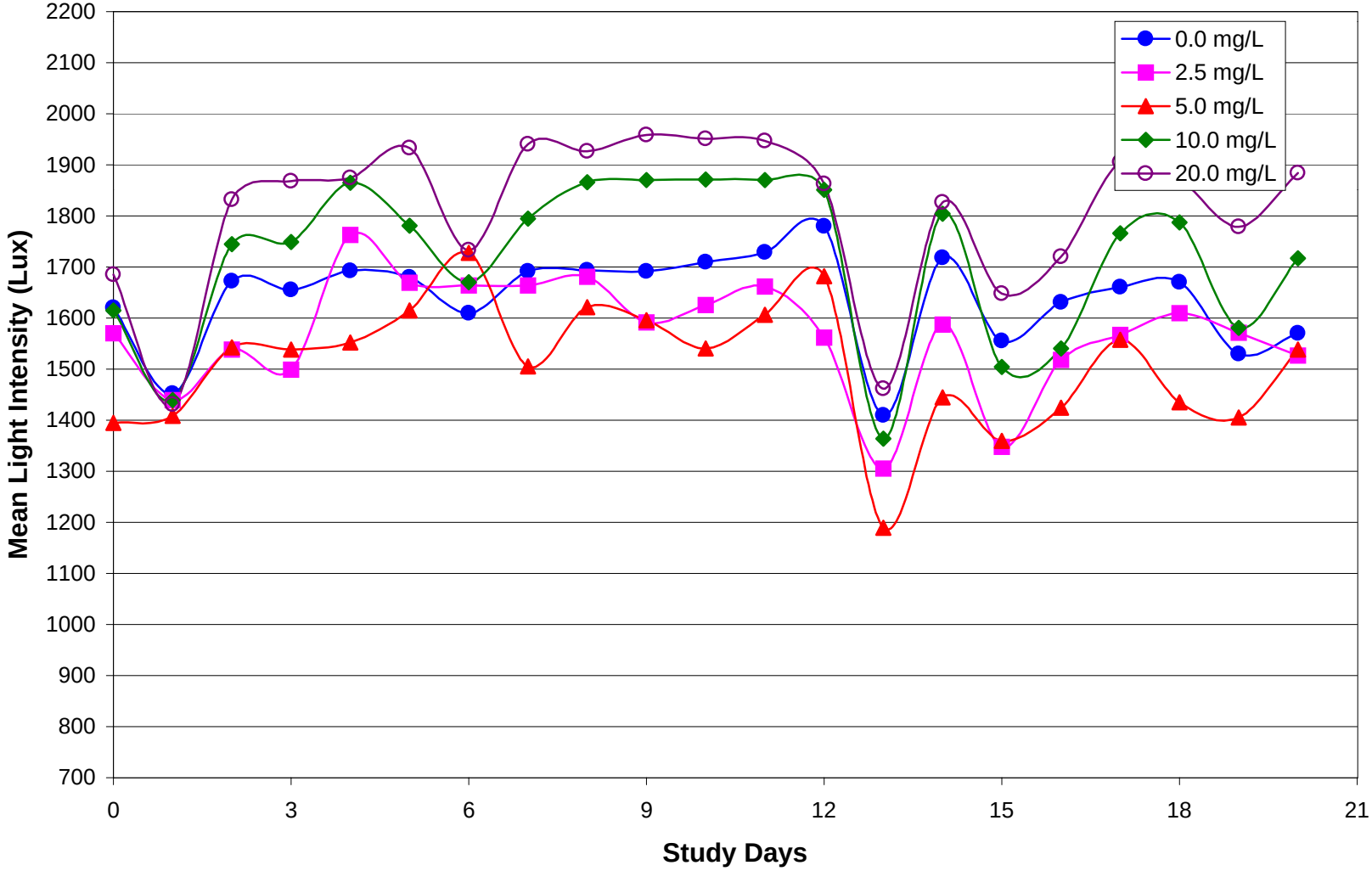
BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average pH for Initial Stage 51 Test



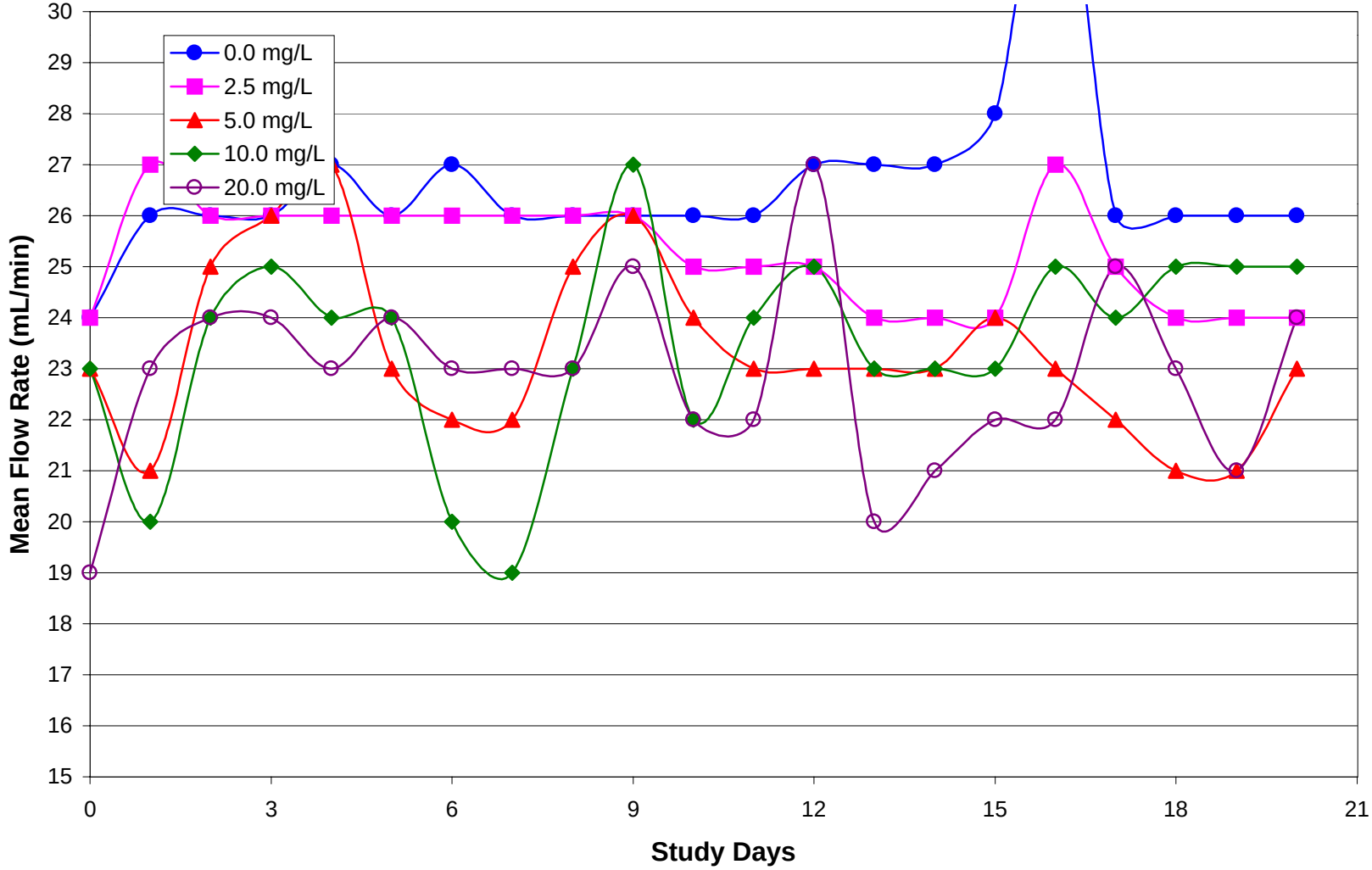
BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average Dissolved Oxygen (DO) for Initial Stage 51 Test



BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average Light Intensity for Initial Stage 51 Test



BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average Flow Rates for Initial Stage 51 Test



WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00051 **Test No: 1**
Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 0.0 mg/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc[1] (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	03/12/04			100.0	21.0	7.5	9.2	1566	26			100.0	21.0	7.5	9.3	1523	26	100.0	21.0	7.5	9.3	1545	26	0.009
1	03/13/04		20	100.0	21.0	7.4	8.8	1737	26		20	100.0	21.0	7.5	8.6	1470	26	100.0	21.0	7.5	8.7	1604	26	
2	03/14/04		20	100.0	21.0	7.1	8.6	1738	26		20	100.0	21.0	7.3	8.6	1425	26	100.0	21.0	7.2	8.6	1582	26	
3	03/15/04		20	100.0	21.0	7.1	8.7	1706	26		20	100.0	21.0	7.3	8.7	1446	26	100.0	21.0	7.2	8.7	1576	26	
4	03/16/04		20	100.0	21.0	7.2	8.4	1785	26		20	100.0	21.0	7.3	8.3	1470	26	100.0	21.0	7.3	8.4	1628	26	
5	03/17/04		20	100.0	21.0	7.2	8.3	1847	27		20	100.0	21.0	7.2	8.3	1487	27	100.0	21.0	7.2	8.3	1667	27	
6	03/18/04		20	100.0	22.0	7.1	8.1	1127	27		20	100.0	22.0	7.2	8.0	1261	27	100.0	22.0	7.2	8.1	1194	27	
7	03/19/04		20	100.0	21.0	7.1	8.5	1351	27		20	100.0	22.0	7.2	8.3	1258	27	100.0	21.5	7.2	8.4	1305	27	0.009
8	03/20/04		20	100.0	22.0	7.1	8.3	1450	28		20	100.0	22.0	7.2	8.2	1267	28	100.0	22.0	7.2	8.3	1359	28	
9	03/21/04		20	100.0	22.0	7.1	7.7	1441	34		20	100.0	22.0	7.2	7.8	1289	34	100.0	22.0	7.2	7.8	1365	34	
10	03/22/04		20	100.0	21.0	7.2	8.2	1677	26		20	100.0	22.0	7.2	8.1	1479	26	100.0	21.5	7.2	8.2	1578	26	
11	03/23/04		20	100.0	21.9	7.2	7.3	1441	26		20	100.0	22.0	7.2	7.4	1365	26	100.0	22.0	7.2	7.4	1403	26	
12	03/24/04		20	100.0	21.4	7.2	7.3	1588	26		20	100.0	21.6	7.3	7.5	1321	26	100.0	21.5	7.3	7.4	1455	26	
13	03/25/04		20	100.0	22.0	7.2	6.8	1758	26		20	100.0	22.0	7.4	7.2	1481	26	100.0	22.0	7.3	7.0	1620	26	
14	03/26/04		20	100.0							20	100.0						100.0						0.009
	Median:				**21.0**	**7.2**	**8.3**	**1633**	**26**				**21.8**	**7.3**	**8.3**	**1436**	**26**		**21.5**	**7.2**	**8.3**	**1560**	**26**	
	Mode:				**21.0**	**7.1**	**8.3**	**1441**	**26**				**22.0**	**7.2**	**8.3**	**1470**	**26**		**21.0**	**7.2**	**8.7**	**#N/A**	**26**	
	Mean:			**100.0**	**21.4**	**7.2**	**8.2**	**1587**	**26.9**			**100.0**	**21.5**	**7.3**	**8.2**	**1396**	**26.9**	**100.0**	**21.5**	**7.2**	**8.2**	**1491**	**26.9**	**0.009**
	SEM:			**0.00**	**0.13**	**0.03**	**0.18**	**54.10**	**0.57**			**0.00**	**0.13**	**0.03**	**0.15**	**26.18**	**0.57**	**0.00**	**0.12**	**0.03**	**0.16**	**38.53**	**0.57**	**0.00**
	% CV:			**0.00**	**10.33**	**4.50**	**23.25**	**508.23**	**41.03**			**0.00**	**10.75**	**4.07**	**19.73**	**262.21**	**41.03**	**0.00**	**9.80**	**4.07**	**21.37**	**373.28**	**41.03**	**0.00**

[1]All measured test concentrations reported by Battelle as less than method detection limit (MDL) [0.0189 mg/L] or none detected (ND) are expressed as 0.009 mg/L (1/2 of MDL).

DAILY DATA

Workorder No: 00051 **Test No: 1**
Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 2.5 mg/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	03/12/04			100.0	21.0	7.4	9.3	1788	26			100.0	21.0	7.5	9.2	1945	26	100.0	21.0	7.5	9.3	1867	26	2.77
1	03/13/04		20	100.0	21.0	7.3	8.8	1704	26		20	100.0	21.0	7.2	8.6	1830	26	100.0	21.0	7.3	8.7	1767	26	
2	03/14/04		20	100.0	21.0	7.3	8.6	1740	26		20	100.0	21.0	7.3	8.5	1817	26	100.0	21.0	7.3	8.6	1779	26	
3	03/15/04		20	100.0	21.0	7.3	8.7	1777	25		20	100.0	21.0	7.2	8.5	1700	25	100.0	21.0	7.3	8.6	1739	25	
4	03/16/04		20	100.0	21.0	7.2	8.1	1767	25		20	100.0	21.0	7.2	8.1	1858	25	100.0	21.0	7.2	8.1	1813	25	
5	03/17/04		20	100.0	21.0	7.2	8.1	1820	25		20	100.0	22.0	7.2	8.1	1752	25	100.0	21.5	7.2	8.1	1786	25	
6	03/18/04		20	100.0	21.0	7.2	7.7	1249	24		20	100.0	22.0	7.2	7.8	1522	24	100.0	21.5	7.2	7.8	1386	24	
7	03/19/04		20	100.0	22.0	7.2	8.0	1350	24		20	100.0	22.0	7.2	8.0	1683	24	100.0	22.0	7.2	8.0	1517	24	2.45
8	03/20/04		20	100.0	22.0	7.2	8.0	1459	24		20	100.0	22.0	7.2	7.8	1556	24	100.0	22.0	7.2	7.9	1508	24	
9	03/21/04		20	100.0	22.0	7.3	7.6	1355	27		20	100.0	22.0	7.3	7.4	1774	27	100.0	22.0	7.3	7.5	1565	27	
10	03/22/04		20	100.0	22.0	7.2	7.9	1799	25		20	100.0	22.0	7.2	7.6	1866	25	100.0	22.0	7.2	7.8	1833	25	
11	03/23/04		20	100.0	21.9	7.1	7.2	1663	24		20	100.0	21.9	7.1	6.9	1812	24	100.0	21.9	7.1	7.1	1738	24	
12	03/24/04		20	100.0	21.5	7.3	7.3	1520	24		20	100.0	21.4	7.2	6.8	1660	24	100.0	21.5	7.3	7.1	1590	24	
13	03/25/04		20	100.0	22.0	7.4	6.8	1726	24		20	100.0	21.9	7.4	6.8	1816	24	100.0	22.0	7.4	6.8	1771	24	
14	03/26/04		20	100.0							20	100.0						100.0						2.15
	Median:				**21.3**	**7.3**	**8.0**	**1715**	**25**				**21.9**	**7.2**	**7.9**	**1793**	**25**		**21.5**	**7.2**	**8.0**	**1753**	**25**	
	Mode:				**21.0**	**7.2**	**8.1**	**#N/A**	**24**				**22.0**	**7.2**	**8.5**	**#N/A**	**24**		**21.0**	**7.2**	**8.1**	**#N/A**	**24**	
	Mean:			**100.0**	**21.5**	**7.3**	**8.0**	**1623**	**24.9**			**100.0**	**21.6**	**7.2**	**7.9**	**1757**	**24.9**	**100.0**	**21.5**	**7.3**	**7.9**	**1690**	**24.9**	**2.455**
	SEM:			**0.00**	**0.13**	**0.02**	**0.18**	**52.29**	**0.27**			**0.00**	**0.13**	**0.03**	**0.19**	**32.05**	**0.27**	**0.00**	**0.12**	**0.02**	**0.19**	**39.40**	**0.27**	**0.18**
	% CV:			**0.00**	**10.60**	**3.16**	**24.07**	**485.71**	**19.97**			**0.00**	**10.30**	**3.78**	**25.70**	**286.12**	**19.97**	**0.00**	**9.66**	**3.34**	**24.73**	**358.62**	**19.97**	**19.84**

DAILY DATA

Workorder No: 00051 **Test No: 1**
Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 5.0 mg/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	03/12/04			100.0	21.0	7.4	9.5	1899	22			100.0	21.0	7.4	9.6	1454	22	100.0	21.0	7.4	9.6	1677	22	4.87
1	03/13/04		20	100.0	21.0	7.3	8.9	1895	25		20	100.0	21.0	7.3	8.6	1375	25	100.0	21.0	7.3	8.8	1635	25	
2	03/14/04		20	100.0	21.0	7.4	9.0	1960	26		20	100.0	21.0	7.4	8.9	1315	26	100.0	21.0	7.4	9.0	1638	26	
3	03/15/04		20	100.0	21.0	7.3	8.9	1966	24		20	100.0	21.0	7.2	8.7	1308	24	100.0	21.0	7.3	8.8	1637	24	
4	03/16/04		20	100.0	21.0	7.2	8.6	1981	23		20	100.0	21.0	7.2	8.3	1383	23	100.0	21.0	7.2	8.5	1682	23	
5	03/17/04		20	100.0	21.0	7.2	8.6	1868	23		20	100.0	21.0	7.2	8.1	1361	23	100.0	21.0	7.2	8.4	1615	23	
6	03/18/04		20	100.0	22.0	7.2	8.2	1323	23		20	100.0	22.0	7.2	7.7	1000	23	100.0	22.0	7.2	8.0	1162	23	
7	03/19/04		20	100.0	22.0	7.2	8.2	1507	23		20	100.0	22.0	7.2	8.0	1258	23	100.0	22.0	7.2	8.1	1383	23	6.09
8	03/20/04		20	100.0	22.0	7.2	8.3	1598	24		20	100.0	22.0	7.2	7.8	1293	24	100.0	22.0	7.2	8.1	1446	24	
9	03/21/04		20	100.0	22.0	7.3	7.5	1706	23		20	100.0	21.0	7.2	7.4	1361	23	100.0	21.5	7.3	7.5	1534	23	
10	03/22/04		20	100.0	22.0	7.2	8.2	1924	22		20	100.0	21.0	7.2	7.6	1391	22	100.0	21.5	7.2	7.9	1658	22	
11	03/23/04		20	100.0	21.8	7.2	7.6	1861	21		20	100.0	21.7	7.1	6.9	1351	21	100.0	21.8	7.2	7.3	1606	21	
12	03/24/04		20	100.0	21.5	7.2	7.8	1709	21		20	100.0	21.1	7.2	6.9	1213	21	100.0	21.3	7.2	7.4	1461	21	
13	03/25/04		20	100.0	21.9	7.4	7.3	1891	23		20	100.0	21.8	7.4	6.6	1375	23	100.0	21.9	7.4	7.0	1633	23	
14	03/26/04		20	100.0							20	100.0						100.0						5.96
	Median:				**21.7**	**7.2**	**8.3**	**1880**	**23**				**21.0**	**7.2**	**7.9**	**1356**	**23**		**21.4**	**7.2**	**8.1**	**1624**	**23**	
	Mode:				**21.0**	**7.2**	**8.2**	**#N/A**	**23**				**21.0**	**7.2**	**6.9**	**1375**	**23**		**21.0**	**7.2**	**#N/A**	**#N/A**	**23**	
	Mean:			**100.0**	**21.5**	**7.3**	**8.3**	**1792**	**23.1**			**100.0**	**21.3**	**7.2**	**7.9**	**1317**	**23.1**	**100.0**	**21.4**	**7.3**	**8.1**	**1555**	**23.1**	**5.640**
	SEM:			**0.00**	**0.13**	**0.02**	**0.17**	**52.79**	**0.37**			**0.00**	**0.12**	**0.03**	**0.23**	**29.26**	**0.37**	**0.00**	**0.11**	**0.02**	**0.20**	**39.18**	**0.37**	**0.39**
	% CV:			**0.00**	**10.35**	**3.12**	**21.98**	**466.58**	**28.83**			**0.00**	**9.74**	**3.48**	**30.01**	**301.66**	**28.83**	**0.00**	**9.26**	**3.21**	**25.74**	**371.86**	**28.83**	**28.09**

DAILY DATA

Workorder No: 00051 **Test No: 1**
Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 10.0 mg/L**

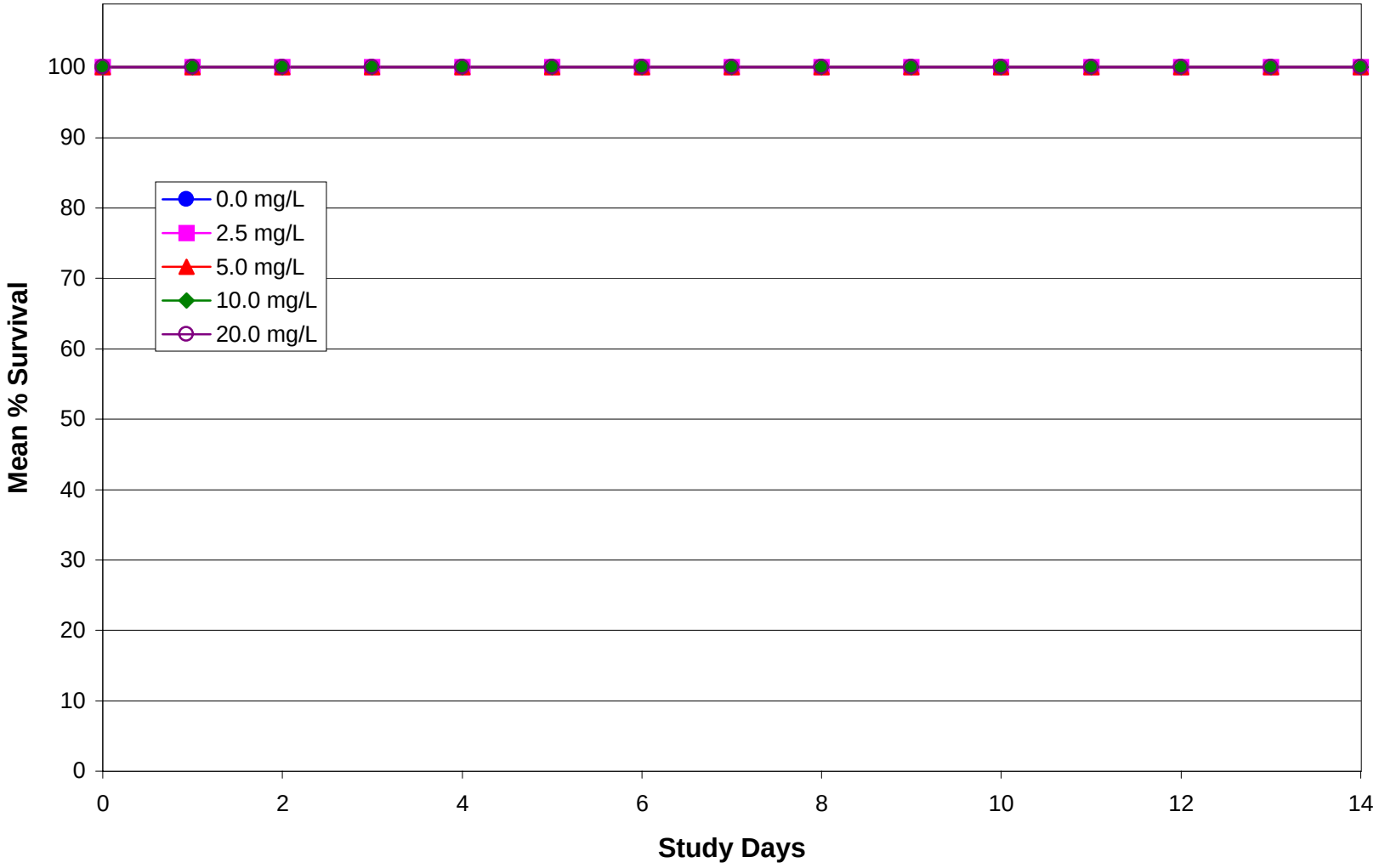
		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	03/12/04			100.0	21.0	7.5	9.7	1457	19			100.0	21.0	7.5	9.5	1974	19	100.0	21.0	7.5	9.6	1716	19	11.48
1	03/13/04		20	100.0	21.0	7.4	8.4	1610	23		20	100.0	21.0	7.3	8.2	1925	23	100.0	21.0	7.4	8.3	1768	23	
2	03/14/04		20	100.0	21.0	7.2	8.7	1625	27		20	100.0	21.0	7.5	8.7	1905	27	100.0	21.0	7.4	8.7	1765	27	
3	03/15/04		20	100.0	21.0	7.3	8.4	1520	22		20	100.0	21.0	7.0	8.3	1865	22	100.0	21.0	7.2	8.4	1693	22	
4	03/16/04		20	100.0	21.0	7.2	8.1	1560	24		20	100.0	21.0	7.2	8.0	1930	24	100.0	21.0	7.2	8.1	1745	24	
5	03/17/04		20	100.0	21.0	7.2	8.1	1560	25		20	100.0	21.0	7.2	8.0	1982	25	100.0	21.0	7.2	8.1	1771	25	
6	03/18/04		20	100.0	22.0	7.2	7.6	1115	23		20	100.0	22.0	7.2	7.8	1248	23	100.0	22.0	7.2	7.7	1182	23	
7	03/19/04		20	100.0	22.0	7.2	8.0	1250	23		20	100.0	22.0	7.1	8.0	1572	23	100.0	22.0	7.2	8.0	1411	23	11.64
8	03/20/04		20	100.0	22.0	7.2	7.9	1344	23		20	100.0	22.0	7.2	7.8	1444	23	100.0	22.0	7.2	7.9	1394	23	
9	03/21/04		20	100.0	22.0	7.2	7.8	1297	25		20	100.0	22.0	7.3	7.8	1782	25	100.0	22.0	7.3	7.8	1540	25	
10	03/22/04		20	100.0	21.0	7.2	7.8	1596	24		20	100.0	22.0	7.2	7.7	1916	24	100.0	21.5	7.2	7.8	1756	24	
11	03/23/04		20	100.0	21.8	7.1	6.9	1465	25		20	100.0	21.8	7.2	7.0	1902	25	100.0	21.8	7.2	7.0	1684	25	
12	03/24/04		20	100.0	21.4	7.3	7.0	1377	25		20	100.0	21.4	7.2	6.9	1908	25	100.0	21.4	7.3	7.0	1643	25	
13	03/25/04		20	100.0	21.9	7.4	6.8	1524	25		20	100.0	21.9	7.4	6.7	1803	25	100.0	21.9	7.4	6.8	1664	25	
14	03/26/04		20	100.0							20	100.0						100.0						12.70
	Median:				**21.2**	**7.2**	**8.0**	**1493**	**24**				**21.6**	**7.2**	**7.9**	**1904**	**24**		**21.5**	**7.2**	**7.9**	**1688**	**24**	
	Mode:				**21.0**	**7.2**	**8.4**	**1560**	**25**				**21.0**	**7.2**	**8.0**	**#N/A**	**25**		**21.0**	**7.2**	**8.1**	**#N/A**	**25**	
	Mean:			**100.0**	**21.4**	**7.3**	**7.9**	**1450**	**23.8**			**100.0**	**21.5**	**7.3**	**7.9**	**1797**	**23.8**	**100.0**	**21.5**	**7.3**	**7.9**	**1623**	**23.8**	**11.939**
	SEM:			**0.00**	**0.13**	**0.03**	**0.20**	**40.91**	**0.50**			**0.00**	**0.13**	**0.04**	**0.19**	**58.85**	**0.50**	**0.00**	**0.12**	**0.03**	**0.20**	**47.69**	**0.50**	**0.38**
	% CV:			**0.00**	**10.29**	**4.04**	**27.08**	**401.98**	**38.72**			**0.00**	**10.37**	**5.20**	**25.75**	**519.46**	**38.72**	**0.00**	**9.92**	**3.95**	**26.36**	**442.83**	**38.72**	**19.18**

DAILY DATA

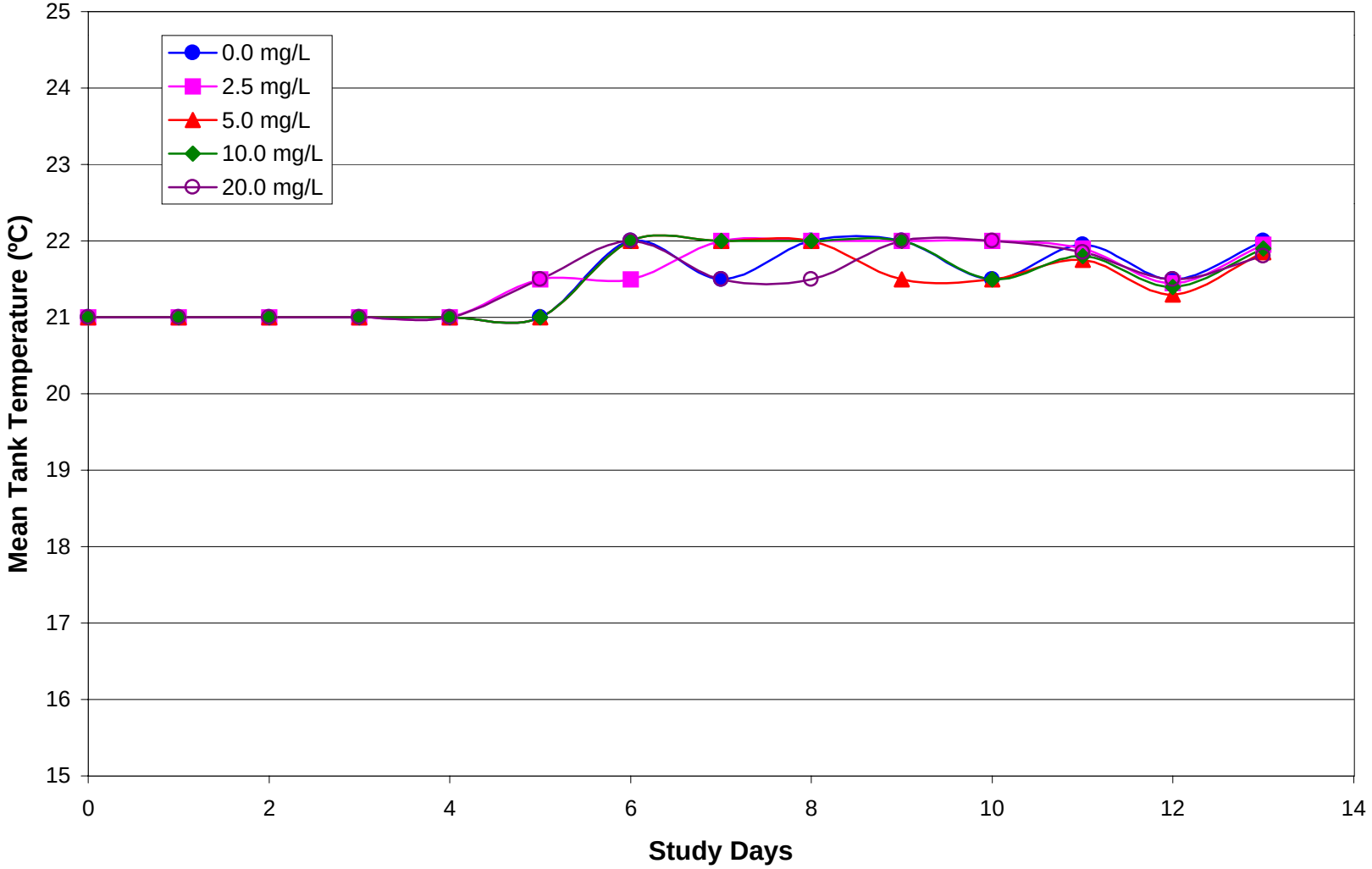
Workorder No: 00051 **Test No: 1**
Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 20.0 mg/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	03/12/04			100.0	21.0	7.2	9.6	1838	23			100.0	21.0	7.4	9.8	1938	23	100.0	21.0	7.3	9.7	1888	23	20.16
1	03/13/04		20	100.0	21.0	7.3	8.9	1862	23		20	100.0	21.0	7.1	8.6	1780	23	100.0	21.0	7.2	8.8	1821	23	
2	03/14/04		20	100.0	21.0	7.3	8.7	1890	25		20	100.0	21.0	7.3	8.7	1865	25	100.0	21.0	7.3	8.7	1878	25	
3	03/15/04		20	100.0	21.0	6.7	8.6	1906	22		20	100.0	21.0	6.7	8.6	1883	22	100.0	21.0	6.7	8.6	1895	22	
4	03/16/04		20	100.0	21.0	7.2	8.2	1889	22		20	100.0	21.0	7.2	8.2	1845	22	100.0	21.0	7.2	8.2	1867	22	
5	03/17/04		20	100.0	21.0	7.2	8.0	1990	27		20	100.0	22.0	7.1	8.2	1768	27	100.0	21.5	7.2	8.1	1879	27	
6	03/18/04		20	100.0	22.0	7.2	7.9	1564	20		20	100.0	22.0	7.1	7.1	1252	20	100.0	22.0	7.2	7.5	1408	20	
7	03/19/04		20	100.0	22.0	7.2	8.1	1333	21		20	100.0	21.0	7.1	7.7	1359	21	100.0	21.5	7.2	7.9	1346	21	20.43
8	03/20/04		20	100.0	22.0	7.2	8.0	1610	22		20	100.0	21.0	7.1	7.9	1543	22	100.0	21.5	7.2	8.0	1577	22	
9	03/21/04		20	100.0	22.0	7.3	8.0	1616	22		20	100.0	22.0	7.2	7.9	1518	22	100.0	22.0	7.3	8.0	1567	22	
10	03/22/04		20	100.0	22.0	7.2	8.2	1845	25		20	100.0	22.0	7.1	8.2	1742	25	100.0	22.0	7.2	8.2	1794	25	
11	03/23/04		20	100.0	21.8	7.2	7.5	1709	23		20	100.0	21.9	7.1	7.5	1680	23	100.0	21.9	7.2	7.5	1695	23	
12	03/24/04		20	100.0	21.4	7.2	6.7	1691	21		20	100.0	21.6	7.3	8.4	1434	21	100.0	21.5	7.3	7.6	1563	21	
13	03/25/04		20	100.0	22.0	7.2	5.0	1821	24		20	100.0	21.6	7.4	8.5	1648	24	100.0	21.8	7.3	6.8	1735	24	
14	03/26/04		20	100.0							20	100.0						100.0						20.71
	Median:				**21.6**	**7.2**	**8.1**	**1830**	**23**				**21.3**	**7.1**	**8.2**	**1711**	**23**		**21.5**	**7.2**	**8.0**	**1764**	**23**	
	Mode:				**21.0**	**7.2**	**8.0**	**#N/A**	**22**				**21.0**	**7.1**	**8.2**	**#N/A**	**22**		**21.0**	**7.2**	**8.2**	**#N/A**	**22**	
	Mean:			**100.0**	**21.5**	**7.2**	**8.0**	**1755**	**22.9**			**100.0**	**21.4**	**7.2**	**8.2**	**1661**	**22.9**	**100.0**	**21.5**	**7.2**	**8.1**	**1708**	**22.9**	**20.437**
	SEM:			**0.00**	**0.13**	**0.04**	**0.29**	**47.24**	**0.50**			**0.00**	**0.13**	**0.05**	**0.17**	**56.38**	**0.50**	**0.00**	**0.11**	**0.04**	**0.19**	**49.77**	**0.50**	**0.16**
	% CV:			**0.00**	**10.52**	**5.45**	**38.38**	**421.96**	**39.22**			**0.00**	**10.15**	**6.51**	**22.40**	**517.62**	**39.22**	**0.00**	**8.88**	**5.56**	**24.93**	**450.60**	**39.22**	**6.07**

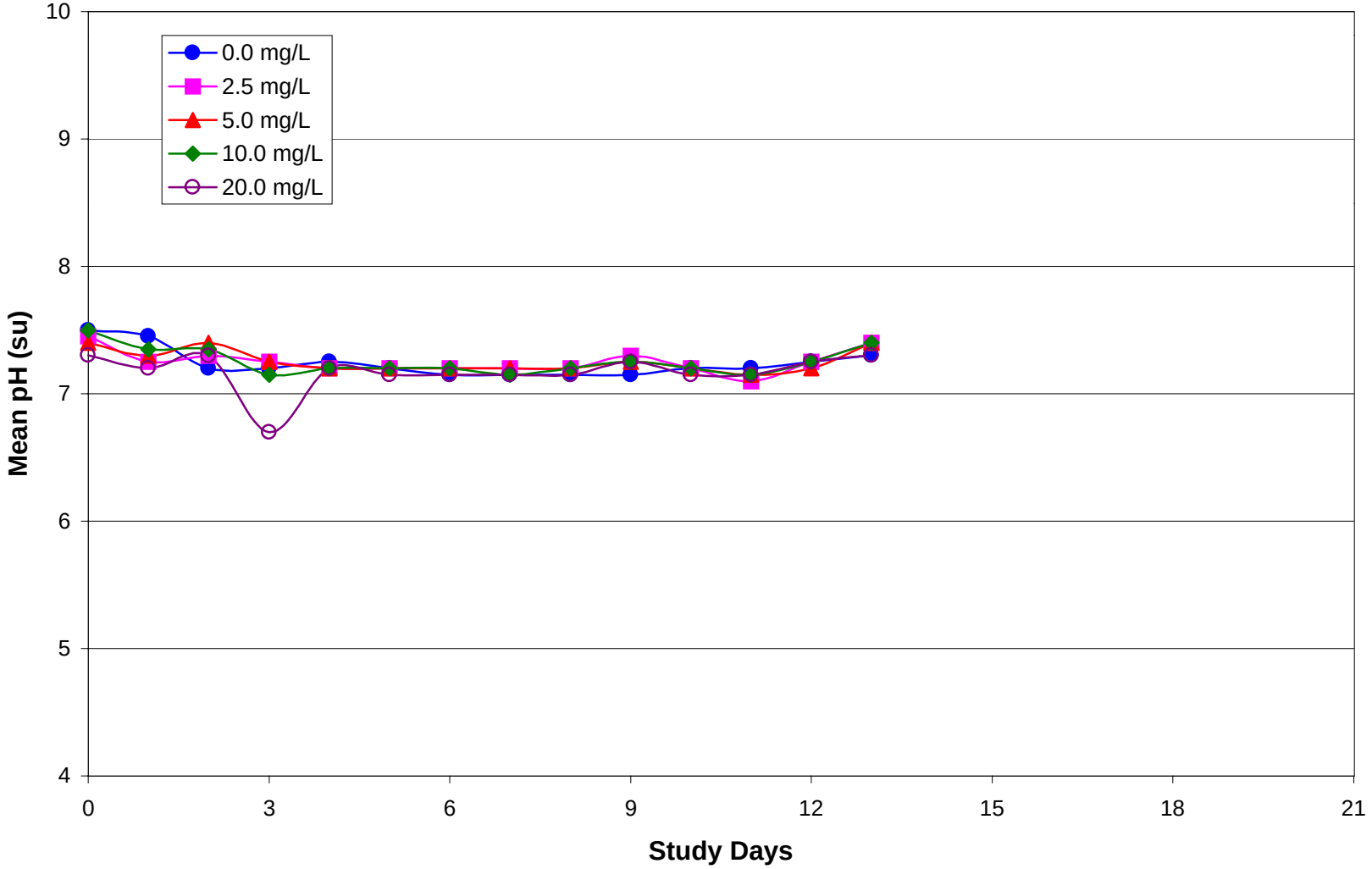
BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average of Larval Survival for Initial Stage 54 Test



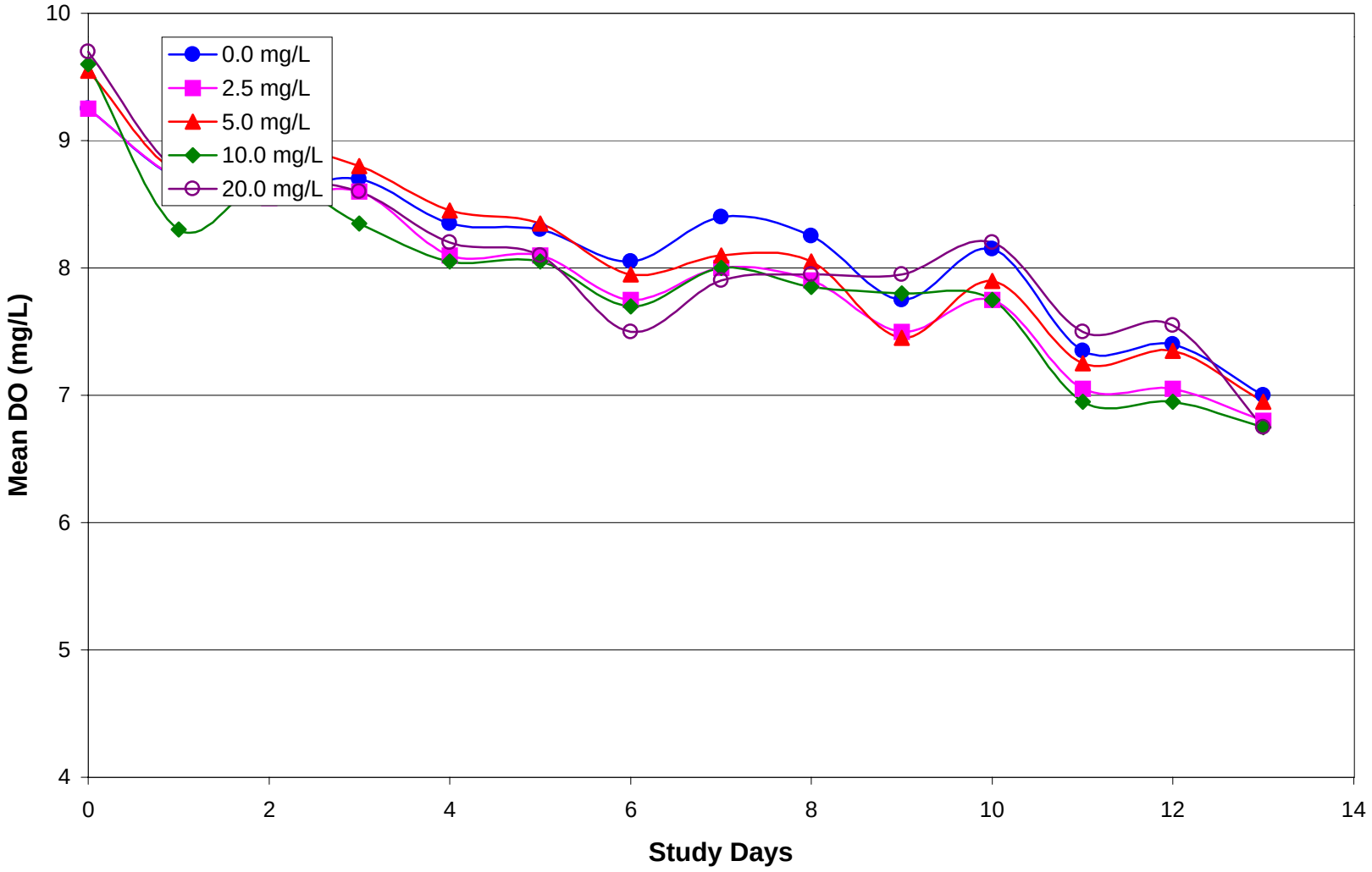
BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average Tank Temperatures for Initial Stage 54 Test



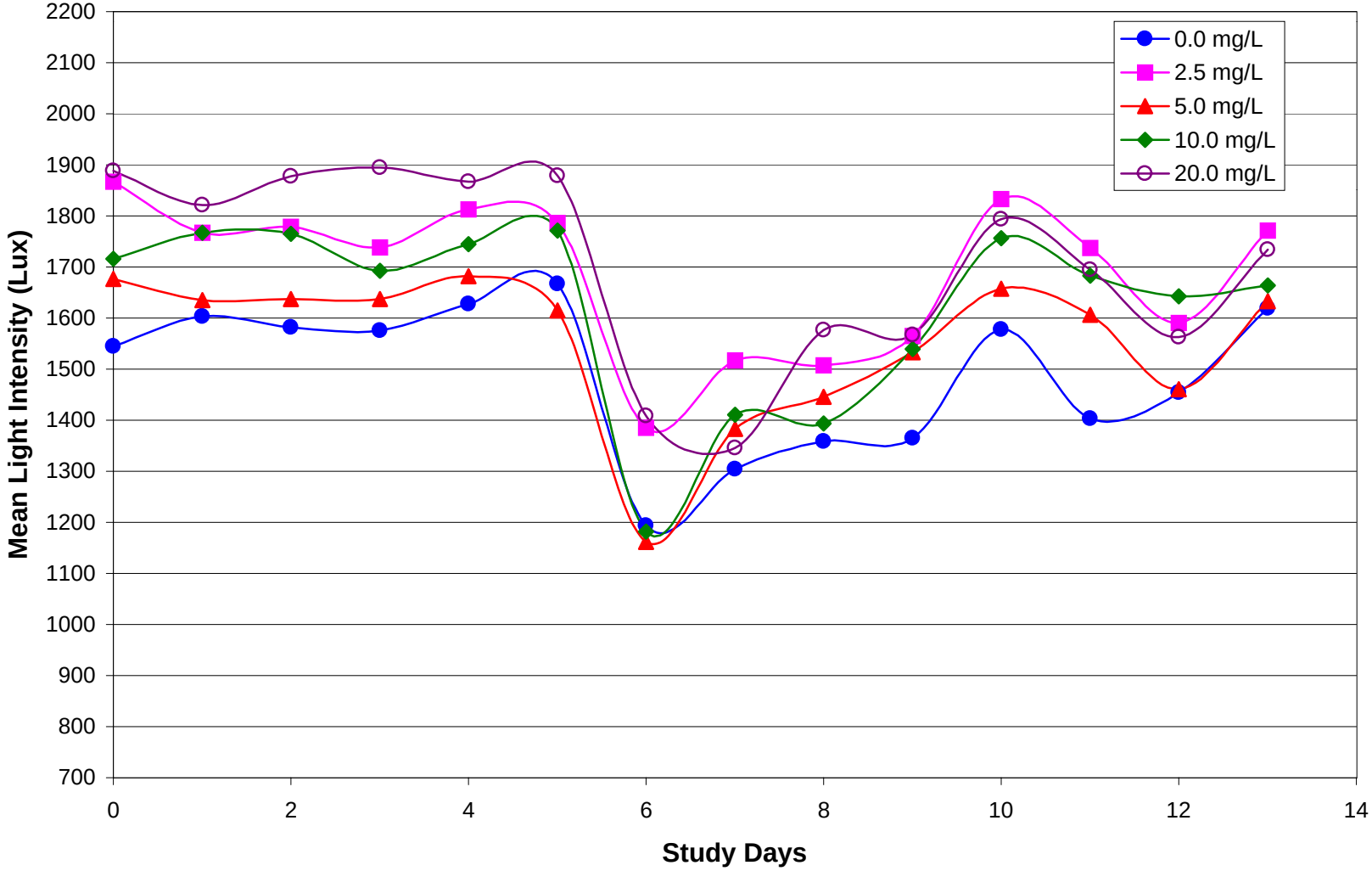
BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average pH for Initial Stage 54 Test



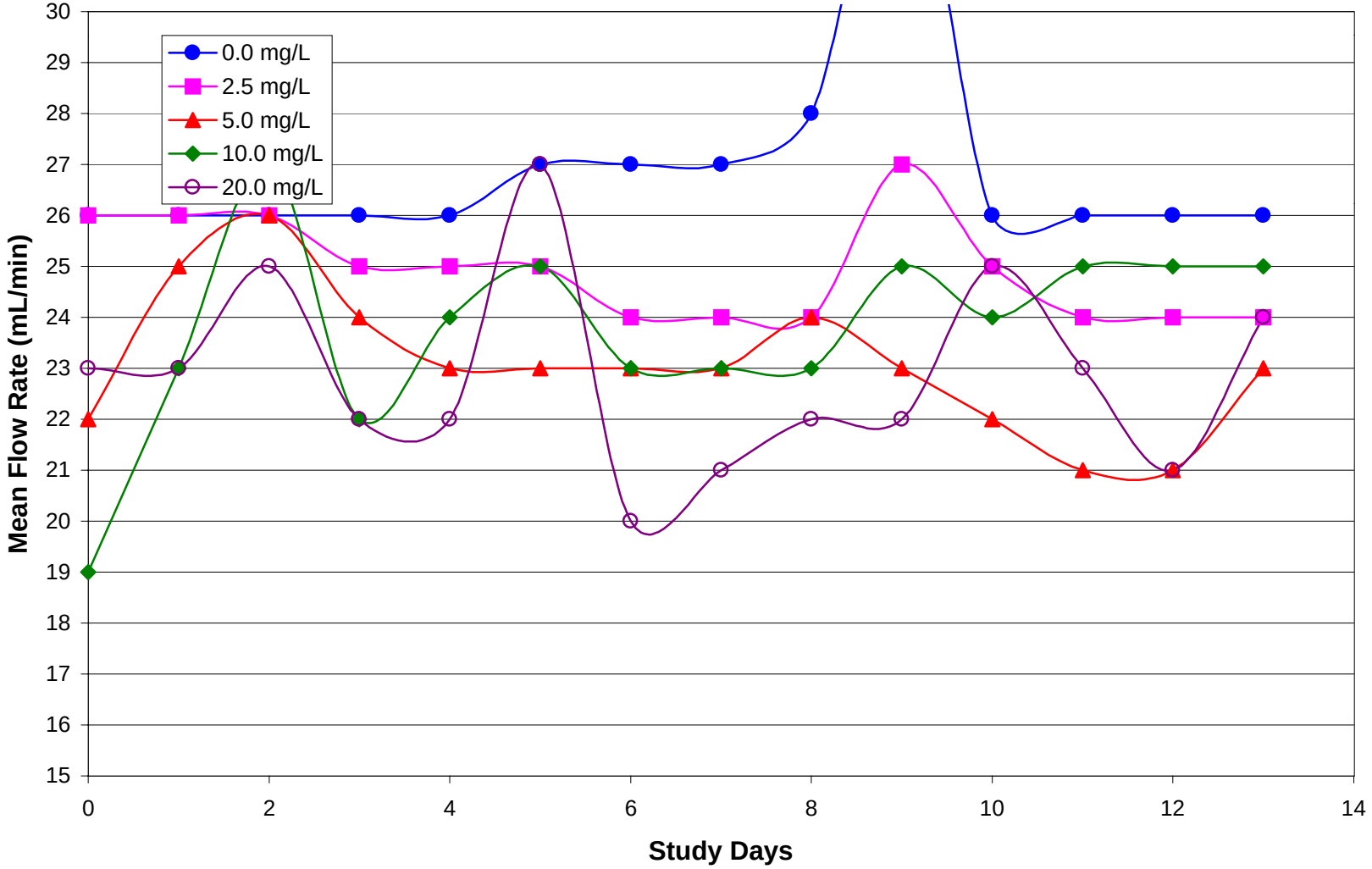
BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average Dissolved Oxygen (DO) for Initial Stage 54 Test



BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average Light Intensity for Initial Stage 54 Test



BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Daily Average Flow Rates for Initial Stage 54 Test



6-n-Propyl-Thiouracil (PTU)
Metamorphosis Assay:
Test Termination Data – Stage and Growth

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00051 **Test No: 1**

Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 0.0 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	001	0.5035	56	Kinked Tail
	002	0.7890	57	
	003	0.8000	59	
	004	0.8204	57	Kinked Tail
	005	0.8074	57	
	006	0.7894	57	Kinked Tail
	007	0.6295	57	Kinked Tail
	008	1.0500	57	Kinked Tail
	009	0.6821	57	
	010	0.6560	57	
	011	0.8838	57	
	012	0.6297	57	
	013	0.7191	57	Kinked Tail
	014	0.6995	57	
	015	0.8679	57	
	016	0.9149	59	
	017	1.0380	59	Kinked Tail
	018	0.7320	57	Kinked Tail
	019	0.8300	57	
	020	0.8949	57	Kinked Tail
	Median:	**0.795**	**57**	
	Mode:		**57**	
	Mean:	**0.787**	**57.250**	
	SEM:	**0.030**	**0.176**	
	%CV:	**15.367**	**10.393**	
B	021	0.5533	60	
	022	0.6451	57	
	023	0.7480	59	Kinked Tail
	024	0.8088	59	Kinked Tail
	025	0.8323	59	
	026	0.8235	59	
	027	0.8797	59	Kinked Tail
	028	0.7333	58	
	029	0.6516	56	
	030	0.6194	57	
	031	0.7621	57	
	032	0.5392	56	Kinked Tail
	033	0.6949	57	
	034	0.5292	56	
	035	0.7674	57	Kinked Tail
	036	0.5080	56	Kinked Tail
	037	0.8026	57	
	038	0.5286	57	
	039	0.5364	57	Kinked Tail
	Median:	**0.695**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.682**	**57.526**	
	SEM:	**0.028**	**0.290**	
	%CV:	**14.956**	**16.659**	
	Median:	**0.748**	**57**	
	Mode:		**57**	
	Total Mean:	**0.736**	**57.385**	
	SEM:	**0.022**	**0.167**	
	%CV:	**16.204**	**13.751**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00051 **Test No: 1**

Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 2.5mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	040	0.8853	57	Kinked Tail
	041	0.6029	57	Kinked Tail
	042	0.7530	57	Kinked Tail
	043	0.5101	56	
	044	0.9983	57	Kinked Tail
	045	0.6073	57	Kinked Tail
	046	0.8615	57	
	047	0.7046	57	Kinked Tail
	048	0.7603	59	
	049	0.6335	57	Kinked Tail
	050	0.8901	57	Kinked Tail
	051	0.5906	57	
	052	0.6657	57	Kinked Tail
	053	0.6419	57	
	054	0.5607	56	
	055	0.9866	59	Kinked Tail
	056	1.1276	59	
	057	0.8110	57	Kinked Tail
	058	0.4245	56	Kinked Tail
	059	0.6264	56	Kinked Tail
	Median:	**0.685**	**57**	
	Mode:		**57**	
	Mean:	**0.732**	**57.100**	
	SEM:	**0.041**	**0.204**	
	%CV:	**21.188**	**12.068**	
B	060	1.0067	59	
	061	0.6142	57	Kinked Tail
	062	0.8571	57	Kinked Tail
	063	0.6768	57	Kinked Tail
	064	0.7163	57	
	065	0.6442	56	Kinked Tail
	066	0.5547	57	Kinked Tail
	067	1.0266	57	
	068	0.6954	58	
	069	0.5598	56	
	070	0.6744	56	Kinked Tail
	071	0.5006	56	
	072	0.7830	57	Kinked Tail
	073	0.9879	59	Kinked Tail
	074	0.6526	56	
	075	0.5822	56	
	076	0.8602	57	Kinked Tail
	077	0.5762	60	
	078	0.8523	59	Kinked Tail
	079	0.6656	56	
	Median:	**0.676**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.724**	**57.150**	
	SEM:	**0.035**	**0.274**	
	%CV:	**18.623**	**16.215**	
	Median:	**0.676**	**57**	
	Mode:		**57**	
	Total Mean:	**0.728**	**57.125**	
	SEM:	**0.027**	**0.169**	
	%CV:	**19.702**	**14.113**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00051 **Test No: 1**

Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 5.0mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	080	0.6440	59	Kinked Tail
	081	0.7074	57	Kinked Tail
	082	0.6332	57	Kinked Tail
	083	0.8393	59	Kinked Tail
	084	0.9423	59	
	085	0.6569	57	
	086	0.6560	57	Kinked Tail
	087	0.7757	59	Kinked Tail
	088	0.7595	57	Kinked Tail
	089	0.7118	57	Kinked Tail
	090	0.4641	56	Kinked Tail
	091	0.8006	57	Kinked Tail
	092	0.6727	59	Kinked Tail
	093	0.8992	59	Kinked Tail
	094	0.8754	57	Kinked Tail
	095	0.8295	59	
	096	0.5999	56	Kinked Tail
	097	0.8021	57	Kinked Tail
	098	0.6273	57	Kinked Tail
	099	0.7230	59	Kinked Tail
	Median:	**0.717**	**57**	
	Mode:		**57**	
	Mean:	**0.731**	**57.700**	
	SEM:	**0.026**	**0.252**	
	%CV:	**13.675**	**14.857**	
B	100	0.8388	57	
	101	0.7028	58	Kinked Tail
	102	0.5824	57	Kinked Tail
	103	0.5452	56	Kinked Tail
	104	0.9140	59	Kinked Tail
	105	0.9087	57	Kinked Tail
	106	0.6192	56	
	107	0.8659	59	Kinked Tail
	108	0.4333	56	Kinked Tail
	109	0.6553	56	Kinked Tail
	110	0.6929	57	
	111	0.9310	57	
	112	0.7987	56	Kinked Tail
	113	0.4393	55	Kinked Tail
	114	0.6525	56	
	115	0.5181	56	Kinked Tail
	116	0.7115	57	Kinked Tail
	117	0.7821	57	
	118	0.9300	59	Small Edema Near Heart
	119	0.9000	57	
	Median:	**0.707**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.721**	**56.900**	
	SEM:	**0.037**	**0.250**	
	%CV:	**19.311**	**14.837**	
	Median:	**0.712**	**57**	
	Mode:		**57**	
	Total Mean:	**0.726**	**57.300**	
	SEM:	**0.022**	**0.187**	
	%CV:	**16.508**	**15.602**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00051 **Test No: 1**

Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 10.0mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	120	0.7486	56	Kinked Tail
	121	0.5804	56	
	122	0.6105	57	Kinked Tail
	123	0.6386	56	Kinked Tail
	124	0.6485	57	Kinked Tail
	125	0.6041	56	Kinked Tail
	126	0.9622	57	
	127	0.7752	57	Kinked Tail
	128	0.8691	58	
	129	0.8610	56	
	130	0.6561	57	
	131	0.5804	56	
	132	0.6179	56	
	133	0.8490	55	Kinked Tail
	134	0.6724	55	Kinked Tail
	135	0.6139	56	Kinked Tail
	136	0.8487	57	
	137	0.9553	58	Kinked Tail
	138	0.8805	54	
	139	0.9014	57	
	Median:	**0.711**	**56**	
	Mode:		**56**	
	Mean:	**0.744**	**56.350**	
	SEM:	**0.030**	**0.221**	
	%CV:	**15.621**	**13.163**	
B	140	0.7850	58	
	141	0.5661	57	
	142	0.7757	57	
	143	0.6838	57	Kinked Tail
	144	0.6550	57	
	145	0.5060	56	
	146	0.8450	58	Kinked Tail
	147	0.7091	58	
	148	0.5131	54	
	149	1.2733	55	
	150	0.7911	54	
	151	0.6351	57	
	152	0.8281	54	Kinked Tail
	153	0.5739	55	
	154	0.6717	55	
	155	0.6894	54	Kinked Tail
	156	0.6411	56	
	157	0.6270	55	
	158	0.7902	55	Kinked Tail
	159	0.7286	55	
	Median:	**0.687**	**56**	**Comments:**
	Mode:		**55**	
	Rep Mean:	**0.714**	**55.850**	
	SEM:	**0.037**	**0.319**	
	%CV:	**19.476**	**19.060**	
	Median:	**0.687**	**56**	
	Mode:		**57**	
	Total Mean:	**0.729**	**56.100**	
	SEM:	**0.024**	**0.195**	
	%CV:	**17.475**	**16.505**	

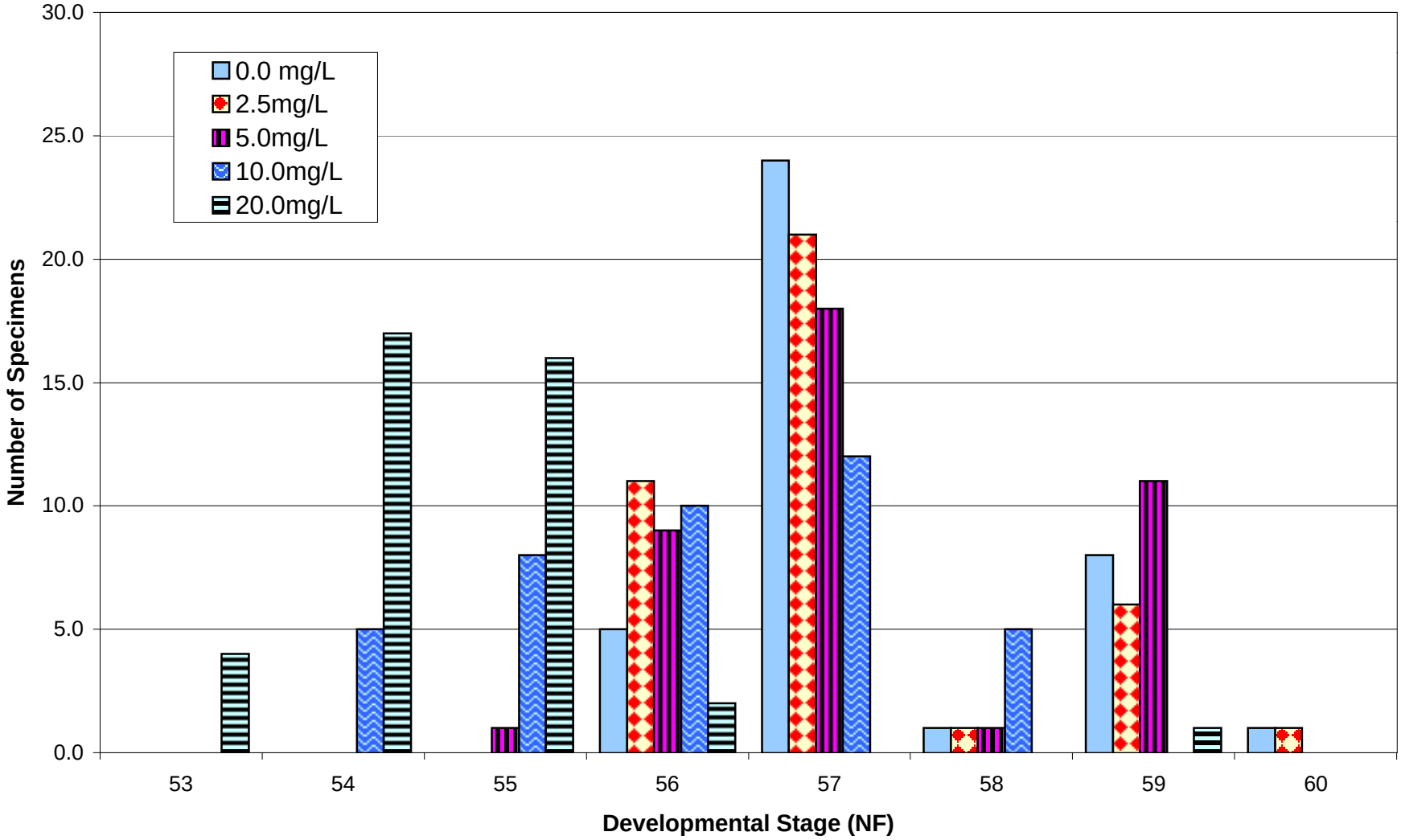
TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00051 **Test No: 1**

Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 20.0mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	160	1.0620	59	Kinked Tail
	161	0.6814	56	
	162	1.1092	55	
	163	0.6475	55	
	164	0.5664	55	
	165	1.1178	55	
	166	0.8084	55	
	167	0.8926	55	
	168	0.8228	55	Kinked Tail
	169	0.7256	55	Kinked Tail
	170	1.0086	55	Kinked Tail
	171	0.8379	55	
	172	0.5660	54	
	173	0.4682	54	Kinked Tail
	174	0.4276	53	Kinked Tail
	175	0.6662	54	
	176	0.4647	54	
	177	0.3855	53	
	178	0.7872	55	
	179	0.3976	53	
	Median:	**0.704**	**55**	
	Mode:		**55**	
	Mean:	**0.722**	**54.750**	
	SEM:	**0.053**	**0.289**	
	%CV:	**27.839**	**17.470**	
B	180	1.3548	55	Kinked Tail
	181	1.0180	55	Kinked Tail
	182	0.7090	56	Kinked Tail
	183	0.7845	55	Kinked Tail
	184	1.1440	55	
	185	0.7392	54	Kinked Tail
	186	1.0869	54	Kinked Tail
	187	0.8168	54	Kinked Tail
	188	0.9769	54	
	189	0.6668	54	Kinked Tail
	190	0.7252	54	Kinked Tail
	191	0.7663	55	Kinked Tail
	192	0.4591	54	Kinked Tail
	193	0.8685	54	
	194	0.7689	54	Kinked Tail
	195	0.6275	54	Kinked Tail
	196	0.5482	54	Kinked Tail
	197	0.5042	53	
	198	0.4620	54	Kinked Tail
	199	0.8170	54	Kinked Tail
	Median:	**0.768**	**54**	**Comments:**
	Mode:		**54**	
	Rep Mean:	**0.792**	**54.300**	
	SEM:	**0.052**	**0.147**	
	%CV:	**26.287**	**8.915**	
	Median:	**0.753**	**54**	
	Mode:		**54**	
	Total Mean:	**0.757**	**54.525**	
	SEM:	**0.037**	**0.164**	
	%CV:	**26.999**	**14.050**	

BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Stage Distribution for Initial Stage 51 Test



TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00051 **Test No: 1**

Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 0.0 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	200	0.7581	57	
	201	0.9473	59	
	202	0.9499	59	
	203	0.6410	57	
	204	0.7710	59	
	205	0.9995	58	
	206	0.5871	60	
	207	0.9254	57	
	208	0.6359	56	
	209	1.2016	59	
	210	0.6681	56	
	211	1.0200	58	
	212	0.7648	56	
	213	0.8488	57	
	214	1.0664	59	
	215	0.9034	58	
	216	0.9614	58	Kinked Tail
	217	0.7207	56	
	218	0.8997	58	
	219	1.2922	59	
	Median:	**0.902**	**58**	
	Mode:		**59**	
	Mean:	**0.878**	**57.800**	
	SEM:	**0.042**	**0.277**	
	%CV:	**20.109**	**16.306**	
D	220	0.6656	58	
	221	0.6608	58	Small Edema Near Heart
	222	0.8268	59	
	223	0.7460	58	
	224	0.6520	56	
	225	0.9814	58	Kinked Tail
	226	0.6226	57	
	227	0.5514	56	
	228	0.9481	58	Kinked Tail
	229	0.8144	59	Kinked Tail
	230	0.8194	58	
	231	0.6966	57	
	232	0.6273	57	
	233	0.5151	59	
	234	0.8281	59	
	235	0.6977	59	
	236	0.8637	59	
	237	1.0500	58	
	238	0.6630	60	
	239	1.2164	59	
	Median:	**0.722**	**58**	**Comments:**
	Mode:		**58**	
	Rep Mean:	**0.772**	**58.100**	
	SEM:	**0.039**	**0.240**	
	%CV:	**20.018**	**14.053**	
	Median:	**0.817**	**58**	
	Mode:		**59**	
	Total Mean:	**0.825**	**57.950**	
	SEM:	**0.030**	**0.182**	
	%CV:	**20.667**	**15.154**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00051 **Test No: 1**

Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 2.5 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	240	0.7650	58	Kinked Tail
	241	0.9133	59	Kinked Tail
	242	0.6663	59	
	243	0.8285	59	Kinked Tail
	244	0.9114	59	Kinked Tail
	245	0.9722	58	Kinked Tail
	246	0.7809	57	
	247	0.9790	59	
	248	0.7851	57	
	249	1.0625	59	
	250	0.8630	59	Kinked Tail
	251	0.7942	58	
	252	1.0775	58	
	253	0.7770	58	
	254	0.6989	59	
	255	0.6999	59	
	256	0.7105	58	
	257	0.8698	58	Kinked Tail
	258	0.8598	58	
	259	0.7516	58	
	Median:	**0.811**	**58**	
	Mode:		**58**	
	Mean:	**0.838**	**58.350**	
	SEM:	**0.027**	**0.150**	
	%CV:	**12.961**	**8.782**	
D	260	0.9175	59	Kinked Tail
	261	0.9542	58	Kinked Tail
	262	0.8467	57	
	263	0.9108	58	Kinked Tail
	264	0.7700	58	Kinked Tail
	265	0.8917	59	Kinked Tail
	266	1.0662	58	Kinked Tail
	267	0.9947	58	Small Edema Near Heart
	268	0.9810	58	Kinked Tail
	269	0.7240	58	
	270	0.7028	57	
	271	0.8286	58	Kinked Tail
	272	0.7778	58	
	273	0.9165	59	
	274	0.8670	58	
	275	0.4828	60	
	276	0.9197	59	
	277	0.8738	58	
	278	1.0080	58	Kinked Tail
	279	0.6466	58	Kinked Tail
	Median:	**0.883**	**58**	**Comments:**
	Mode:		**58**	
	Rep Mean:	**0.854**	**58.200**	
	SEM:	**0.031**	**0.156**	
	%CV:	**15.025**	**9.121**	
	Median:	**0.861**	**58**	
	Mode:		**58**	
	Total Mean:	**0.846**	**58.275**	
	SEM:	**0.020**	**0.107**	
	%CV:	**13.886**	**8.893**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00051 **Test No: 1**

Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 5.0 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	280	0.8535	57	Kinked Tail
	281	0.5404	58	
	282	0.7680	57	Kinked Tail
	283	0.8804	59	Kinked Tail
	284	1.0486	58	Kinked Tail
	285	0.8614	58	Small Edema Near Stomach
	286	1.0097	58	
	287	0.9867	59	
	288	0.8145	58	Small Edema Near Heart
	289	0.6577	59	
	290	0.9899	58	
	291	0.7878	58	
	292	0.7363	58	
	293	0.7913	58	Kinked Tail
	294	0.7992	56	
	295	0.7665	56	
	296	0.4258	60	
	297	0.4263	60	Kinked Tail
	298	0.7689	57	
	299	0.8816	59	
	Median:	**0.795**	**58**	
	Mode:		**58**	
	Mean:	**0.790**	**58.050**	
	SEM:	**0.039**	**0.246**	
	%CV:	**19.478**	**14.425**	
D	300	1.0236	59	
	301	0.7327	59	
	302	0.9331	59	
	303	0.8670	57	Kinked Tail
	304	0.7980	59	Kinked Tail
	305	1.0963	57	Kinked Tail
	306	0.9794	57	
	307	0.8265	57	
	308	1.0527	57	
	309	1.0325	57	
	310	0.7223	59	
	311	0.8977	56	
	312	0.9485	58	
	313	0.8322	57	
	314	0.8565	58	Kinked Tail
	315	0.7976	59	Kinked Tail
	316	0.7307	56	
	317	1.0325	58	Kinked Tail
	318	1.0813	58	Kinked Tail
	319	1.0024	58	
	Median:	**0.915**	**58**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.912**	**57.750**	
	SEM:	**0.027**	**0.228**	
	%CV:	**12.812**	**13.416**	
	Median:	**0.855**	**58**	
	Mode:		**58**	
	Total Mean:	**0.851**	**57.900**	
	SEM:	**0.025**	**0.167**	
	%CV:	**17.391**	**13.895**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00051 **Test No: 1**

Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 10.0 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	320	1.0058	58	
	321	0.9463	58	Kinked Tail
	322	0.7739	58	Kinked Tail
	323	0.6609	57	
	324	0.8763	57	
	325	0.7643	58	
	326	0.6380	56	
	327	0.9200	57	
	328	0.7518	56	
	329	0.8610	58	
	330	0.8558	56	
	331	0.5997	57	
	332	0.7497	58	
	333	0.8492	58	Kinked Tail
	334	1.0918	57	Kinked Tail
	335	0.8346	57	
	336	1.2156	58	
	337	0.7145	57	Kinked Tail
	338	0.7497	56	
	339	1.3631	57	Kinked Tail
	Median:	**0.842**	**57**	
	Mode:		**58**	
	Mean:	**0.861**	**57.200**	
	SEM:	**0.043**	**0.172**	
	%CV:	**20.641**	**10.152**	
D	340	0.5663	56	
	341	0.7485	57	Kinked Tail
	342	0.7492	57	Kinked Tail
	343	0.9510	57	
	344	0.7772	57	
	345	0.9597	56	Kinked Tail
	346	0.8719	57	
	347	0.7196	59	
	348	0.7160	57	
	349	0.8443	57	
	350	1.1302	59	Kinked Tail
	351	0.8648	59	Kinked Tail
	352	0.9336	57	
	353	0.8433	56	
	354	0.7065	57	
	355	0.6758	57	Kinked Tail
	356	1.2766	57	
	357	0.7701	56	
	358	1.0583	59	
	359	0.9895	59	
	Median:	**0.844**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.858**	**57.300**	
	SEM:	**0.038**	**0.242**	
	%CV:	**18.384**	**14.280**	
	Median:	**0.844**	**57**	
	Mode:		**57**	
	Total Mean:	**0.859**	**57.250**	
	SEM:	**0.028**	**0.147**	
	%CV:	**19.296**	**12.249**	

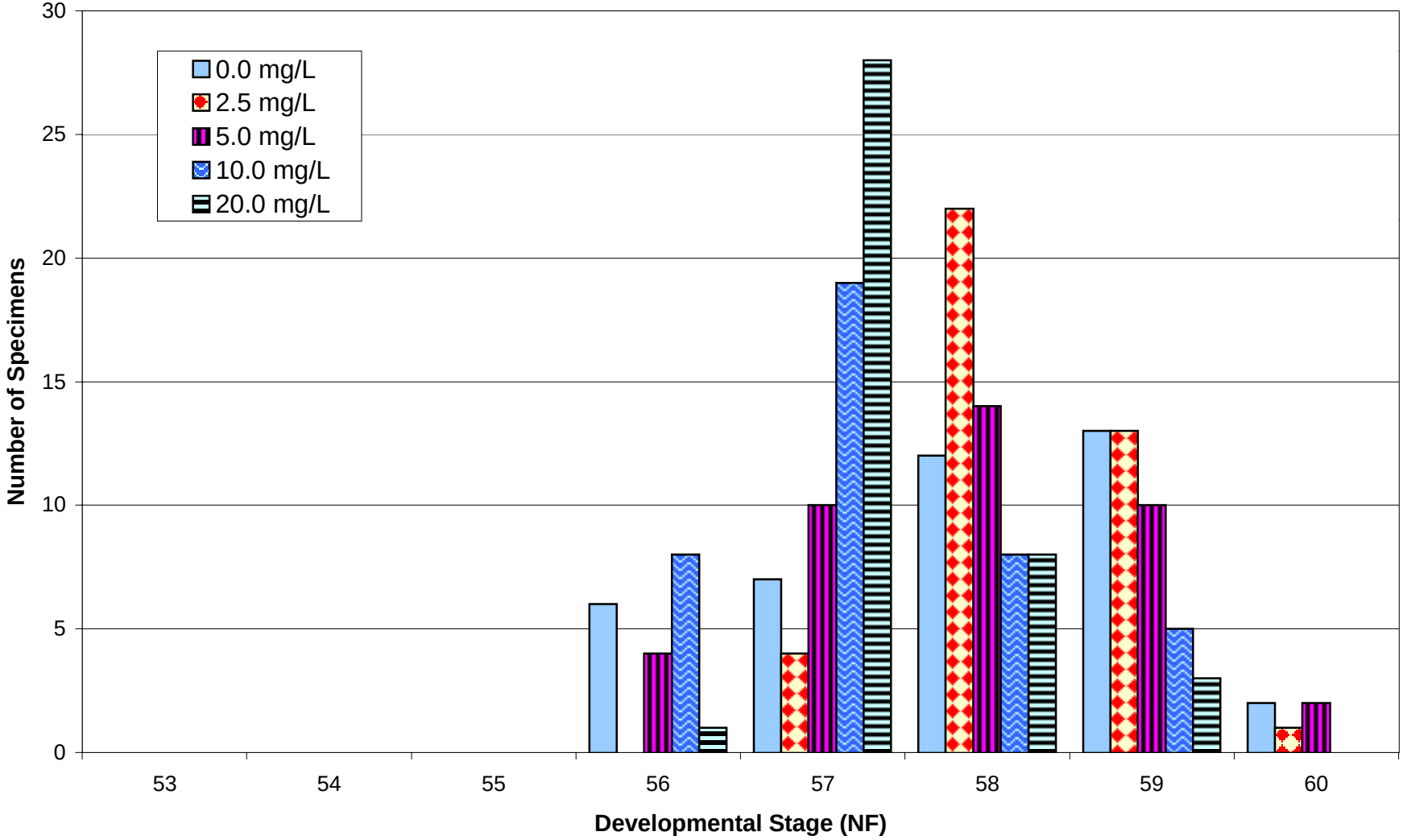
TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00051 **Test No: 1**

Test Chemical: 6-n-Propyl-2-thiouracil (PTU) **Target Conc: 20.0 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	360	0.9508	57	
	361	1.0403	57	Kinked Tail
	362	1.1043	57	
	363	0.8854	57	
	364	0.8013	57	
	365	0.8722	57	
	366	0.7958	57	
	367	0.7022	56	
	368	0.6325	57	
	369	0.8997	59	
	370	0.9570	58	
	371	0.8249	58	Kinked Tail
	372	1.0764	58	
	373	0.8542	57	
	374	0.8942	59	Kinked Tail
	375	0.8284	58	Kinked Tail
	376	0.8931	57	
	377	0.7114	58	
	378	0.8798	58	Kinked Tail
	379	0.8507	57	Kinked Tail
	Median:	**0.876**	**57**	
	Mode:		**57**	
	Mean:	**0.873**	**57.450**	
	SEM:	**0.027**	**0.170**	
	%CV:	**12.692**	**10.016**	
D	380	0.8635	57	
	381	1.1330	57	
	382	0.8604	57	
	383	0.6606	57	
	384	0.8862	57	
	385	0.7588	57	Kinked Tail
	386	0.9135	57	
	387	0.6871	57	
	388	0.9063	59	
	389	1.1396	58	
	390	0.8681	57	Kinked Tail
	391	0.6972	57	
	392	0.8126	57	
	393	0.7722	57	Kinked Tail
	394	0.9021	57	
	395	0.8575	57	
	396	1.1633	58	Kinked Tail
	397	1.1998	57	
	398	0.7997	57	
	399	0.8305	57	
	Median:	**0.862**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.886**	**57.200**	
	SEM:	**0.035**	**0.117**	
	%CV:	**16.810**	**6.917**	
	Median:	**0.866**	**57**	
	Mode:		**57**	
	Total Mean:	**0.879**	**57.325**	
	SEM:	**0.022**	**0.104**	
	%CV:	**14.733**	**8.662**	

BATT02-00051
6-n-Propyl-2-thiouracil (PTU)
Stage Distribution for Initial Stage 54 Test



6-n-Propyl-Thiouracil (PTU) Metamorphosis Assay: Statistical Data – Stage and Growth

One Way Analysis of Variance

Data source: BATT02-00051_PTU_Stage 51_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0mg/L	39	0	57	57	57.75
2.5mg/L	40	0	57	56	57
5.0mg/L	40	0	57	56.5	59
10.0mg/L	40	0	56	55	57
20.0mg/L	40	0	54	54	55

H = 95.869 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
20.0mg/L vs 0.0mg/L	103.194	7.963	Yes	
10.0mg/L vs 0.0mg/L	47.769	3.686	Yes	**LOEC**
2.5mg/L vs 0.0mg/L	11.519	0.889	No	
5.0mg/L vs 0.0mg/L	5.394	0.416	Do Not Test	**NOEC**

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00051_PTU_Stage 51_Growth

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.011)

Group Name	N	Missing	Mean	Std Dev	SEM
0.0mg/L	39	0	0.736	0.139	0.0223
2.5mg/L	40	0	0.728	0.168	0.0266
5.0mg/L	40	0	0.726	0.141	0.0222
10.0mg/L	40	0	0.729	0.149	0.0236
20.0mg/L	40	0	0.757	0.235	0.0371

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.0262	0.00654	0.225	0.924
Residual	194	5.629	0.029		
Total	198	5.655			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.924).

Power of performed test with alpha = 0.050: 0.049

The power of the performed test (0.049) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

One Way Analysis of Variance

Data source: BATT02-00051_PTU_Stage 54_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0mg/L	40	0	58	57	59
2.5mg/L	40	0	58	58	59
5.0mg/L	40	0	58	57	59
10.0mg/L	40	0	57	57	58
20.0mg/L	40	0	57	57	58

H = 34.939 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
10.0mg/L vs 0.0mg/L	40	3.091	Yes	**LOEC**
20.0mg/L vs 0.0mg/L	38.388	2.966	Yes	
2.5mg/L vs 0.0mg/L	19.412	1.5	No	
5.0mg/L vs 0.0mg/L	3.525	0.272	Do Not Test	**NOEC**

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00051_PTU_Stage 54_Growth

Normality Test: Failed (P = 0.009)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0mg/L	40	0	0.817	0.664	0.949
2.5mg/L	40	0	0.861	0.768	0.919
5.0mg/L	40	0	0.855	0.768	0.988
10.0mg/L	40	0	0.844	0.749	0.949
20.0mg/L	40	0	0.866	0.8	0.91

H = 3.414 with 4 degrees of freedom. (P = 0.491)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.491)

6-n-Propyl-Thiouracil (PTU)
Test Chemical Analytical Data from Battelle:
Purity / Stability Report

Battelle
Putting Technology To Work

Chemical Repository Services for the EDSP

EPA Contract No. 68-W-01-023

Chemistry Report for WA 3-11
Propylthiouracil in Water

February 10, 2004

Prepared By:

Michael E. Cobb. — 2-10-04 (Date)
Chemical Repository Study Director

Approved By:

Richard M. Ecker — 2/10/04 (Date)
Director, Marine Sciences Laboratory

Battelle Marine Sciences Laboratory
1529 West Sequim Bay Road
Sequim, WA 98382

Submitted to:

Douglas J. Fort, Ph.D.

Fort Environmental Laboratories

Stillwater, Oklahoma 74074

Chemistry Report for WA 3-11
Propylthiouracil in Water

Reviewed by: Mary E Lynn

Mary Lynn, EDSP Quality Assurance Representative
Windward Company, Gig Harbor

Date: 2/10/04

Personnel participating in this study:

Analyst: Tim Fortman

Statistician: Valerie Cullinan

Chemical Repository Study Director: Michael Cobb

Chemistry Report for WA 3-11
Propylthiouracil in Water

Parameter	Chemical
Compound Name	Propylthiouracil
CAS #	51-52-5
Central File No.	CF-2151
Initial Receipt Date	10/10/03
Expiration Date	10/10/08
Manufacturer	Sigma, Inc.
Lot Number	063K0144
Battelle Study #	WA 3-11-01-01
Method	SW 846, 8316 Modified

Executive Summary

The chemical purity of propylthiouracil determined by HPLC by the manufacturer was 100%. The purity result from Battelle-Sequim by HPLC was determined to be 99.9%. Stability testing was conducted on two concentrations of propylthiouracil: a stock solution with a target concentration of 700 mg/L and a dilution with a target concentration of 1.2 mg/L. In both cases, the observed concentrations were greater than the target. Thus, the average Day 0 concentrations for the stock and dilution solutions (818 mg/L and 1.57 mg/L) were used to evaluate stability. Though the stock solution concentration and the diluted solution concentration were above the target levels, the validity and conclusions of the study were in no way affected as the stability evaluation was based on the Day 0 analyzed concentration. Based on the final regression model and the lower 95% confidence limit of the slope, the stock solution concentration of propylthiouracil was expected to stay greater than or equal to 90% of the average Day 0 concentration for up to an estimated 45 days. The dilution concentration of propylthiouracil was expected to stay greater than or equal to 90% of the average Day 0 concentration for up to an estimated 50 days. Thus, stability testing of both the propylthiouracil stock and dilution solutions in water were considered stable at both the 818 mg/L and 1.57 mg/L concentrations for the required testing/holding period of 3 weeks.

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1.0 INTRODUCTION

The goal of the Battelle-Sequim, Marine Sciences Laboratory (MSL) Chemical Repository for the Endocrine Disruptor Screening Program (EDSP) is to provide the participating laboratory with requested chemicals of documented quality at required concentrations and in a matrix appropriate for different toxicological tests. The EDSP Chemical Repository supplies the manufacturer's information regarding purity and stability, the material safety data sheet (MSDS) chemical information, and independent analysis of purity and stability in a matrix specified by the Purity and Stability Testing Plan made in collaboration with the requesting Principal Investigator. Additional analyses associated with the in-life studies are also provided when requested. This report is the product of such a request.

Under Work Assignment (WA) 3-11 and Battelle-Sequim Study Number WA 3-11-01-01, Douglas J. Fort, Ph.D. from Fort Environmental Laboratories, requested purity and stability testing of propylthiouracil (Figure 1). Electronic files submitted to the EDSP Data Coordination Center in support of this work assignment were CRF_WA-3-11_propylthiouracil-water.doc, WA 3-11 6-n-propyl-2thiouracilpurity-stability test plan1.doc, DSUM_WA-3-11_PTU-water.xls, and DAF_WA-3-11_PTU-water.doc.

2.0 GENERAL METHODS

Methods of standard operation of the Chemical Repository (Repository) are addressed in a series of Repository specific MSL SOPs (reference MSL-R-001 through 014) as well as additional MSL SOPs as required. These procedures address chemical procurement including procurement of controlled substances, when applicable, which have unique permitting, ordering, handling, inventory, and storage requirements; chemical receipt and chain of custody, chemical log-in and labeling, inventory, chemical storage; stock solution preparation, documentation and archiving; test solution preparation, documentation and shipping; chemical disposal, and repository maintenance over time. The quality assurance (QA) requirements for procurement of chemicals for use in the Chemical Repository are addressed in procedure, MSL-A-012, Procurement. Each purchase requisition receives QA review to determine what is being ordered and which specific requirements apply.

2.1 Chemical Procurement

As requested by Douglas J. Fort, Ph.D., propylthiouracil, (CAS No. 51-52-5) was purchased for purity and stability analysis and used as the first test chemical in a frog metamorphosis assay (Figure 1). Propylthiouracil was purchased from Sigma, Inc. and lot number 063K0144 was received on 10/10/03 with an expiration date of 10/10/08 (Table 1). The chemical was left in the original container, logged in to the Chemical Management System (CMS) and given a CMS barcode and unique log in number (CF-2151) as per SOP MSL-R-001-00. The chemical was stored in a clean, dry location at room temperature.

EDSP Chemical Request Form

For EPA WA: 3-11

Study Director:

Name: Douglas J. Fort, Ph.D.

Affiliation: Fort Environmental Laboratories

Location: Stillwater, Oklahoma 74074

Telephone number: 405-624-6771

Bioassay Information:

Proposed Bioassay: Frog Metamorphosis Assay Demonstration

Test Chemical: 6-Propyl-2-thiouracil (PTU)

Carrier(s) NA

Concentrations/Dilution Series: 2.5, 5.0, 10.0, 20.0 mg/L

*Consider if analysis method detection limit which may be determined in Purity

In *vitro or in vivo* tests? *In vivo*

Organism to be tested: *Xenopus Laevis* (South African Clawed Frog)

Method of test solution administration: Flow-through exposure/diluter system

Planned/proposed test duration: 14 and 21 days

Chemical Information:

Chemical Name: 6-Propyl-2-thiouracil (PTU)

CAS: 51-52-5

Any known purity information: may refer to attached documentation

Any know stability information: Contact Dr. Joe Tietge, EPA Duluth, MN

Desired purity (%) for test? ≥ 98%

Figure 1. EDSP Requisition Form for Propylthiouracil

Table 1. Chemical Procurement Information

Parameter	Chemical
Compound Name	Propylthiouracil
CAS #	51-52-5
Central File No.	CF-2151
Initial Receipt Date	10/10/03
Expiration Date	10/10/08
Manufacturer	Sigma, Inc.
Lot Number	063K0144
Manufacturer's Purity	100%
Storage Conditions	Brown glass, dry place, room temperature
Battelle Study #	WA 3-11-01-01
Method	SW 846, 8316 Modified

2.2 Chemical Purity

Chemical purity was verified by chromatographic analysis through comparison of the peak area of the principal peak (PTU) with the total areas of all peaks discerned (PTU peak and contaminant peaks) reported as % Purity = (PTU Area/(PTU Area + Contaminant Peak Areas)) X 100. This result was then compared to the manufacturer's certificate of analysis/purity (Appendix A). No statistical analyses were performed for the verification of chemical purity. General methods are documented in the procedure, EDSP.D-012-01, Chemical Repository Summary Displays and Statistical Analyses for the EDSP Data Coordination Center (DCC).

Purity verification was conducted on a high performance liquid chromatography system with a UV absorbance detector (HPLC-UV) at the 276 nm wavelength by running a solution of about 8000 ng/mL in 68% Methanol: 32% 100mM potassium phosphate buffer, pH adjusted to 3 with phosphoric acid (v:v). A water blank was also analyzed on the HPLC. The purity was determined by first identifying the peaks in the chromatogram of the propylthiouracil that were the same as the peaks in the analysis of the blank. The areas associated with these common peaks were then eliminated by inhibiting integration or other methods and the remaining peaks were reported as a percentage of the total peak area. The percentage associated with the largest peak represented the purity of propylthiouracil. The HPLC was set up with an autosampler. The autosampler was set to inject 450 μL of the matrix dilution. One replicate was analyzed.

2.3 Preparation of Stock Matrices for Stability Analysis

A general study plan for stability testing based on the WA 3-11 request from Douglas J. Fort, Ph.D. was developed as the stability test protocol and is presented in Appendix B. A single stock solution was prepared to arrive at the chemical concentrations requested for stability analysis (Table 2). All samples were analyzed in triplicate so that a mean concentration and relative standard deviation (RSD) could be determined. General methods are documented in EDSP.D-012-01.

Propylthiouracil stock matrices were prepared on 11/14/03 for testing as described in Table 2. Briefly, for the 700 mg/L (stock) propylthiouracil, 0.7022 g was

weighed into an amber 1 liter bottle with ~1 liter of MSL well water. The solution was agitated with a stir bar and stir plate until all of the propylthiouracil was dissolved. For the 1.2 mg/L propylthiouracil, 1.8 ml of the stock solution was added to another 1 liter amber ashed bottle and approximately 1liter MSL well water was

Table 2. Stock Matrix Composition for Stability Testing

Study and Duration	Test Chemical	Target Concentration	Sample ID	Stock Matrix
WA 3-11-01-01 3 Weeks	Propylthiouracil	700 mg/L Stock Solution	2151-2a-1-1 Stk	0.7022 g in ~1 L water
		1.2 mg/L Diluted Solution	2151-2a-1-1 Dil	1.8 mL of stock in ~1 L water

added. The solution was hand agitated to disperse the solution. Bottles were labeled and stored at room temperature for the duration of the test.

2.4 Analytical Chemistry for Stability Testing

Chemical stability was evaluated under storage conditions and matrix specifications as requested by the participating laboratory. At initiation and at each time period throughout the duration of the test, the chemical concentration was determined by chromatographic analysis. Triplicate aliquots were tested. The frequency of determinations and the duration of testing were determined by the requesting principal investigator and the chemists based on *a priori* knowledge about chemical stability. General methods are documented in EDSP.D-012-01.

Propylthiouracil stock solution was sampled by taking 0.01 mL of the sample and placing it into a 1.8-mL autosampler vial with 0.99 mL of 68:32 Methanol:buffer (v:v). The solution was agitated with a transfer pipette and then transferred to an HPLC autosampler vial. Propylthiouracil diluter solution was sampled by taking 0.1 mL of the sample and placing it into a 1.8-mL autosampler vial with 0.9 mL of 68:32 Methanol:buffer (v:v). The solution was agitated with a transfer pipette and then transferred to an HPLC autosampler vial. These vials were then analyzed by HPLC with ultraviolet/visible wavelengths (HPLC-UV/VIS) using a wavelength of 276 nm for quantification. A water blank was prepared the same way as the diluter sample and then analyzed by HPLC for quantification. Initial calibration verification and continuing calibration verification (ICV/CCV) samples were analyzed to demonstrate on-going calibration accuracy.

2.5 Statistical Analysis of Stability

Log linear degradation curves were fit to the data to describe the chemical concentration over time and their dependence on storage conditions and solvent matrix. Lack of fit and residual plots were evaluated to determine the form of the regression. Power calculations based on the observed variability were used to determine the sensitivity of the test to detect degraded concentrations. General methods are documented in SOP EDSP.D-012-01.

3.0 RESULTS

3.1 Chemical Purity

Battelle-Sequim conducted a HPLC purity scan on the propylthiouracil. The chromatogram, after solvent blank correction, showed one large peak that had the appropriate retention time for propylthiouracil and several very small peaks. The area of the propylthiouracil peak was 99.9% of the total area of all peaks in the chromatogram. Chemical purity of propylthiouracil determined by the manufacturer was 100% (Appendix A).

3.2 Analytical Chemistry for Stability Testing

Chemical stability testing was initiated on 11/14/03. Chemical concentration was determined 9 times over a period of 24 days. The analytical and quality control (QC) results are presented in Appendix C. One water blank was analyzed with every batch for QC purposes. There were no detectable concentrations of propylthiouracil in the blanks. ICV/CCV results ranged from 97.3% to 103%. All QC data were within acceptance criteria. The MDL was determined to be 0.0189 mg/L.

3.3 Statistical Results of Stability Trial

All of the observed concentrations for the stock solution of propylthiouracil were greater than the target concentration of 700 mg/L. Thus, the average Day 0 concentration of 818 mg/L was used to evaluate stability. A plot of the stock solution against time suggested very little chemical decay (Figure 2). None of the data points were less than 90% of the average Day 0 concentration. Based on the final regression model and the lower 95% confidence limit of the slope, the concentration of propylthiouracil was expected to stay greater than or equal to 90% of the average Day 0 concentration for up to an estimated 45 days (Table 3). Thus, this stock solution was considered stable for the required 3-week testing/holding period. The complete statistical analysis is presented in Appendix D.

All of the observed concentrations for the diluted solution of propylthiouracil were greater than the target concentration of 1.2 mg/L. Thus, the average Day 0 concentration of 1.57 mg/L was used to evaluate stability. All observations of the diluted solution of propylthiouracil were greater than 90% of the average day zero concentration (Figure 2). Based on the final regression model and the lower 95% confidence limit of the slope, the concentration of propylthiouracil was expected to stay greater than or equal to 90% of the average Day 0 concentration for an estimated 50 days (Table 3). Thus, this stock solution was considered stable for the required testing/holding period of 3 weeks. The complete statistical analysis is presented in Appendix D.

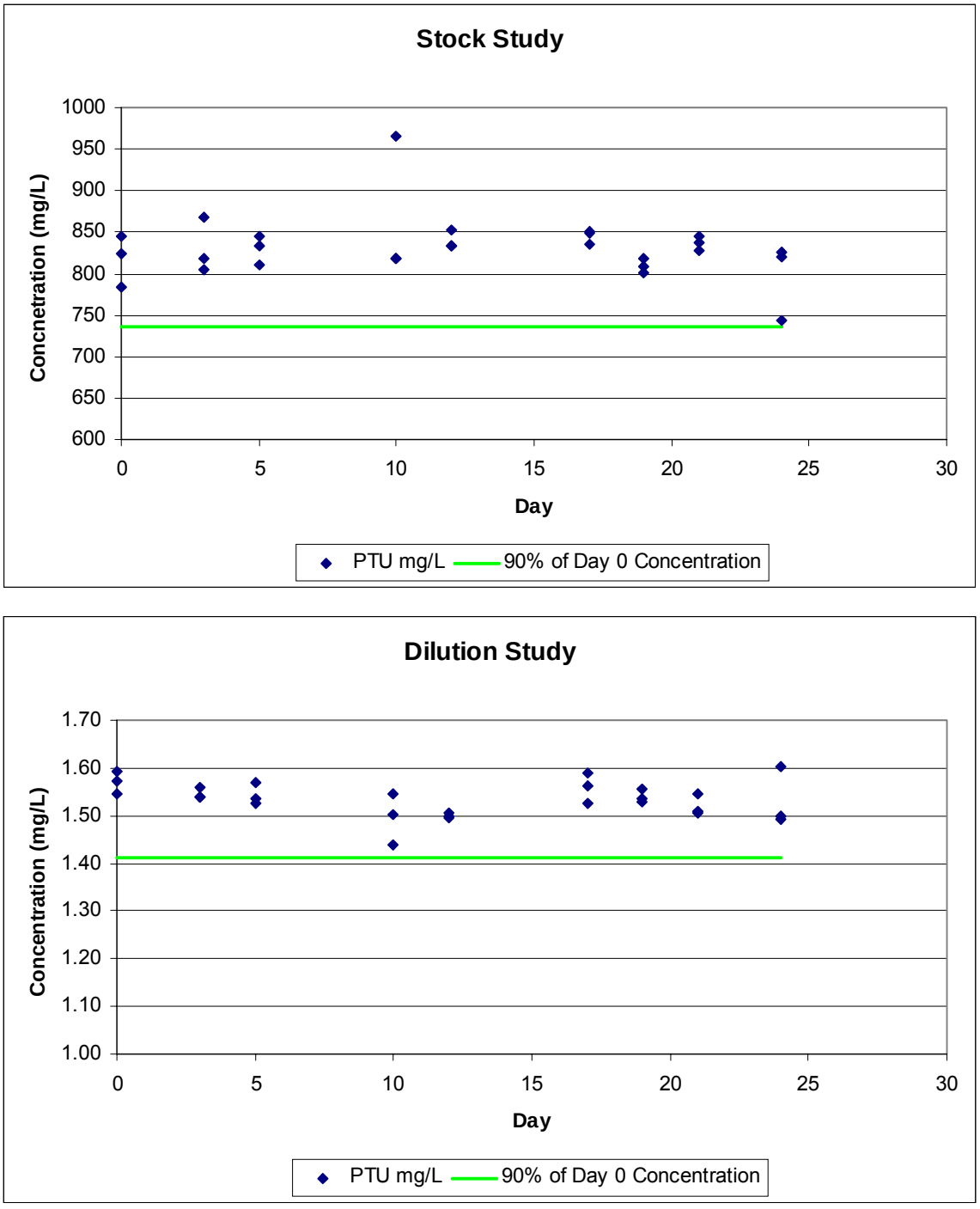


Figure 2. Observed concentration of propylthiouracil (PTU) with a target concentration of 700 mg/L (above) and 1.2 mg/L (below) against time

Table 3. Summary of Statistical Results for Propylthiouracil

WA 3-11-01-01 Statistical Analysis conducted by Valerie Cullinan Using Minitab Version 13.32, Minitab Inc., 1999.	2151-2a-1-1 Stk PTU 700 mg/L	2151-2a-1-1 Dil PTU 1.2 mg/L
Average Day 0 Concentration (mg/L)	818	1.57
Number of determinations	1	1
Number of weeks tested	3	3
Number of replicates per day	3	3
Number of outliers removed	0	0
Number of observations removed	0	0
Overall Mean Concentration	830	1.53
95% Upper CL	842	1.55
error degrees of freedom	26	26
1-sample t-test of Ho: μ >= Target	NS [a]	S [b]
estimated intercept of ln(concentration) against time	6.7298	0.4354
estimated slope of ln(concentration) against time	-0.0007	-0.0006
standard error of slope	0.0010	0.0006
error degrees of freedom	25	25
Significance test of lack-of-fit for final model	NS	NS
Significance test of Ho: $\beta = 0$ vs. H1: $\beta \neq 0$	NS	NS
Lower 95% CL of β	-0.003	-0.002
Upper 95% CL of β	0.001	0.001
Maximum Percent Loss (using LCL) over 8 days	2.3%	1.4%
Mean Percent Loss (using bhat)	0.6%	0.5%
LN(90% of Target)	6.6012	0.3461
Number of days until at 90% of Target (using LCL)	45	50
Conclusion using Target Concentration:	**Stable for 3 wks**	**Stable for 3 wks**

[a] Not Significant at $\alpha = 0.05$
[b] Significant at $\alpha = 0.05$

4.0 CONCLUSIONS

The chemical purity determined by Battelle-Sequim was 99.9% and by the manufacturer was 100%. Stability testing of propylthiouracil in water concluded that the chemical was stable at both the 818 mg/L and 1.57 mg/L concentrations for a testing/holding period of 3 weeks.

APPENDIX A

MANUFACTURER'S CERTIFICATE OF ANALYSIS/PURITY

SIGMA-ALDRICH

Certificate of Analysis

Product Name	6-Propyl-2-thiouracil
Product Number	P3755
CAS Number	51-52-5
Molecular Formula	$C_7H_{10}N_2OS$
Molecular Weight	170.2

TEST	SPECIFICATION	LOT 063K0144 RESULTS
APPEARANCE	WHITE TO WHITE WITH A YELLOW CAST POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS TO SLIGHTLY HAZY FAINT YELLOW SOLUTION IN 10% AMMONIA AT 100 MG/1 ML	CLEAR COLORLESS
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONFORMS
PURITY BY SODIUM HYDROXIDE TITRATION	99% MINIMUM	99%
PURITY BY HPLC	99% MINIMUM	100%
QC ACCEPTANCE DATE		JUNE 2003

Lori Schulz, Manager
Analytical Services

APPENDIX B

PURITY AND STABILITY TESTING PLAN

CHEMICAL INFORMATION

Chemical Name: 6-n-Propyl-2-thiouracil (MSL CF-2151, CMS 219750)

CAS Number: 51-52-5

Lot Number: 063K0144, stored at RT in MSL 5 Rm 219

Expiration date: 10/08

Manufacturer's Purity Information: >99% (from certificate of analysis)

Manufacturer's Stability Information: Protect from light, avoid strong oxidizing agents, strong acids, and strong bases (*from MSDS*)

MSL Purity Results:

Purity (%): To be determined at MSL by HPLC

MDL: Will be determined as part of stability study.

Calibration Standard available: Yes, there is material from a separate Mfg. in inventory – CMS # 174438, lot # GF01, >99% purity

Name: 6-n-Propyl-2-thiouracil

CAS: 51-52-5

BIOASSAY INFORMATION

Study Director:

Name: Douglas J. Fort, Ph.D.
Affiliation: Fort Environmental Laboratories
Location: Stillwater, Oklahoma 74074
Telephone number: 405-624-6771
Email: djfort@fortlabs.com

Proposed Bioassay: EDSP WA 3-11 Frog Metamorphosis Assay Demonstration

Test Chemical: 6-n-Propyl-2-thiouracil (PTU)

CAS: 51-52-5

Carrier(s): PTU to be dissolved in well water

Concentrations/Dilution Series: 0.00, 2.50, 5.00, 10.0, 20.0 mg/L

In vitro or in *vivo tests*? *In vivo*

Organism to be tested: *Xenopus laevis* (South African Clawed Frog)

Method of test solution administration: Flow-through exposure/diluter system

Planned/Proposed test duration: 3 weeks

Solubility: Prior to initiation of the stability testing, solubility of the PTU, in the carrier, will be demonstrated at levels sufficient to allow accurate production of the stock solution.

Design of Stock Solution Stability Test: A stock solution of approximately 700 mg/L of PTU will be prepared for stability testing. The solution will be stored in a glass container at ambient room temperature in the dark for three weeks, sampled twice weekly in triplicate and analyzed weekly by HPLC.

# of replicates: 3

Duration: 3 weeks, sampling twice weekly with triplicate samples taken each time

Other Factors:

Temperature regime(s): ambient °C
Test container type: glass
Light or dark: Dark except when container is removed for sampling or handling
Other: None

Carrier(s): PTU will be dissolved in well water.

Test Chemical	Test Chemical Concentration and Method	
	Aqueous Stock (mg/L)	Detection Method
PTU	700[e]	HPLC

[e]**Estimated stock concentration:** The estimated detection limit based on the sensitivity on the equipment is *TBD*, which is about *TBD* times (depends on the volume of sample extracted) below the target stock concentration of 700 mg/L. For assessment of stock solution stability and preparation, we expect sample volume to be *TBD*. From these, *TBD* ul aliquots will be removed for analysis. This would be diluted to give an approximate concentration of *TBD* ug/L for analysis.

Design of stability test carried out in preparation for receipt of samples collected to monitor Diluter system performance: An aliquot of the stock solution will be diluted to 5.00 mg/L (TBD) in well water for verification of storage condition for samples to be collected and tested for monitoring Diluter system performance. The 5.00 mg/L (TBD) sample will be stored in glass at 4 °C in the dark for three weeks. All aspects of the stability testing will be identical to that of the stock solution above, save sampling event

will occur once per week instead of twice. Testing will be carried out in parallel with the stock solution stability testing.

Personal Protective Equipment: All personnel must wear a lab coat, eye protection and Nitrile gloves when handling PTU or water spiked with PTU. Disposal of all wastewater must comply with the wastewater treatment plan prepared for WA 3-11.

Statistical testing: Regression analysis will be used to test for a significant slope of the chemical concentration recovered versus time. The concentration recovered will be transformed to the natural logarithm to reduce heterogeneity of variances between times. Residual plots will be evaluated to determine the need for fitting polynomials.

Resulting records package:

Manufacturer's certificate of analysis or purity
MSDS
Records:

- Date sample received;
- Date(s) sample analyzed;
- Sample matrix;
- Electronic file identification codes (when applicable to identify instrument data files);
- Data summary reports;
  - Chemical repository confirmatory test results of chemical identity and purity;
  - Chemical repository test results of lot-to-lot variation in chemical purity;
  - Chemical repository periodic assessment results of changes in purity of stock solutions and dilutions and generation of degradation products
- QC data reports;
- Data qualifying flags; and
- Dilution factor(s).

Prepared by Michael Cobb

Distribution;
Project File
Tim Fortman
Waverly Thorsen
Deborah Coffey
Val Cullinan
Whitney Hansen
Eric Crecelius

The following differences from the Stability Test Plan occurred:

The target concentrations were listed as 700 and 5.0 mg/L in the propylthiouracil stability test plan. The actual concentrations determined were approximately 800 and 1.5 mg/L. The differences were due to two factors.

First, there was a change in the dose concentration range of the amphibian study, documented and received from Doug Fort, increasing the dose concentration range from the original 1.25 – 10.0 mg/L, up to 2.5 – 20.0 mg/L. The study had been initiated prior to receipt of this change order. The 5.0 mg/L initially estimated as the target dilution test concentration in the test plan was accompanied with "TBD." Upon further discussion (prior to receipt of the dose concentration change order) we decided to bracket both the dose and stock solution concentration range in the study by selection of a dilution concentration at the bottom of the originally specified range (1.25 mg/L).

The factor resulting in the increased test concentration level from 700 to ~800 mg/L was due to use of a one liter bottle to measure the (approximate) volume of water diluent rather than use of a class A graduated cylinder - a practice that will not be repeated.

Neither of these factors have any impact on the conclusions of the study since Day 0 analyzed concentrations were used to evaluate stability.

Signed:_____ _____
Tim Fortman, Analyst **Date**

Signed:_____ _____
Michael Cobb, Study Director **Date**

APPENDIX C

ANALYTICAL RESULTS OF STABILITY TESTING

Table C1. Propylthiouracil concentration in Water (mg/L)

Target Conc.	Sample ID	Sample Date	Analysis Date	Propylthiouracil (mg/L)	Average (mg/L)	Recovery[a]	RSD
700 mg/L	2151-2a-1-1 Stk R-1	11/14/2003	11/17/2003	825		101%	
700 mg/L	2151-2a-1-1 Stk R-2	11/14/2003	11/17/2003	845	818	103%	4%
700 mg/L	2151-2a-1-1 Stk R-3	11/14/2003	11/17/2003	784		96%	
700 mg/L	2151-2a-1-2 Stk R-1	11/17/2003	11/17/2003	818		100%	
700 mg/L	2151-2a-1-2 Stk R-2	11/17/2003	11/17/2003	805	830	98%	4%
700 mg/L	2151-2a-1-2 Stk R-3	11/17/2003	11/17/2003	867		106%	
700 mg/L	2151-2a-1-3 Stk R-1	11/19/2003	11/19/2003	810		99%	
700 mg/L	2151-2a-1-3 Stk R-2	11/19/2003	11/19/2003	834	830	102%	2%
700 mg/L	2151-2a-1-3 Stk R-3	11/19/2003	11/19/2003	846		103%	
700 mg/L	2151-2a-1-4 Stk R-1	11/24/2003	11/26/2003	966		118%	
700 mg/L	2151-2a-1-4 Stk R-2	11/24/2003	11/26/2003	817	868	100%	10%
700 mg/L	2151-2a-1-4 Stk R-3	11/24/2003	11/26/2003	819		100%	
700 mg/L	2151-2a-1-5 Stk R-1	11/26/2003	11/26/2003	834		102%	
700 mg/L	2151-2a-1-5 Stk R-2	11/26/2003	11/26/2003	852	840	104%	1%
700 mg/L	2151-2a-1-5 Stk R-3	11/26/2003	11/26/2003	834		102%	
700 mg/L	2151-2a-1-6 Stk R-1	12/1/2003	12/4/2003	849		104%	
700 mg/L	2151-2a-1-6 Stk R-2	12/1/2003	12/4/2003	851	845	104%	1%
700 mg/L	2151-2a-1-6 Stk R-3	12/1/2003	12/4/2003	835		102%	
700 mg/L	2151-2a-1-7 Stk R-1	12/3/2003	12/4/2003	808		99%	
700 mg/L	2151-2a-1-7 Stk R-2	12/3/2003	12/4/2003	801	809	98%	1%
700 mg/L	2151-2a-1-7 Stk R-3	12/3/2003	12/4/2003	818		100%	
700 mg/L	2151-2a-1-8 Stk R-1	12/5/2003	12/8/2003	828		101%	
700 mg/L	2151-2a-1-8 Stk R-2	12/5/2003	12/8/2003	838	837	102%	1%
700 mg/L	2151-2a-1-8 Stk R-3	12/5/2003	12/8/2003	845		103%	
700 mg/L	2151-2a-1-9 Stk R-1	12/8/2003	12/8/2003	743		91%	
700 mg/L	2151-2a-1-9 Stk R-2	12/8/2003	12/8/2003	821	797	100%	6%
700 mg/L	2151-2a-1-9 Stk R-3	12/8/2003	12/8/2003	826		101%	
1.2 mg/L	2151-2a-1-1 Dil R-1	11/14/2003	11/17/2003	1.57		100%	
1.2 mg/L	2151-2a-1-1 Dil R-2	11/14/2003	11/17/2003	1.59	1.57	101%	1%
1.2 mg/L	2151-2a-1-1 Dil R-3	11/14/2003	11/17/2003	1.55		99%	
1.2 mg/L	2151-2a-1-2 Dil R-1	11/17/2003	11/17/2003	1.54		98%	
1.2 mg/L	2151-2a-1-2 Dil R-2	11/17/2003	11/17/2003	1.56	1.55	99%	1%
1.2 mg/L	2151-2a-1-2 Dil R-3	11/17/2003	11/17/2003	1.54		98%	
1.2 mg/L	2151-2a-1-3 Dil R-1	11/19/2003	11/19/2003	1.53		97%	
1.2 mg/L	2151-2a-1-3 Dil R-2	11/19/2003	11/19/2003	1.57	1.54	100%	1%
1.2 mg/L	2151-2a-1-3 Dil R-3	11/19/2003	11/19/2003	1.54		98%	
1.2 mg/L	2151-2a-1-4 Dil R-1	11/24/2003	11/26/2003	1.50		96%	
1.2 mg/L	2151-2a-1-4 Dil R-2	11/24/2003	11/26/2003	1.55	1.50	98%	4%
1.2 mg/L	2151-2a-1-4 Dil R-3	11/24/2003	11/26/2003	1.44		92%	
1.2 mg/L	2151-2a-1-5 Dil R-1	11/26/2003	11/26/2003	1.50		96%	
1.2 mg/L	2151-2a-1-5 Dil R-2	11/26/2003	11/26/2003	1.50	1.50	95%	0%
1.2 mg/L	2151-2a-1-5 Dil R-3	11/26/2003	11/26/2003	1.50		95%	
1.2 mg/L	2151-2a-1-6 Dil R-1	12/1/2003	12/4/2003	1.59		101%	
1.2 mg/L	2151-2a-1-6 Dil R-2	12/1/2003	12/4/2003	1.56	1.56	100%	2%
1.2 mg/L	2151-2a-1-6 Dil R-3	12/1/2003	12/4/2003	1.53		97%	
1.2 mg/L	2151-2a-1-7 Dil R-1	12/3/2003	12/4/2003	1.53		97%	

Target Conc.	Sample ID	Sample Date	Analysis Date	Propylthiouracil (mg/L)	Average (mg/L)	Recovery[a]	RSD
1.2 mg/L	2151-2a-1-7 Dil R-2	12/3/2003	12/4/2003	1.56	1.54	99%	1%
1.2 mg/L	2151-2a-1-7 Dil R-3	12/3/2003	12/4/2003	1.54		98%	
1.2 mg/L	2151-2a-1-8 Dil R-1	12/5/2003	12/8/2003	1.51		96%	
1.2 mg/L	2151-2a-1-8 Dil R-2	12/5/2003	12/8/2003	1.54	1.52	98%	1%
1.2 mg/L	2151-2a-1-8 Dil R-3	12/5/2003	12/8/2003	1.50		96%	
1.2 mg/L	2151-2a-1-9 Dil R-1	12/8/2003	12/8/2003	1.50		95%	
1.2 mg/L	2151-2a-1-9 Dil R-2	12/8/2003	12/8/2003	1.49	1.53	95%	4%
1.2 mg/L	2151-2a-1-9 Dil R-3	12/8/2003	12/8/2003	1.60		102%	
Blank	2151-2a-1 Blank 1		11/17/2003	0.0189 U	0.0189 U		
Blank	2151-2a-1 Blank 2		11/17/2003	0.0189 U			
Blank	2151-2a-1 Blank 3		11/19/2003	0.0189 U			
Blank	2151-2a-1 Blank 4		11/26/2003	0.0189 U	0.0189 U		
Blank	2151-2a-1 Blank 5		11/26/2003	0.0189 U			
Blank	2151-2a-1 Blank 6		12/4/2003	0.0189 U	0.0189 U		
Blank	2151-2a-1 Blank 7		12/4/2003	0.0189 U			
Blank	2151-2a-1 Blank 8		12/8/2003	0.0189 U	0.0189 U		
Blank	2151-2a-1 Blank 9		12/8/2003	0.0189 U			

[a] Recovery is based on the Average Day 0 Concentration
U Sample values below method detection limit (MDL)
Calculations are produced before rounding.

Table C2. ICV/CCV Data for Propylthiouracil Concentration in Water

Analysis Date	Sample Name	Propylthiouracil (μg/mL)	Recovery[a]	PD
11/17/2003	PP-1267 ICV	0.7947	99.3%	0.66%
11/17/2003	PP-1266 C CCV	0.8159	102%	1.99%
11/17/2003	PP-1266C CCV	0.8102	101%	1.28%
11/17/2003	PP-1266 C CCV	0.8019	100%	0.23%
11/17/2003	PP-1266 C CCV	0.8123	102%	1.54%
11/19/2003	PP-1266 C CCV	0.7782	97.3%	2.73%
11/19/2003	PP-1266 C CCV	0.8228	103%	2.85%
11/26/2003	PP-1267 ICV	0.7866	98.3%	1.67%
11/26/2003	PP-1266 C CCV	0.7982	99.8%	0.22%
11/26/2003	PP-1266 C CCV	0.7980	99.8%	0.25%
11/26/2003	PP-1266 C CCV	0.8089	101%	1.12%
12/4/2003	PP-1267 ICV	0.7874	98.4%	1.57%
12/4/2003	PP-1266 C CCV	0.8042	101%	0.52%
12/4/2003	PP-1266 C CCV	0.8123	102%	1.54%
12/4/2003	PP-1266 C CCV	0.8134	102%	1.67%
12/8/2003	PP-1267 ICV	0.7889	98.6%	1.38%
12/8/2003	PP-1266 C CCV	0.8069	101%	0.87%
12/8/2003	PP-1266 C CCV	0.8041	101%	0.52%
12/8/2003	PP-1266 C CCV	0.8098	101%	1.23%

[a] Recovery based on 0.80 μg/mL
PD = Deviation from 100% recovery.
Calculations are produced before rounding.

Calibration Standards for PP-1266 and PP-1267

Calibrations were performed using a six-point calibration, standards were labeled PP-1266 A thru F. This mix is used for PTU analysis by HPLC-UV/Vis. These standards were made by serial dilutions of standards PP-1264 and PP-1265.

- PTU standard PP-1264 was made by weighing 0.0500 g of the neat material (PTU; Sigma P3755-25G; Lot #063K0144; expiration date 10/08) into a 25 mL volumetric flask. This was then diluted to the mark with Methanol.
- PTU standard PP-1265 was made by measuring 1 mL of PP-1264 into a 10 mL volumetric flask. This was then diluted to the mark with methanol:phosphate buffer mix.

The PP-1266 series (A through F) was made as follows.

- Solution A, 2 mL of PP-1265 added to a 50 mL volumetric flask and diluted to the mark with a methanol:phosphate buffer mix.
- Solution B, 1 mL of PP-1265 added to a 50 mL volumetric flask and diluted to the mark with a methanol:phosphate buffer mix.
- Solution C, 0.2 mL of PP-1265 added to a 50 mL volumetric flask and diluted to the mark with a methanol:phosphate buffer mix.
- Solution D, 0.1 mL of PP-1265 added to a 50 mL volumetric flask and diluted to the mark with a methanol:phosphate buffer mix.
- Solution E, 0.02 mL of PP-1265 added to a 50 mL volumetric flask and diluted to the mark with a methanol:phosphate buffer mix.
- Solution F, 0.01 mL of PP-1265 added to a 50 mL volumetric flask and diluted to the mark with a methanol:phosphate buffer mix.

The Methanol:Phosphate Buffer Mix used is a mix of 68% Methanol with 32% Phosphate buffer. The buffer is made by weighing 13.6 g KH_2PO_4 onto 4 liters of DI water and adjusting the pH to 3 with Phosphoric acid.

The ICV standard PP-1267 (A and B) was made in a 2 step process. PP-1267 A was made by weighing 0.0501 grams of PTU (TCI P0533; Lot #GF-01; expiration date 10/04) into a 25-mL volumetric flask and diluted to the mark with methanol. PP-1267 B was made by measuring 0.02 mL of PP-1267A into a 50 mL volumetric flask and diluting to the mark with the methanol:buffer described above.

APPENDIX D

STATISTICAL REPORT

WA-3-11
Statistical Analysis conducted by Valerie Cullinan
Using Minitab Version 13.32, Minitab Inc., 1999.

1/8/2004 3:16:36 PM

Analysis of PTU – 818 mg/L Stock Stability Study

- Test to determine if the data are from a population with mean of 818 mg/L.

Macro performs a one-sample t-test for mu less than TARGET &
What is the target value for X 3
DATA> 818

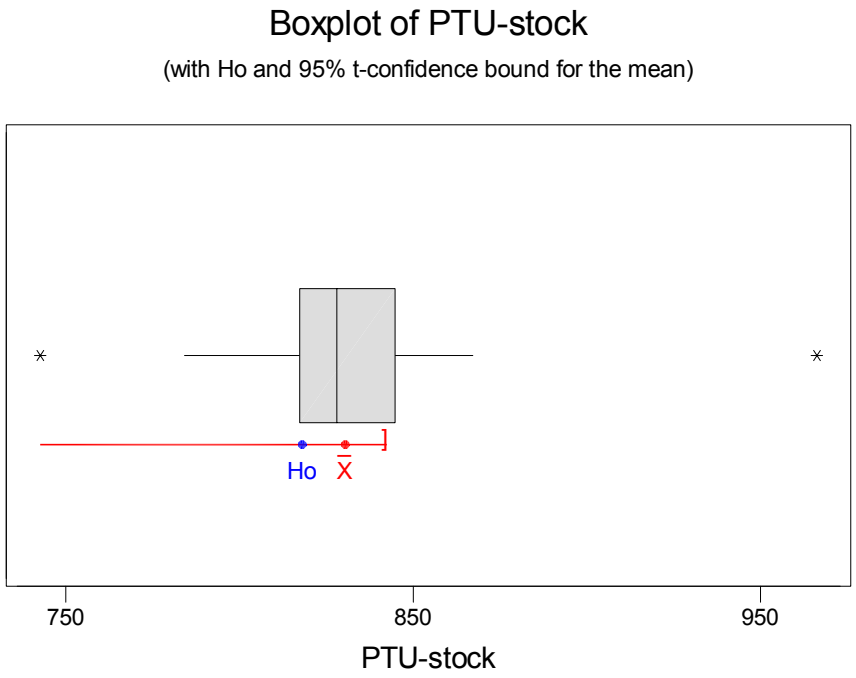
One-Sample T: PTU-stock

Test of mu = 818 vs mu < 818

Variable	N	Mean	StDev	SE Mean
PTU-stock	27	830.36	36.60	7.04

Variable	95.0% Upper Bound	T	P
PTU-stock	842.37	1.75	0.954

t Boxplot of PTU-stock



Outliers are < Median-3*IQD OR > Median+3*IQD

Boundary for outliers are values < 745.563 and > 910.798

Day Rep High Value

10 1 966.309

Day Rep Low Value

24 1 742.626

Do you want to remove any outliers? (yes OR no)
n

- Transform data to natural logarithm and conduct regression analysis.

Day	Rep	Ln(Concentration)
0	1	6.7150
0	2	6.7388
0	3	6.6645
3	1	6.7066
3	2	6.6914
3	3	6.7655
5	1	6.6965
5	2	6.7267
5	3	6.7400
10	1	6.8735
10	2	6.7059
10	3	6.7081
12	1	6.7257
12	2	6.7476
12	3	6.7266
17	1	6.7438
17	2	6.7469
17	3	6.7278
19	1	6.6948
19	2	6.6865
19	3	6.7074
21	1	6.7192
21	2	6.7306
21	3	6.7391
24	1	6.6102
24	2	6.7102
24	3	6.7171

Conducts Simple Linear Regression

Regression Analysis: PTU-stock versus Day

The regression equation is
PTU-stock = 6.73 - 0.00071 Day

Predictor	Coef	SE Coef	T	P	
Constant	6.72975	0.01538	437.69	0.000	
Day	-0.000713	0.001046	-0.68	0.501	NS

S = 0.04348 R-Sq = 1.8% R-Sq(adj) = 0.0%

Analysis of Variance

Source	DF	SS	MS	F	P	
Regression	1	0.000880	0.000880	0.47	0.501	
Residual Error	25	0.047257	0.001890			
Lack of Fit	7	0.013754	0.001965	1.06	0.429	NS
Pure Error	18	0.033504	0.001861			
Total	26	0.048137				

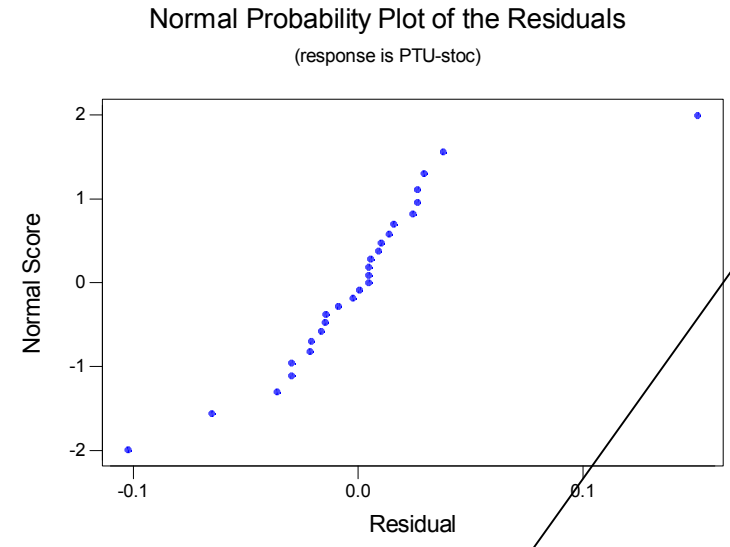
Unusual Observations

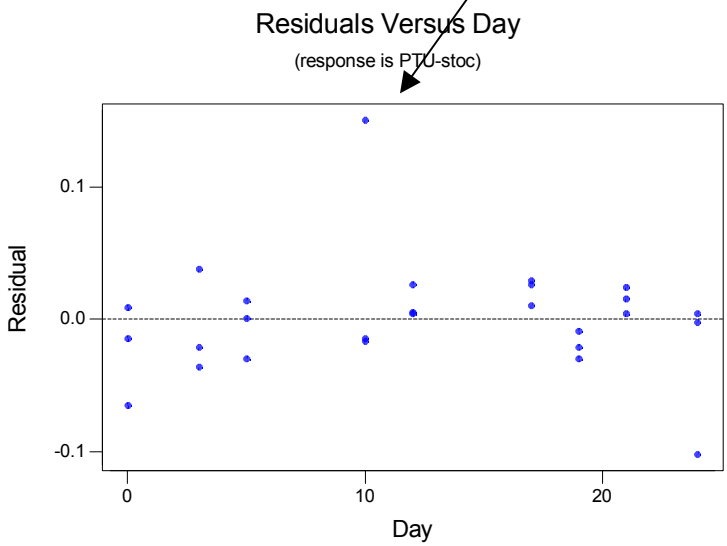
Obs	Day	PTU-stoc	Fit	SE Fit	Residual	St Resid
10	10.0	6.87348	6.72262	0.00872	0.15087	3.54R
25	24.0	6.61019	6.71263	0.01480	-0.10244	-2.51R

R denotes an observation with a large standardized residual

Normplot of Residuals for PTU-stoc

Could remove this data point.





Do you want to remove any data points? (yes OR no)
n

Should a quadratic be fit? (yes OR no)
n

Power analysis for t-test of slope less than zero

Power and Sample Size

1-Sample t Test

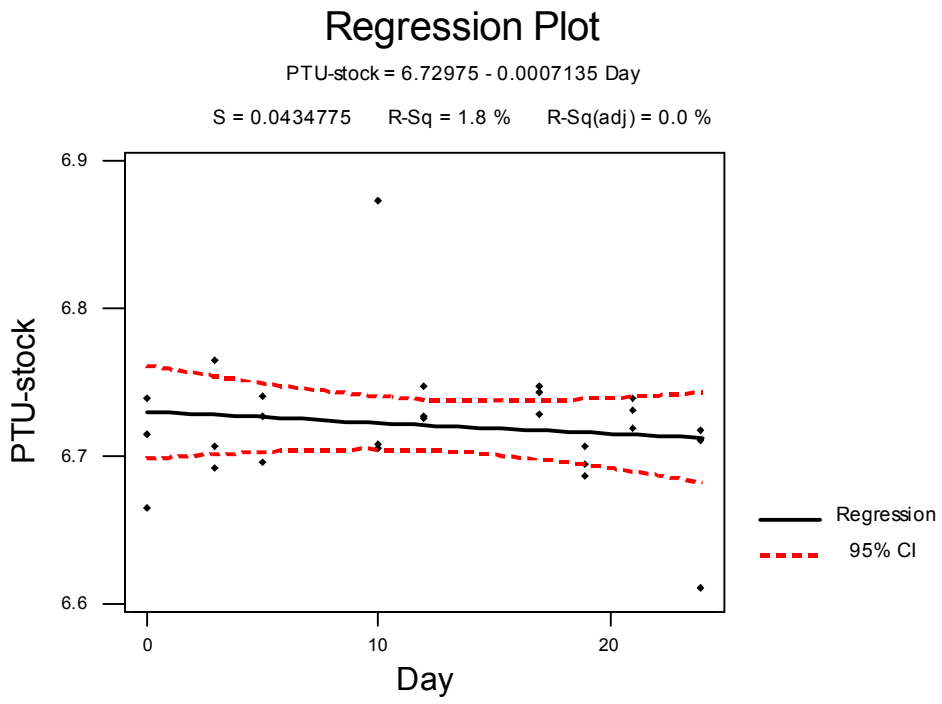
Testing mean = null (versus < null)
Calculating power for mean = null + difference
Alpha = 0.05 Sigma = 0.0434775

Sample
 Size Power Difference
   25 0.9900   -0.0356

- That means we would detect a mean of 6.6713 as significantly less than ln(818) = 6.7069 or a change of 789 from 818 = 3.5% loss.

Fit 95% confidence bands about the fitted simple linear model

Fitted Line Plot: PTU-stock versus Day



- **Conclusion – stable for 3 weeks.**

Analysis of PTU – 1.6 mg/L Dilution Stability Study

Test to determine if the data are from a population with mean of 1.6 mg/L.

Macro performs a one-sample t-test for mu less than TARGET &
What is the target value for X 4
DATA> 1.6

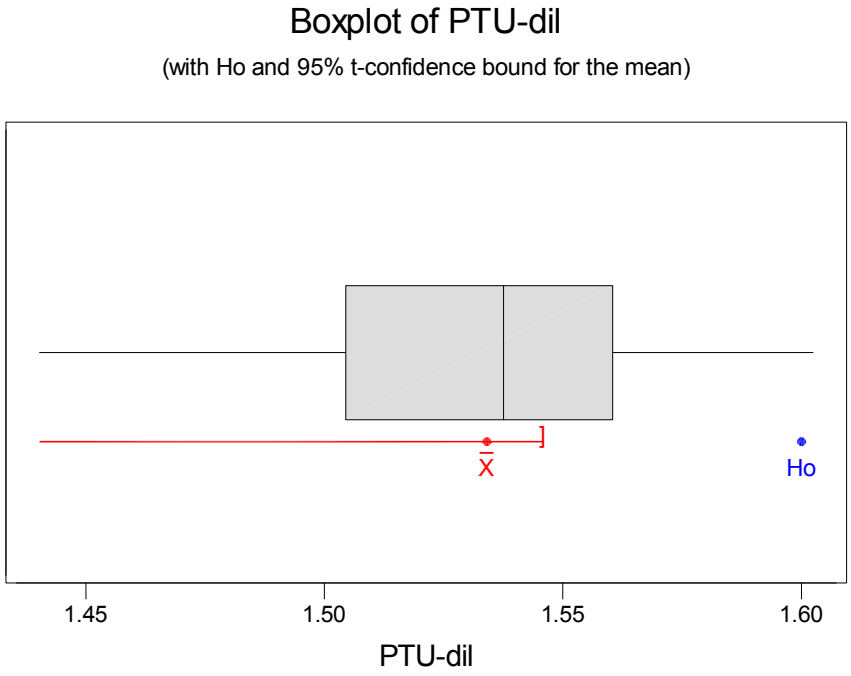
One-Sample T: PTU-dil

Test of mu = 1.6 vs mu < 1.6

Variable	N	Mean	StDev	SE Mean
PTU-dil	27	1.53410	0.03635	0.00700

Variable	95.0% Upper Bound	T	P
PTU-dil	1.54603	-9.42	0.000

t Boxplot of PTU-dil



Outliers are < Median-3*IQD OR > Median+3*IQD
Boundary for outliers are values < 1.3695 and > 1.7055
No outliers

- Transform data to natural logarithm and conduct regression analysis.

Day	Rep	Ln(Concentration)
0	1	0.4525
0	2	0.4653
0	3	0.4363
3	1	0.4319
3	2	0.4449
3	3	0.4306
5	1	0.4221
5	2	0.4506
5	3	0.4301
10	1	0.4074
10	2	0.4359
10	3	0.3649
12	1	0.4084
12	2	0.4049
12	3	0.4033
17	1	0.4626
17	2	0.4471
17	3	0.4227
19	1	0.4245
19	2	0.4417
19	3	0.4302
21	1	0.4107
21	2	0.4346
21	3	0.4085
24	1	0.4045
24	2	0.3993
24	3	0.4715

Conducts Simple Linear Regression

The regression equation is
PTU-dil = 0.435 -0.000623 Day

Predictor	Coef	SE Coef	T	P	
Constant	0.435350	0.008380	51.95	0.000	
Day	-0.0006225	0.0005700	-1.09	0.285	NS

S = 0.02370 R-Sq = 4.6% R-Sq(adj) = 0.7%

Analysis of Variance

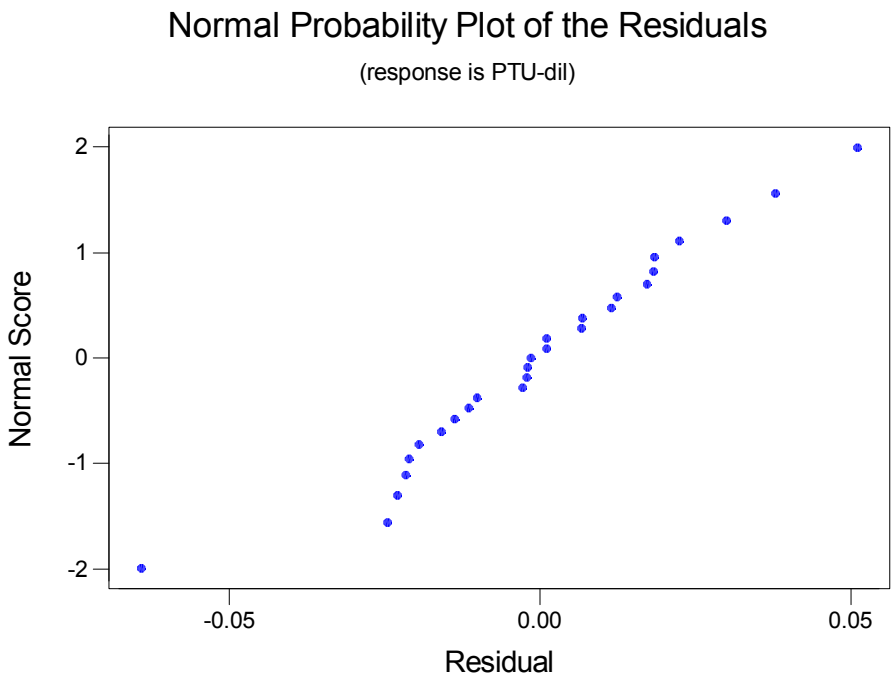
Source	DF	SS	MS	F	P	
Regression	1	0.0006696	0.0006696	1.19	0.285	
Residual Error	25	0.0140363	0.0005615			
Lack of Fit	7	0.0058602	0.0008372	1.84	0.140	NS
Pure Error	18	0.0081761	0.0004542			
Total	26	0.0147060				

Unusual Observations

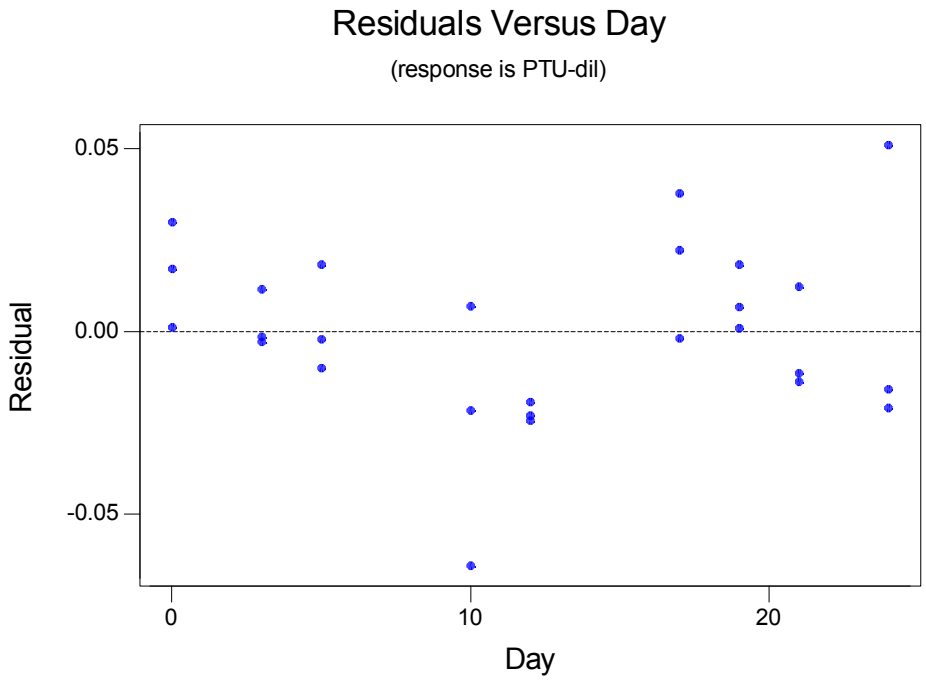
Obs	Day	PTU-dil	Fit	SE Fit	Residual	St Resid
12	10.0	0.36485	0.42912	0.00475	-0.06427	-2.77R
27	24.0	0.47150	0.42041	0.00806	0.05109	2.29R

R denotes an observation with a large standardized residual

Normplot of Residuals for PTU-dil



Residuals from PTU-dil vs Day



Break to allow evaluation of lack-of-fit and residuals
* Entering PAUSE mode. Type RESUME or R to leave PAUSE.
%MTB > r

Do you want to remove any data points? (yes OR no)
n
Should a quadratic be fit? (yes OR no)
n
Power analysis for t-test of slope less than zero

Power and Sample Size

1-Sample t Test

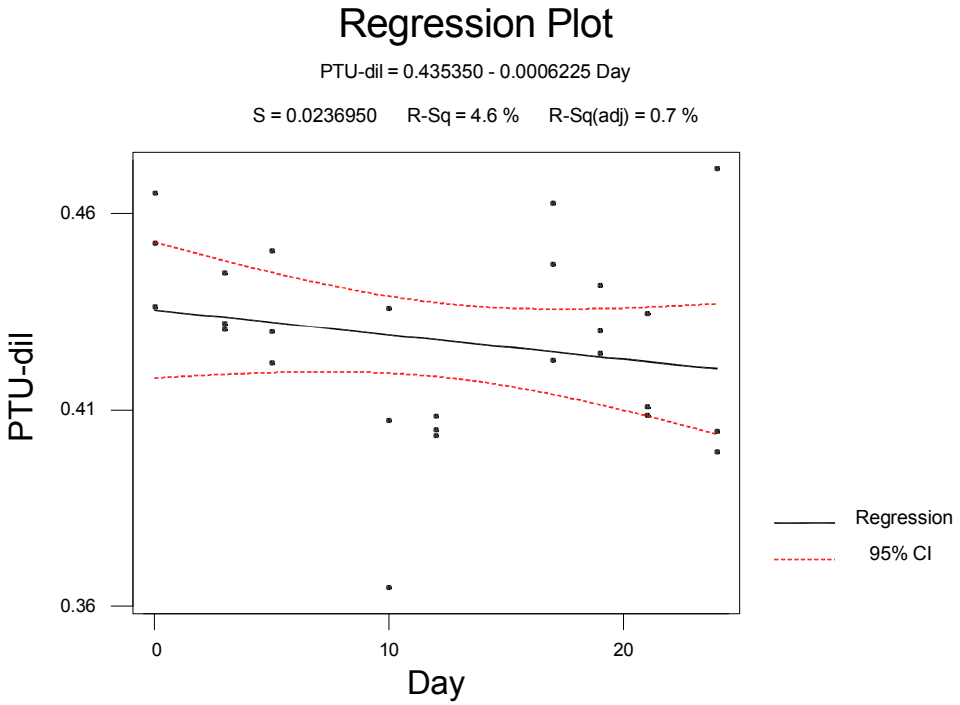
Testing mean = null (versus < null)
Calculating power for mean = null + difference
Alpha = 0.05 Sigma = 0.0236950

Sample Size	Power	Difference
25	0.9900	-0.0194

- That means we would detect a mean of 0.451 as significantly less than ln(1.6) = 0.47 or a change of 1.57 from 1.6 = 1.9% loss.

Fit 95% confidence bands about the fitted simple linear model

Fitted Line Plot: PTU-dil versus Day



- **Conclusion – stable for 3 weeks.**

6-n-Propyl-Thiouracil (PTU)
Test Chemical Analytical Data from Battelle:
In-Life Test Chemical Analysis Report

COPY

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE. USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-8771 Fax 405-377-3388
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	John Hay Fu
P.O. #:	

Lab Use Only
Batch #
BATT02-00051

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only Temp	pH
002	Deck Tap H2O	3/5/04 1600	1	G	Wet Ice	PT 4		
003	2.5 m/L PT 4	↓	1	↓	↓	↓		
004	5.0 m/L PT 4		1					
005	10.0 m/L PT 4		1					
006	20.0 m/L PT 4		1					
007	700 m/L PT 4	↓	2	↓	↓	↓		

SPECIAL INSTRUCTIONS: Please send back Ice Chest

DATE:
INITIALS:

			Date/Time
John Hay Fu	3/5/04 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

COPY

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE. USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road

Stillwater, OK 74074

405-624-6771 Fax 405-377-3388

mail@fortlabs.com

Client:	Ba Helk
Submit Report To:	Doug Fort
Sampler's Signature:	John Hayes
P.O. #	

Lab Use Only
Client Code / P.O. #
BATT02 - 00051

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only: pH	Temp	pH
008	Deck Tap H_2O	3/12/04 1600	1	G	Blue Ice	PTH			
009	2.5 mg/L PTH	↓	1	↓	↓	↓			
010	5.0 mg/L PTH		1						
011	10.0 mg/L PTH		1						
012	20.0 mg/L PTH		1						
013	700 mg/L PTH	↓	2	↓	↓	↓			

SPECIAL INSTRUCTIONS: Please send back Ice chest

DATE:
INITIALS:

Relinquished By	Date-Time	Received By	Date-Time
John Hayes	3/15/04 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

COPY

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	BsHelk
Submit Report To:	Doug Fort
Sampler's Signature:	
P.O. #:	

FOR LAB ONLY
Lab Number: 4064
BATTQ2-00051

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only ID	Temp	pH
014	DeCh Tap H_2O	3/19/04 1000	1	G	Blue Ice	PTU			
015	2.5 m/L PTU		1						
016	5.0 m/L PTU		1						
017	10.0 m/L PTU		1						
018	20.0 m/L PTU		1						
019	700 m/L PTU	↓ ↓	2	↓	↓	↓			

SPECIAL INSTRUCTIONS: Please return Ice Chest

DATE:
INITIALS:

Relinquished By	Date/Time	Received By	Date/Time
John Kelly	3/23/04 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

COPY

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE. USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	John Wenz Thomas
P O #:	

BATQ2-00051

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
020	DeCl2 Tap H2O	3/26/04 1600	1	G	Blue Ice	PTL
021	PTL 2.5 ml/L		1			
022	PTL 5.0 ml/L		1			
023	PTL 10.0 ml/L		1			
024	PTL 200 ml/L		1			
025	PTL 700 ml/L		2			

SPECIAL INSTRUCTIONS: Please return Ice Chest.

DATE:
INITIALS:

John Wenz Thomas	3/29/04 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

6-n-Propyl-Thiouracil (PTU)
Thyroid Histology Data from EPL:
Sample Identification Key
Thyroid Pathology Report

EPL Sample Key for 6-n-Propyl-2-Thiouracil (PTU)

FEL Sample Number	Conc. Set Number	Individual Specimen Number[1]	Conc. Replicate Number	Initial Specimen Stage	PTU Conc. (mg/L)
026	1	041, 044, 051, 053, 057 067, 072, 073, 075, 076	A B	51	2.5
027	2	168, 169, 170, 172, 178 181, 194, 195, 196, 199	A B	51	20.0
028	3	086, 087, 089, 095, 099 105, 111, 116, 117, 118	A B	51	5.0
029	4	002, 003, 006, 007, 009 023, 024, 026, 027, 038	A B	51	0.0
030	5	122, 125, 129, 132, 137 145, 146, 149, 156, 157	A B	51	10.0
031	6	240, 252, 253, 254, 257 264, 267, 272, 277, 278	C D	54	2.5
032	7	362, 364, 368, 373, 377 382, 385, 386, 387, 389	C D	54	20.0
033	8	321, 322, 325, 332, 337 341, 343, 344, 347, 349	C D	54	10.0
034	9	207, 209, 214, 216, 219 221, 222, 223, 227, 232	C D	54	0.0
035	10	281, 283, 293, 294, 295 303, 305, 309, 310, 313	C D	54	5.0

[1] Individual specimens for thyroid histology were randomly selected using *Principles and Procedures of Statistics, A Biometrical Approach*, 2nd Ed., by Robert G.D. Steel and James H. Torrie, McGraw-Hill Book Co., USA, 1980.

EPL®

EXPERIMENTAL PATHOLOGY LABORATORIES, INC.
P.O. BOX 474 HERNDON, VIRGINIA 20172-0474 (703) 471-7060 Fax: (703) 471-8447

June 10, 2004

Douglas J. Fort, Ph.D.
Fort Environmental Laboratories, Inc.
1414 South Sangre Road
Stillwater, OK 74074

Dear Dr. Fort:

Enclosed is a copy of the draft pathology report for Battelle, Study Number BATT02-00051, "Amphibian Metamorphosis Assay Demonstration Study," (EPL Project Number 237-008).

Upon approval, a final pathology report will be issued.

Sincerely,

Marilyn J. Wolfe

MARILYN J. WOLFE, DVM, PhD, Diplomate, ACVP
Veterinary Pathologist

MJW/cb
Enclosure

EPL®

FINAL DRAFT

EXPERIMENTAL PATHOLOGY LABORATORIES, INC.

BATTELLE
STUDY NUMBER BATT02-00051
EPL PROJECT NUMBER 237-008

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

PATHOLOGY REPORT

Submitted by:

Experimental Pathology Laboratories, Inc.
P.O. Box 474
Herndon, VA 20172-0474
(703) 471-7060

Submitted to:

Battelle
Columbus, OH 43201

June 10, 2004

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PATHOLOGY SUMMARY

BATTELLE
STUDY NUMBER BATT02-00051
EPL PROJECT NUMBER 237-008

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

PATHOLOGY SUMMARY

A histomorphologic evaluation was performed on the thyroid glands of *Xenopus laevis* (South African clawed frog) that were exposed to various concentrations of 6-n-propylthiouracil (PTU) to determine the differential sensitivity of exposing Stage 51 and Stage 54 larvae and to establish the necessary duration of exposure. Stage 51 larval frogs were exposed to PTU for 21 days and Stage 54 larval frogs were exposed to PTU for 14 days. The experimental designs were as follows:

Stage 51 Frog Larvae Exposed for 21 Days

Group/Replicate	PTU Conc. (mg/L)	No. of Frogs/Group	Individual Frog Numbers
029A	0.0	5	002, 003, 006, 007, 009
029B	0.0	5	023, 024, 026, 027, 038
026A	2.5	5	041, 044, 051, 053, 057
026B	2.5	5	067, 072, 073, 075, 076
028A	5.0	5	086, 087, 089, 095, 099
028B	5.0	5	105, 111, 116, 117, 118
030A	10.0	5	122, 125, 129, 132, 137
030B	10.0	5	145, 146, 149, 156, 157
027A	20.0	5	168, 169, 170, 172, 178
027B	20.0	5	181, 194, 195, 196, 199

Stage 54 Frog Larvae Exposed for 14 Days

Group/Replicate	PTU Conc. (mg/L)	No. of Frogs/Group	Individual Frog Numbers
034C	0.0	5	207, 209, 214, 216, 219
034D	0.0	5	221, 222, 223, 227, 232
031C	2.5	5	240, 252, 253, 254, 257
031D	2.5	5	264, 267, 272, 277, 278
035C	5.0	5	281, 283, 293, 294, 295
035D	5.0	5	303, 305, 309, 310, 313
033C	10.0	5	321, 322, 325, 332, 337
033D	10.0	5	341, 343, 344, 347, 349
032C	20.0	5	362, 364, 368, 373, 377
032D	20.0	5	382, 385, 386, 387, 389

MATERIALS AND METHODS

Slides were prepared by Experimental Laboratories, Inc. (EPL®). Head samples were trimmed from the frog larvae and placed in cassettes with the cut side down so that the heads were sectioned in a transverse plane from caudal to rostral. Three step sections (32 microns apart) were taken from each block at the level of the thyroid glands and were mounted on one slide. The slides were examined by a technician to assure that a sufficient amount of thyroid tissue was present bilaterally in the sections. If necessary to meet these criteria, additional step sections were cut from the block. The slides were stained with hematoxylin and eosin.

Microscopic findings for thyroid glands from each frog are listed in the Histopathology Incidence Tables. The changes are graded from 1 to 5 depending upon severity (1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high). Non-gradable changes are designated

as present (P). All lesions are summarized by group on the Summary Incidence Tables. The sexes of the frogs were not reported.

RESULTS

Stage 51 *Xenopus laevis* exposed to PTU for 21 days

There were exposure-related changes in the thyroid gland which included follicular cell hyperplasia and diffuse enlargement of the thyroid gland. At the two highest exposure concentrations (10.0 and 20.0 mg/L), the colloid in some frogs had a variable appearance among the follicles. In some follicles the colloid was homogeneous and in others the colloid had a foamy appearance. Several frogs at 20.0 mg/L PTU had colloid with only a foamy appearance. Colloid in the thyroid follicles was noticeably decreased in two frogs exposed to 20.0 mg/L PTU. Hyperplasia of the follicular cells occurred in the majority of the frogs at the two highest exposure concentrations (10.0 mg/L and 20.0 mg/L) of PTU. The severity of follicular cell hyperplasia varied from minimal to severe/high at 10.0 mg/L PTU and from mild to severe/high at 20.0 mg/L PTU. One frog each at 2.5 mg/L and 5.0 mg/L PTU had follicular cell hyperplasia (mild and minimal in severity respectively). Diffuse enlargement of the thyroid glands occurred most frequently at the three highest exposure concentrations of PTU. The severity of enlargement was minimal to mild at 5.0 mg/L PTU, minimal to severe/high at 10.0 mg/L, and mild to severe/high at 20.0 mg/L. See Table 1 for a tabulation of the prevalence of selected changes of the thyroid glands described above in Stage 51 frogs.

Table 1

Prevalence with Replicates Combined of Selected Changes in Thyroid Glands of Stage 51 Frogs After Exposure to Various Concentrations of PTU for 21 Days

Group	29 A & B	26 A & B	28 A & B	30 A & B	27 A & B
Exposure Conc.	0 mg/L	2.5 mg/L	5.0 mg/L	10.0 mg/L	20.0 mg/L
Thyroid (No. Examined)	(10)	(10)	(10)	(10)	(10)
Colloid, Decreased	0	0	0	0	2
Enlargement, Diffuse	0	1	7	7	6
Hyperplasia, Follicular Cells	0	1	1	6	10
Interfollicular Pigment, Bilateral	7	7	9	6	8

Interfollicular pigment was present bilaterally in the majority of the thyroid glands at all exposure concentrations and varied from minimal to moderate in severity. There was no relationship of the prevalence or severity of this change to exposure concentrations of PTU.

See Appendix A for photographs of thyroid glands from the Stage 51 frogs. One or two frogs from each of the exposure concentrations are illustrated by both a low (1.25x) and high (10x) magnification photograph. The photographs are annotated to illustrate various features of thyroid glands and changes that resulted from exposure to PTU. Frog number 51-170 (20.0 mg/L PTU) and frog number 51-129 (10.0 mg/L PTU) each have one additional photograph taken at medium magnification (4x) to show the thyroid glands in their entirety. The

thyroid glands were so enlarged that only a portion of the glands could be photographed at 10x magnification.

RESULTS

Stage 54 *Xenopus laevis* exposed to PTU for 14 days

There were exposure-related changes in the thyroid gland which included follicular cell hyperplasia, distension of thyroid follicles, and diffuse enlargement of the thyroid glands. At the two highest exposure concentrations of PTU (10.0 mg/L and 20.0 mg/L) the colloid in most of the frogs had a light eosinophilic foamy appearance in contrast to a more homogeneous appearance of the colloid in frogs of the other exposure concentrations. Minimal follicular cell hyperplasia occurred in one frog at 2.5 mg/L exposure concentration. The severity of this change in nine of ten frogs at 5.0 mg/L was minimal to mild. At the two highest exposure concentrations follicular cell hyperplasia occurred in 100% of the frogs and the severity was usually moderate to moderately severe.

Distension of thyroid follicles and diffuse enlargement of the thyroid glands occurred most commonly in Stage 54 frogs exposed to 5.0, 10.0, and 20.0 mg/L PTU. Thyroid enlargement varied in severity from minimal to moderate in affected frogs from these groups with most of the frogs with moderate thyroid enlargement from groups exposed to the two highest concentrations of PTU. Distended thyroid follicles were usually minimal to mild in the affected frogs. Two frogs at the 10.0 mg/L exposure concentration had moderate distended follicles. See Table 2 for a tabulation of the prevalence of selected changes in the thyroid glands described above in Stage 54 frogs.

Table 2

Prevalence with Replicates Combined of Selected Changes in Thyroid Glands of Stage 54 Frogs After Exposure to Various Concentrations of PTU for 14 Days

Group	34 C & D	31 C & D	35 C & D	33 C & D	32 C & D
Exposure Conc.	0 mg/L	2.5 mg/L	5.0 mg/L	10.0 mg/L	20.0 mg/L
Thyroid (No. Examined)	(10)	(10)	(10)	(10)	(10)
Distension, Thyroid Follicles	0	2	6	8	6
Enlargement, Diffuse	0	0	4	9	5
Hyperplasia, Follicular Cells	0	1	9	10	10
Interfollicular Pigment, Bilateral	9	9	8	7	7

Interfollicular pigment was present unilaterally or bilaterally in the majority of thyroid glands at all exposure concentrations and varied in severity from minimal to moderate. There was no relationship of the prevalence or severity of this finding to the exposure concentration of PTU.

See Appendix B for photographs of thyroid glands from Stage 54 frogs. One frog from each of the exposure concentrations is illustrated by photographs taken at a low magnification (1.25x) and a high magnification (10x). The photographs are annotated to illustrate various features of thyroid glands and changes that resulted from exposure to various concentrations of PTU.

DISCUSSION

Propylthiouracil is a known inhibitor of thyroid hormone synthesis. It acts by inhibiting thyroid peroxidase and thus prevents the oxidation of inorganic iodide

(I) to molecular (reactive) iodine (I_2),[1] an essential step in the synthesis of thyroid hormone. A decrease in circulating thyroid hormone which would result from exposure to PTU is a signal for the pituitary gland to release thyrotropin, a hormone which stimulates the thyroid gland and results in hypertrophy and hyperplasia of follicular cells.[2]

Effects of PTU on the thyroid glands of the African clawed frogs were apparent both in frogs that were exposed at Stage 51 for 21 days and Stage 54 for 14 days. At the highest exposure concentration of PTU, all the frogs were affected with thyroid follicular cell hyperplasia irrespective of the developmental stage of the frog at the start of the test or the duration of the test. However, hyperplasia of follicular cells occurred with greater frequency among Stage 54 frogs exposed to 5.0 and 10.0 mg/L PTU than among Stage 51 frogs at the same exposure concentrations. The severity of the change was less (usually minimal) among both Stage 51 and Stage 54 frogs at the 5.0 mg/L exposure concentration than at the two high concentrations of PTU.

There was a difference in appearance of the colloid in frogs exposed to the two highest concentrations of PTU as compared to the colloid in frogs exposed to PTU concentrations lower than 10.0 mg/L. The colloid at the two highest concentrations of PTU had a light eosinophilic foamy appearance as compared to colloid with a compact, homogeneous appearance as occurred in frogs at exposure concentrations lower than 10.0 mg/L. Foamy colloid was the dominant colloid among Stage 54 frogs at the two highest exposure concentrations. In Stage 51 frogs, thyroid follicles with foamy colloid occurred less frequently and were often present along with follicles containing homogeneous colloid in the same thyroid gland. Colloid contains thyroglobulin, a high molecular weight glycoprotein. Upon proteolysis, active thyroid hormone is released from thyroglobulin. The foamy appearance of the colloid in thyroid glands that are

inhibited in hormone production by a high concentration of PTU may reflect the loss of thyroid hormone as it is being utilized but not replaced. It is not known why the foamy colloid was present more commonly in Stage 54 frogs than in Stage 51 frogs.

CONCLUSIONS

Exposure of *Xenopus laevis* to PTU resulted in changes in the thyroid glands that included follicular cell hyperplasia, diffuse enlargement of the thyroid glands, distension of thyroid follicles, and colloid with a light eosinophilic foamy appearance. Although most of these changes were most common in both Stage 51 frogs and Stage 54 frogs at the two highest exposure concentrations, one or more of these changes occurred also at the 2.5 and 5.0 mg/L exposure concentrations among frogs of both stages. Follicular cell hyperplasia was most common and most severe at the 10.0 and 20.0 mg/L exposure concentrations in both Stage 51 frogs exposed to PTU for 21 days and Stage 54 frogs exposed to PTU for 14 days. At the 5.0 mg/L exposure concentration, the prevalence of follicular cell hyperplasia was greater among the Stage 54 frogs than the Stage 51 frogs although the severity usually was minimal. Under the conditions of this test, it appears that the Stage 54 frogs might be slightly more sensitive to the effects of PTU with regard to follicular cell hyperplasia.

MARILYN J. WOLFE, DVM, PhD, Diplomate, ACVP — Date
Veterinary Pathologist

MJW/cb

REFERENCES

[1] Capen, C. C., DeLellis, R. A., and Yarrington, J. T. (1991). Endocrine system. *In* "Handbook of Toxicologic Pathology," (W.M. Haschek and C.G. Rousseaux, eds.), pp. 718-719. Academic Press, San Diego.

[2] Capen, C. C. (1991). The Endocrine Glands. *In* "Pathology of Domestic Animals," (K. V. F. Jubb, P. C. Kennedy, and N. Palmer, eds.), pp. 315. Academic Press, San Diego.

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Amphibian Metamorphosis Assay Demonstration Study

Client Study: BATT02-00051 EPL Project Coordinator: Marilyn J. Wolfe

EPL Project Number: 237-008 EPL Pathologist: Marilyn J. Wolfe

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 Project Coordinator and Management are indicated below.

	Dates	
Area Inspected	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management _____

Date of last quarterly facility inspection 5/04

_____ _____

EPL Quality Assurance Unit Date

SUMMARY INCIDENCE TABLES

21 Days – Stage 51

SUMMARY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

	GROUP 029A	GROUP 029B	GROUP 026A	GROUP 026B	GROUP 028A	GROUP 028B
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Colloid, Decreased						
Colloid, Light Eosinophilic						
Homogeneous	5	5	5	5	5	5
Colloid, Light Eosinophilic,						
Foamy						
Distension, Thyroid Follicles					1	
Enlargement, Diffuse			1		5	2
Hyperplasia, Follicular Cells			1		1	
Interfollicular Pigment,						
Bilateral	4	3	4	3	5	4
Interfollicular Pigment,						
Unilateral						

SUMMARY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

	GROUP 030A	GROUP 030B	GROUP 027A	GROUP 027B		
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)		
Colloid, Decreased			2			
Colloid, Light Eosinophilic Homogeneous	5	5	4	2		
Colloid, Light Eosinophilic, Foamy	1	1	2	5		
Distension, Thyroid Follicles			1			
Enlargement, Diffuse	4	3	4	2		
Hyperplasia, Follicular Cells	3	3	5	5		
Interfollicular Pigment, Bilateral	3	3	4	4		
Interfollicular Pigment, Unilateral						

HISTOPATHOLOGY INCIDENCE TABLES

21 Days – Stage 51

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

	GROUP 029A						GROUP 029B						GROUP 026A							
ANIMAL	51-002	51-003	51-006	51-007	51-009		51-023	51-024	51-026	51-027	51-038		51-041	51-044	51-051	51-053	51-057			
THYROID																				
Colloid, Decreased																				
Colloid, Light Eosinophilic																				
Homogeneous	P	P	P	P	P		P	P	P	P	P		P	P	P	P	P			
Colloid, Light Eosinophilic,																				
Foamy																				
Distension, Thyroid Follicles																				
Enlargement, Diffuse																	2			
Hyperplasia, Follicular Cells																	2			
Interfollicular Pigment,																				
Bilateral	3	2	2		2		2	2			2		2		2	2	2			
Interfollicular Pigment,																				
Unilateral																				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

	GROUP 026B						GROUP 028A						GROUP 028B							
ANIMAL	51-067	51-072	51-073	51-075	51-076		51-086	51-087	51-089	51-095	51-099		51-105	51-111	51-116	51-117	51-118			
THYROID																				
Colloid, Decreased																				
Colloid, Light Eosinophilic																				
Homogeneous	P	P	P	P	P		P	P	P	P	P		P	P	P	P	P			
Colloid, Light Eosinophilic, Foamy																				
Distension, Thyroid Follicles								2												
Enlargement, Diffuse							2	1	2	2	2			1			2			
Hyperplasia, Follicular Cells								1												
Interfollicular Pigment, Bilateral	2		2		2		2	2	2	2	2		2	2	2	2				
Interfollicular Pigment, Unilateral																				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

	GROUP 030A						GROUP 030B						GROUP 027A						
ANIMAL	51-112	51-112	51-112	51-113	51-113		51-114	51-114	51-114	51-115	51-115		51-116	51-116	51-117	51-117	51-117		
THYROID																			
Colloid, Decreased														4			2		
Colloid, Light Eosinophilic																			
Homogeneous	P	P	P	P	P		P	P	P	P	P			P	P	P	P		
Colloid, Light Eosinophilic,																			
Foamy			P						P				P		P				
Distension, Thyroid Follicles																2			
Enlargement, Diffuse	2		5	2	2		1	2	4				4	4	5		2		
Hyperplasia, Follicular Cells		1	5		2		1	1	5				4	4	5	3	4		
Interfollicular Pigment,																			
Bilateral	3			2	2		3			2	2		1	2	1	2			
Interfollicular Pigment,																			
Unilateral																			

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

GROUP
027B

ANIMAL	51-181	51-194	51-195	51-196	51-199															
THYROID																				
Colloid, Decreased																				
Colloid, Light Eosinophilic																				
Homogeneous	P	P																		
Colloid, Light Eosinophilic,																				
Foamy	P	P	P	P	P															
Distension, Thyroid Follicles																				
Enlargement, Diffuse	4				2															
Hyperplasia, Follicular Cells	4	2	3	2	3															
Interfollicular Pigment,																				
Bilateral	2		2	2	1															
Interfollicular Pigment,																				
Unilateral																				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

SUMMARY INCIDENCE TABLES

14 Days – Stage 54

SUMMARY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

	GROUP 034C	GROUP 034D	GROUP 031C	GROUP 031D	GROUP 035C	GROUP 035D
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Colloid, Decreased						
Colloid, Light Eosinophilic Homogeneous	5	5	5	5	5	5
Colloid, Light Eosinophilic, Foamy						
Distension, Thyroid Follicles				2	2	4
Enlargement, Diffuse						4
Hyperplasia, Follicular Cells			1		4	5
Interfollicular Pigment, Bilateral	5	4	4	5	3	5
Interfollicular Pigment, Unilateral					1	

SUMMARY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

	GROUP 033C	GROUP 033D	GROUP 032C	GROUP 032D		
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)		
Colloid, Decreased						
Colloid, Light Eosinophilic						
Homogeneous		1				
Colloid, Light Eosinophilic,						
Foamy	5	4	5	5		
Distension, Thyroid Follicles	5	3	2	4		
Enlargement, Diffuse	4	5	2	3		
Hyperplasia, Follicular Cells	5	5	5	5		
Interfollicular Pigment,						
Bilateral	4	3	3	4		
Interfollicular Pigment,						
Unilateral	1					

HISTOPATHOLOGY INCIDENCE TABLES

14 Days – Stage 54

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

	GROUP 034C						GROUP 034D						GROUP 031C							
ANIMAL	54-2207	54-2209	54-2214	54-2216	54-2219		54-2321	54-2322	54-2323	54-2327	54-2332		54-2404	54-2452	54-2553	54-2554	54-2557			
THYROID																				
Colloid, Decreased																				
Colloid, Light Eosinophilic																				
Homogeneous	P	P	P	P	P		P	P	P	P	P		P	P	P	P	P			
Colloid, Light Eosinophilic,																				
Foamy																				
Distension, Thyroid Follicles																				
Enlargement, Diffuse																				
Hyperplasia, Follicular Cells														1						
Interfollicular Pigment,																				
Bilateral	2	2	2	2	2		3	2		3	2		2	2	2		2			
Interfollicular Pigment,																				
Unilateral																				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

	GROUP 031D						GROUP 035C						GROUP 035D							
ANIMAL	54-2264	54-2267	54-2272	54-2277	54-2278		54-2281	54-2283	54-2293	54-2294	54-2295		54-3003	54-3005	54-3009	54-3010	54-3013			
THYROID																				
Colloid, Decreased																				
Colloid, Light Eosinophilic																				
Homogeneous	P	P	P	P	P		P	P	P	P	P		P	P	P	P	P			
Colloid, Light Eosinophilic,																				
Foamy																				
Distension, Thyroid Follicles	1				2			1			1		2	1		2	1			
Enlargement, Diffuse													1	1		2	1			
Hyperplasia, Follicular Cells							1		1	2	2		1	1	1	1	1			
Interfollicular Pigment,																				
Bilateral	2	2	2	2	2			3	3		2		3	2	3	3	3			
Interfollicular Pigment,																				
Unilateral										2										

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

	GROUP 033C						GROUP 033D						GROUP 032C							
ANIMAL	54-321	54-322	54-325	54-323	54-337		54-341	54-343	54-344	54-347	54-349		54-362	54-364	54-368	54-373	54-377			
THYROID																				
Colloid, Decreased																				
Colloid, Light Eosinophilic																				
Homogeneous									P											
Colloid, Light Eosinophilic,																				
Foamy	P	P	P	P	P		P	P		P	P		P	P	P	P	P			
Distension, Thyroid Follicles	1	1	3	3	2			2		1	1		2	1						
Enlargement, Diffuse	3	1	2		1		2	3	1	3	3		2	2						
Hyperplasia, Follicular Cells	4	3	3	2	3		3	4	2	4	4		3	3	3	3	1			
Interfollicular Pigment,																				
Bilateral		2	2	2	2			3	3		2		1	2		2				
Interfollicular Pigment,																				
Unilateral	2																			

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00051
14 & 21 Day Sacrifices
Xenopus laevis

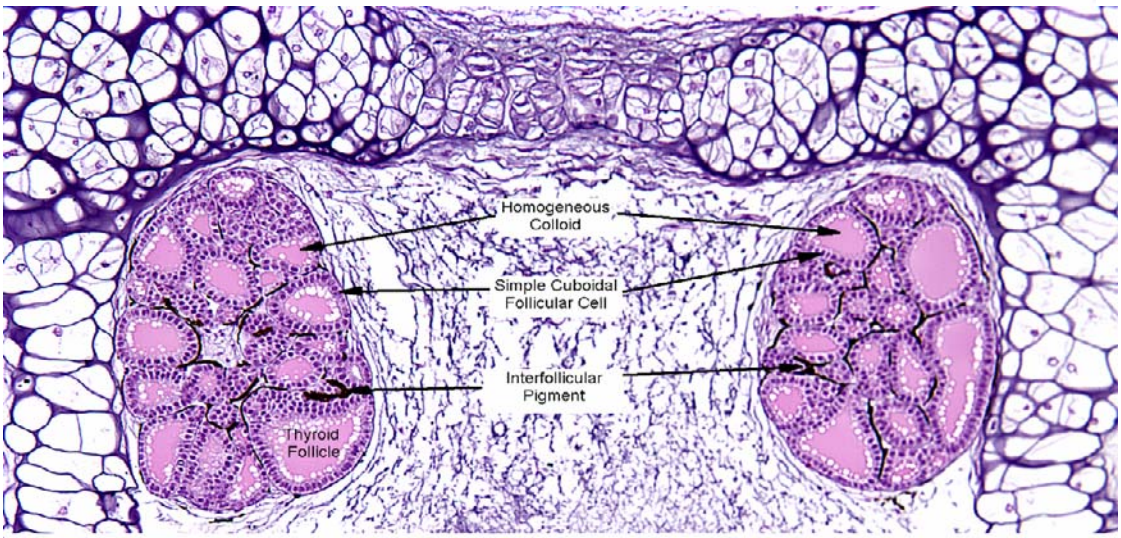
	GROUP 032D																			
ANIMAL	54-382	54-385	54-386	54-387	54-389															
THYROID																				
Colloid, Decreased																				
Colloid, Light Eosinophilic Homogeneous																				
Colloid, Light Eosinophilic, Foamy	P	P	P	P	P															
Distension, Thyroid Follicles	2	1	2		2															
Enlargement, Diffuse		2	2		3															
Hyperplasia, Follicular Cells	3	4	3	3	4															
Interfollicular Pigment, Bilateral		2	1	2	2															
Interfollicular Pigment, Unilateral																				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

APPENDIX A: PHOTOGRAPHS – STAGE 51 FROGS

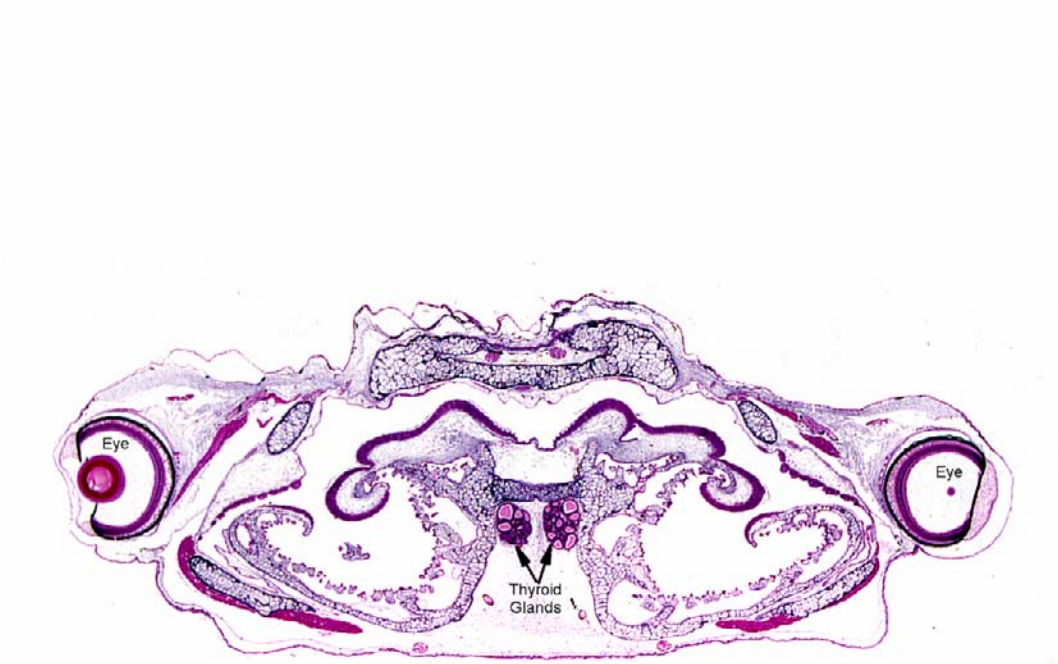


Z10438 *Xenopus laevis* 51-024 Group 029B
BATT02-00051 0 mg/L PTU H&E 1.25x

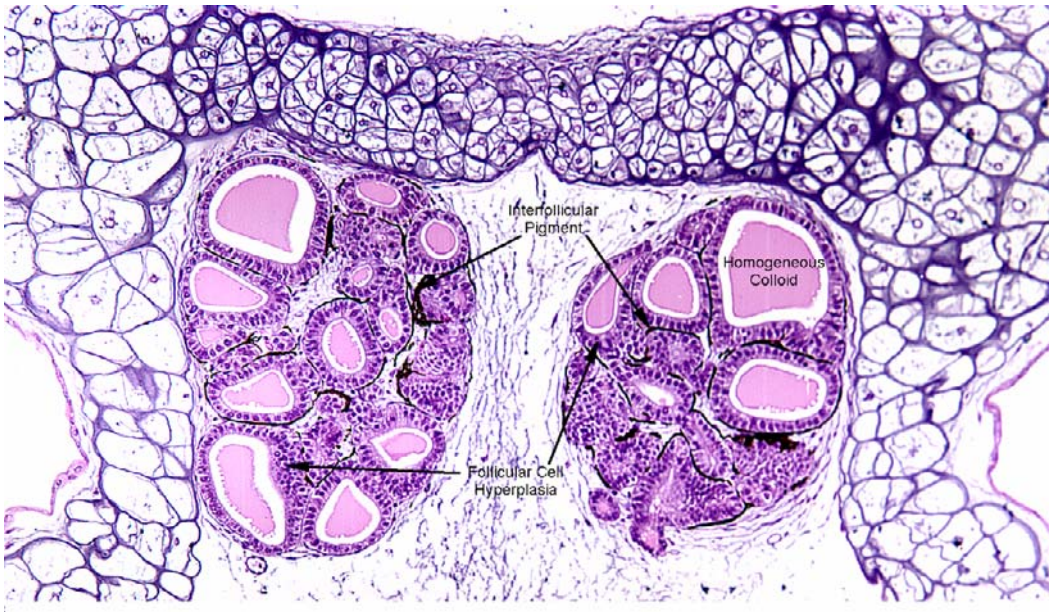


Z10463 *Xenopus laevis* 51-024 Group 029B
BATT02-00051 0 mg/L PTU H&E 10x

Z10438 and Z10463. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* control. The colloid is homogeneous and light eosinophilic and the follicles are lined by simple cuboidal epithelium.

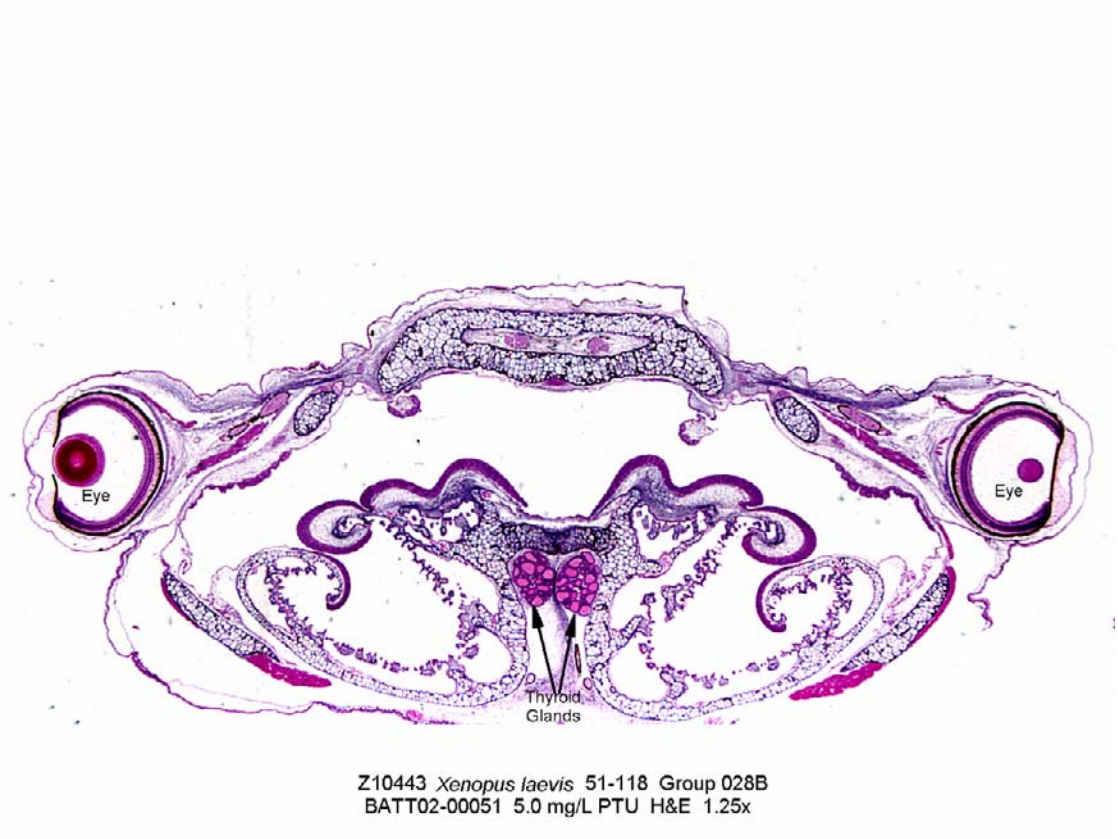


Z10442 *Xenopus laevis* 51-057 Group 026A
BATT02-00051 2.5 mg/L PTU H&E 1.25x

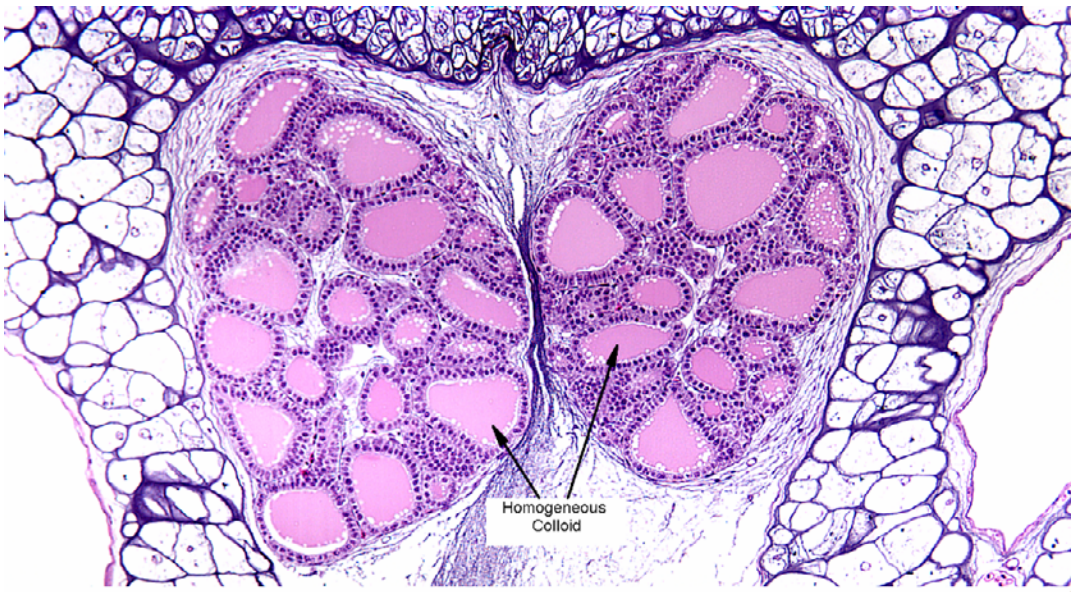


Z10452 *Xenopus laevis* 51-057 Group 026A
BATT02-00051 2.5 mg/L PTU H&E 10x

Z10442 and Z10452. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* exposed to 2.5 mg/L PTU for 21 days. There is mild follicular cell hyperplasia and mild diffuse enlargement of the thyroid glands.



Z10443 *Xenopus laevis* 51-118 Group 028B
BATT02-00051 5.0 mg/L PTU H&E 1.25x

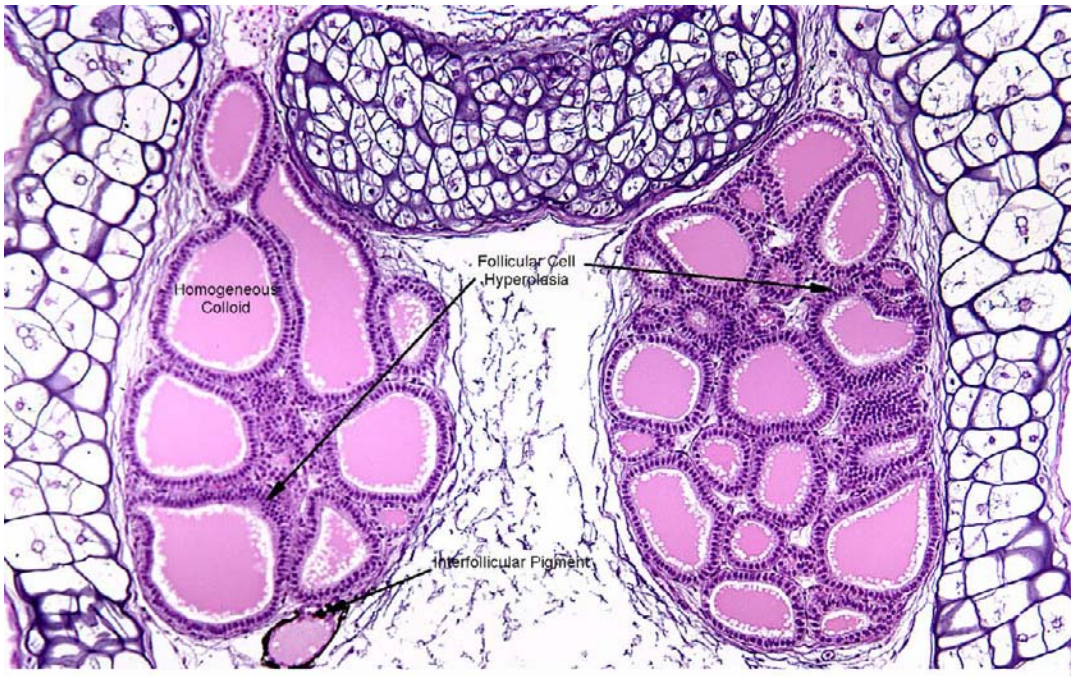


Z10453 *Xenopus laevis* 51-118 Group 028B
BATT02-00051 5.0 mg/L PTU H&E 10x

Z10443 and Z10453. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* exposed to 5.0 mg/L PTU for 21 days. There is mild diffuse enlargement of the thyroid gland. The colloid is homogeneous and there is no interfollicular pigment.

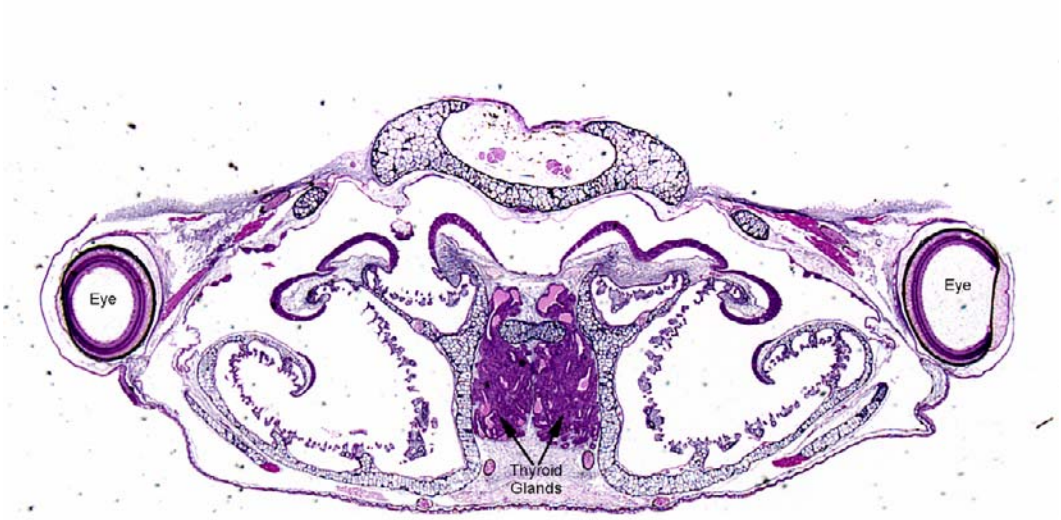


Z10445 *Xenopus laevis* 51-146 Group 030B
BATT02-00051 10.0 mg/L PTU H&E 1.25x



Z10455 *Xenopus laevis* 51-146 Group 030B
BATT02-00051 10.0 mg/L PTU H&E 10x

Z10445 and 10455. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* exposed to 10.0 mg/L PTU for 21 days. There is mild diffuse enlargement of the thyroid glands with minimal follicular cell hyperplasia. There is no interfollicular pigment.

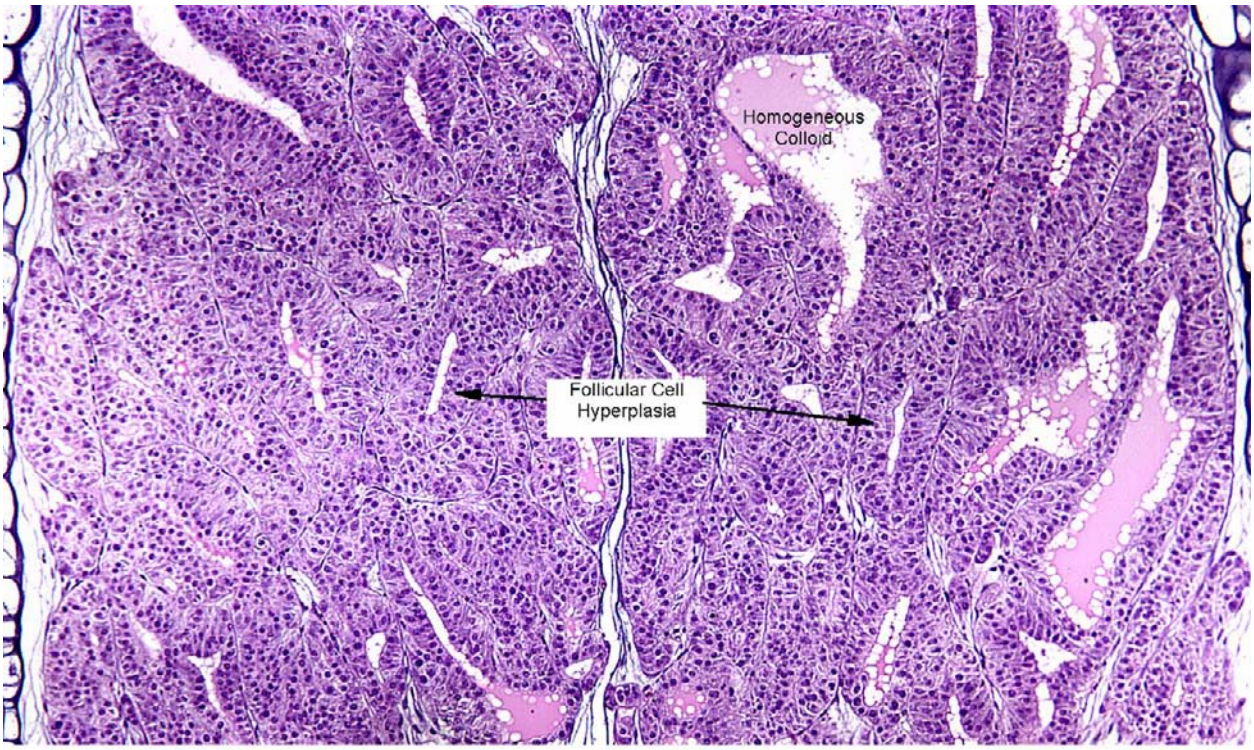


Z10444 *Xenopus laevis* 51-129 Group 030A
BATT02-00051 10.0 mg/L PTU H&E 1.25x



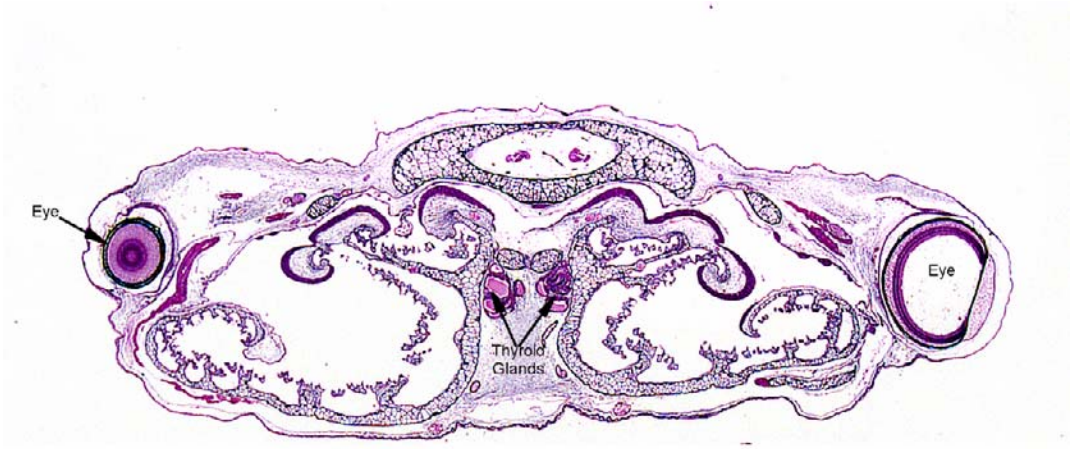
Z10464 *Xenopus laevis* 51-129 Group 030A
BATT02-00051 10.0 mg/L PTU H&E 4x

Z10444 and 10464. Low (1.25x) and medium (4x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* exposed to 10.0 mg/L PTU for 21 days. There is severe diffuse enlargement of the thyroid glands and severe follicular cell hyperplasia. Compare to frog No. 51-146 from the same exposure concentration (Z10445 and 10455).

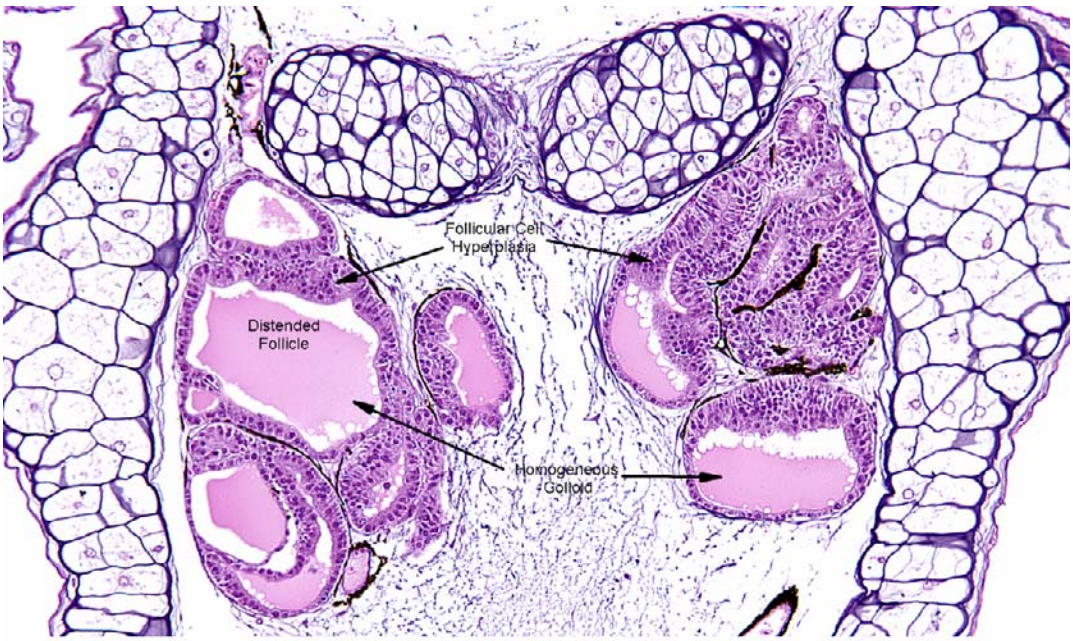


Z10454 *Xenopus laevis* 51-129 Group 030A
BATT02-00051 10.0 mg/L PTU H&E 10x

Z10454. High (10x) magnification photomicrograph of thyroid glands from Stage 51 *Xenopus laevis* exposed to 10.0 mg/L PTU for 21 days. There is severe follicular cell hyperplasia. This is a portion of the thyroid glands shown in their entirety in the previous two photographs (Z10444 and 10464).



Z10446 *Xenopus laevis* 51-172 Group 027A
BATT02-00051 20.0 mg/L PTU H&E 1.25x



Z10457 *Xenopus laevis* 51-172 Group 027A
BATT02-00051 20.0 mg/L PTU H&E 10x

Z10446 and Z10457. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* exposed to 20.0 mg/L PTU for 21 days. There is mild distension of follicles and moderate follicular cell hyperplasia. Compare to frog No. 51-170 in the following photographs that has severe follicular cell hyperplasia.

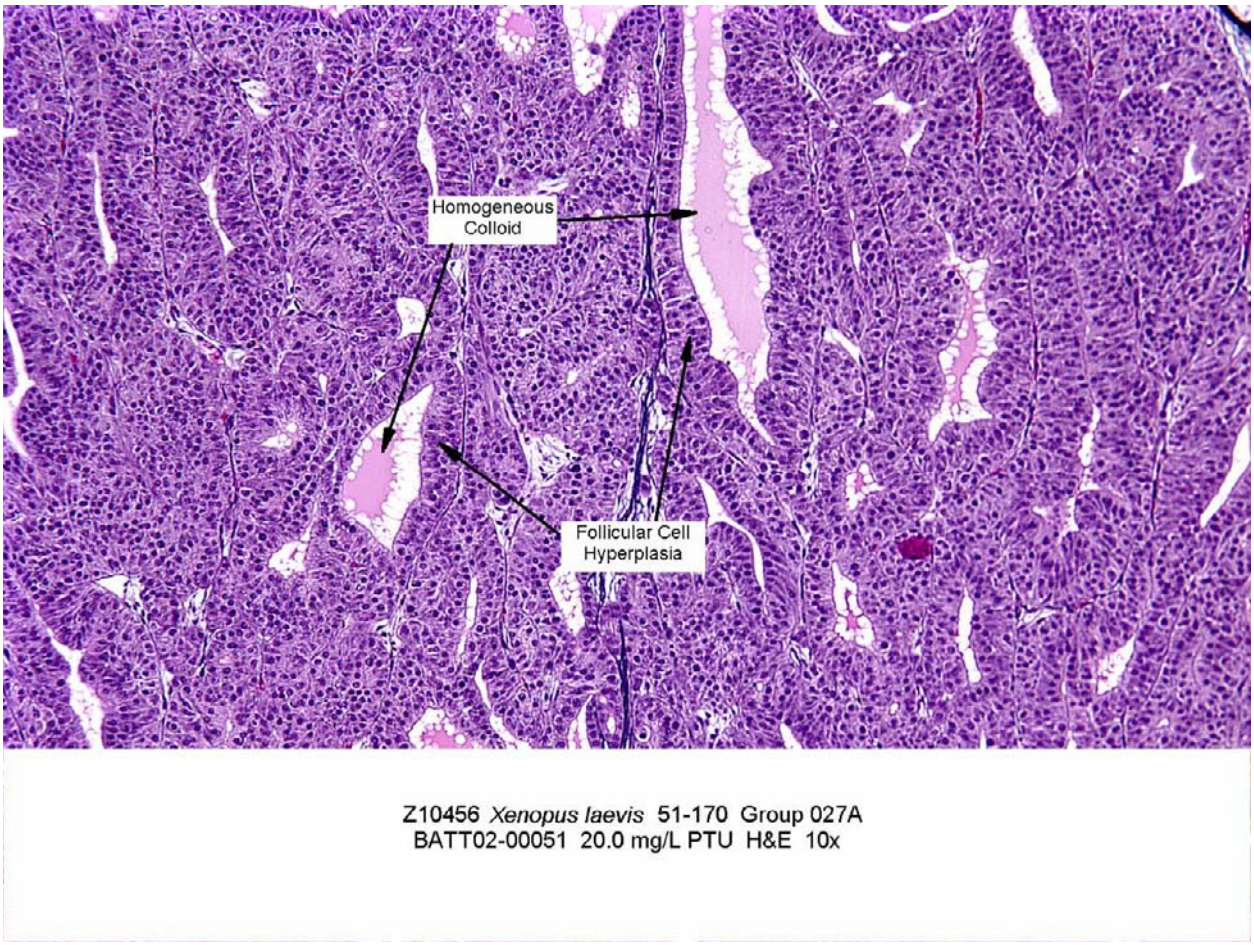


Z10439 *Xenopus laevis* 51-170 Group 027A
BATT02-00051 20.0 mg/L PTU H&E 1.25x



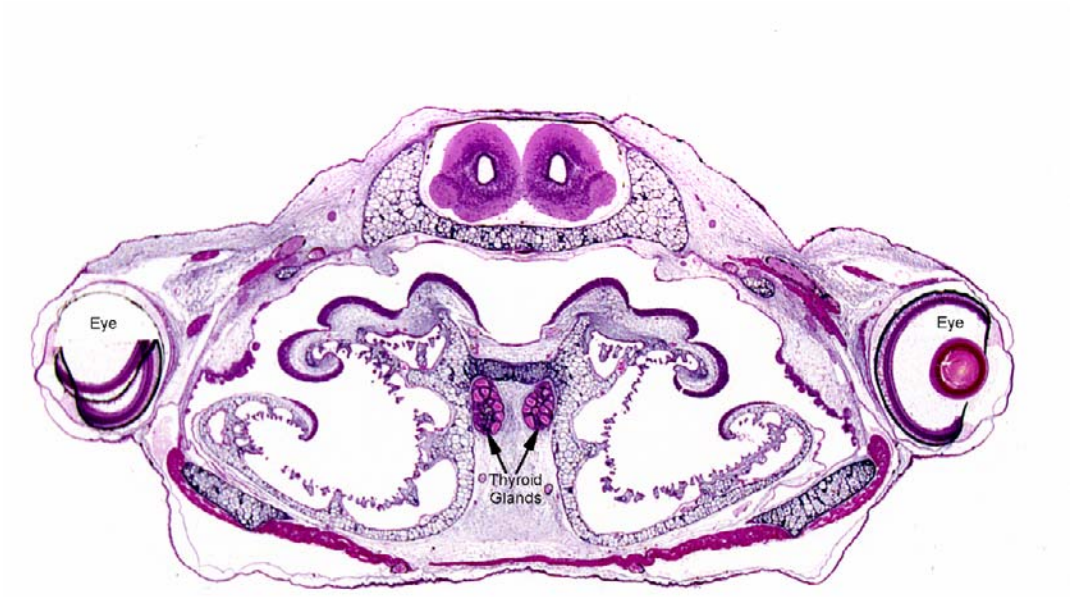
Z10440 *Xenopus laevis* 51-170 Group 027A
BATT02-00051 20.0 mg/L PTU H&E 4x

Z10439 and Z10440. Low (1.25x) and medium (4x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* exposed to 20.0 mg/L PTU for 21 days. There is severe diffuse enlargement of the thyroid glands and severe follicular cell hyperplasia.

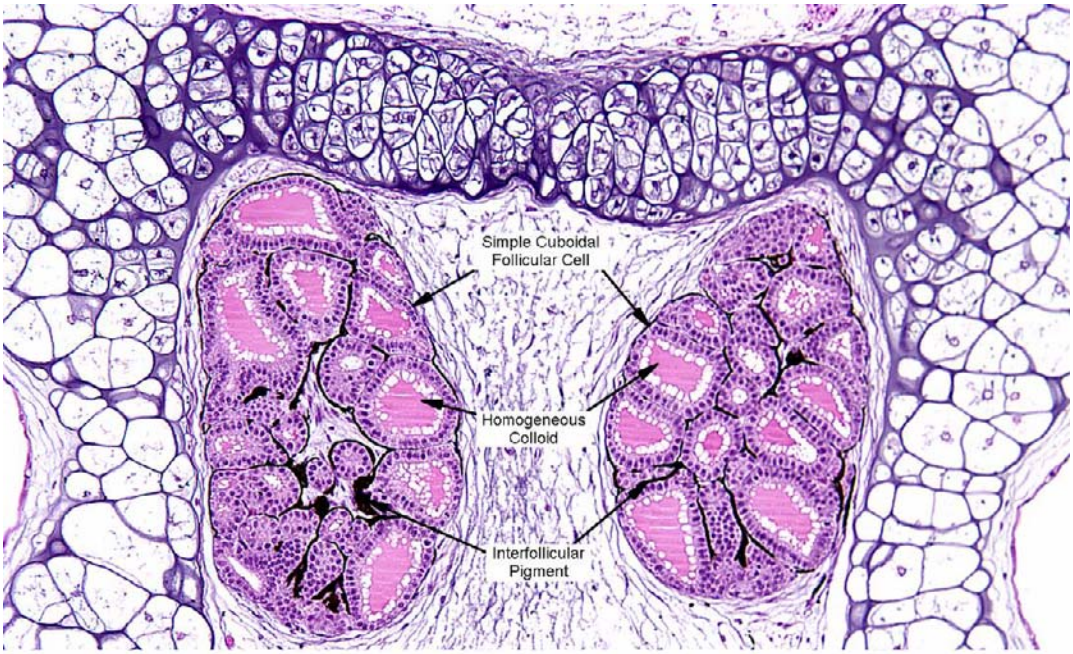


Z10456. High (10x) magnification photomicrograph of thyroid glands from Stage 51 *Xenopus laevis* exposed to 20.0 mg/L PTU for 21 days. There is severe follicular cell hyperplasia. This is a portion of the thyroid glands shown in their entirety in the previous two photographs (Z10439 and Z10440).

APPENDIX B: PHOTOGRAPHS – STAGE 54 FROGS



Z10447 *Xenopus laevis* 54-222 Group 034D
BATT02-00051 0 mg/L PTU H&E 1.25x

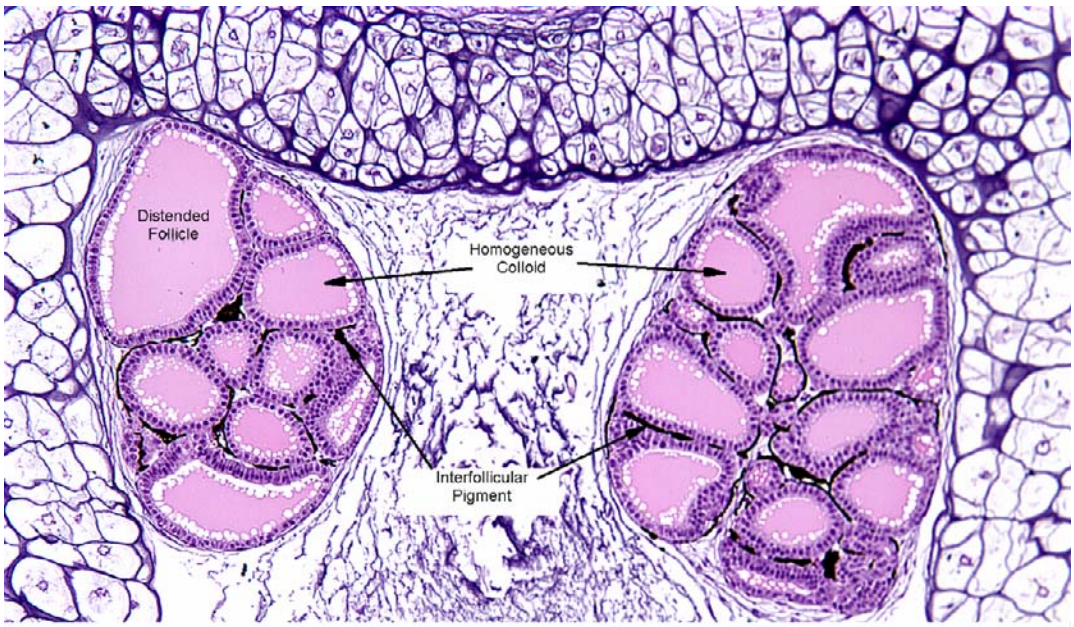


Z10458 *Xenopus laevis* 54-222 Group 034D
BATT02-00051 0 mg/L PTU H&E 10x

Z10447 and Z10458. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 54 control *Xenopus laevis*. The colloid is light eosinophilic and homogeneous. The follicles are lined by simple cuboidal epithelium.

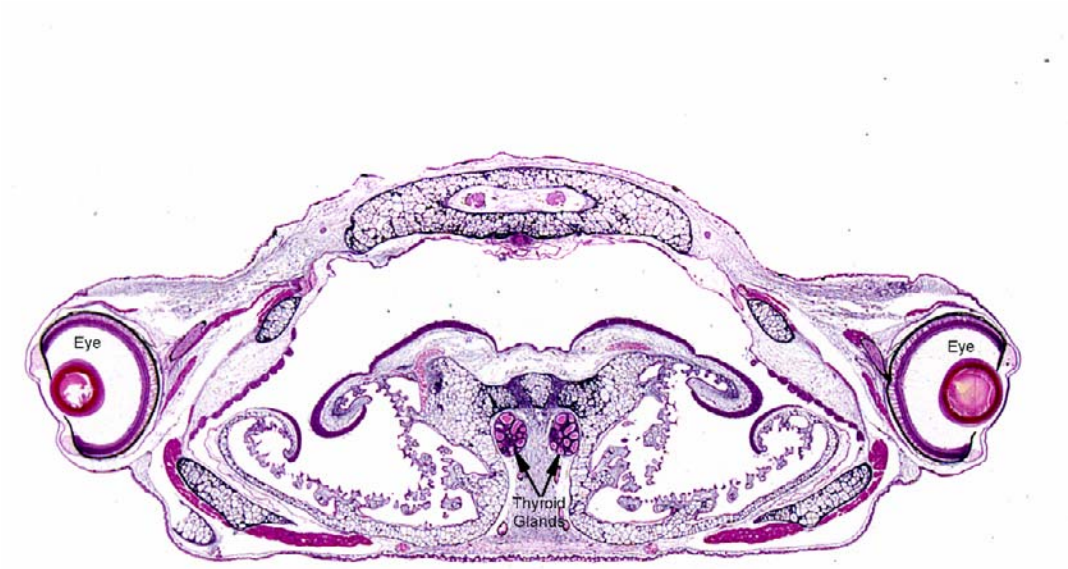


Z10448 *Xenopus laevis* 54-278 Group 031D
BATT02-00051 2.5 mg/L PTU H&E 1.25x

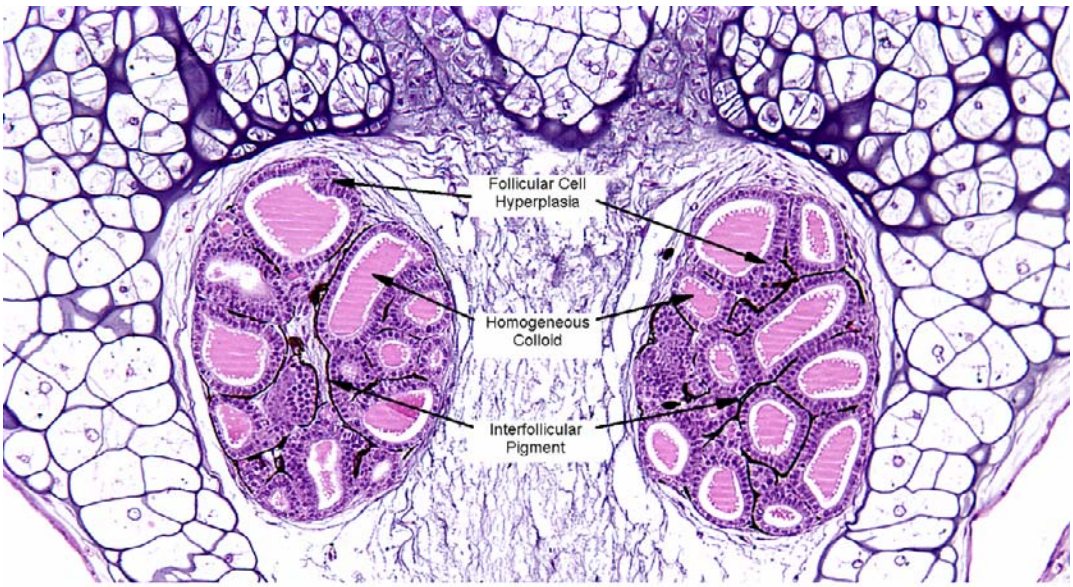


Z10459 *Xenopus laevis* 54-278 Group 031D
BATT02-00051 2.5 mg/L PTU H&E 10x

Z10448 and Z10459. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* exposed to 2.5 mg/L PTU for 14 days. The features are similar to those of the control frogs (Photographs Z10447 and Z10458) except for mild distension of a few thyroid follicles.



Z10449 *Xenopus laevis* 54-295 Group 035C
BATT02-00051 5.0 mg/L PTU H&E 1.25x

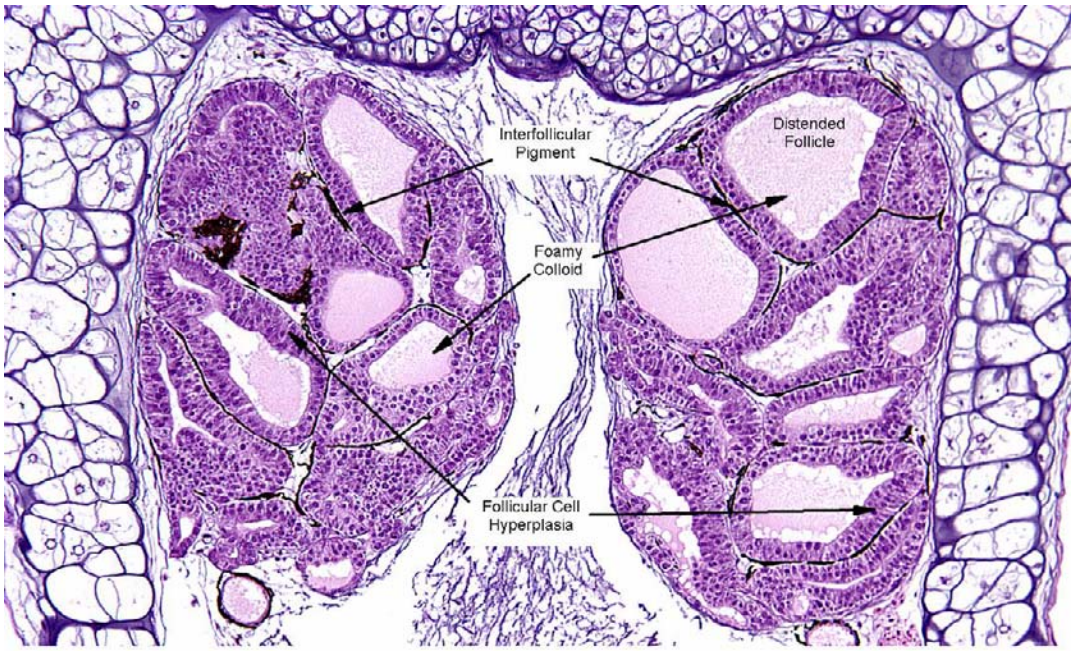


Z10460 *Xenopus laevis* 54-295 Group 035C
BATT02-00051 5.0 mg/L PTU H&E 10x

Z10449 and Z10460. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* exposed to 5.0 mg/L PTU for 14 days. There is mild follicular cell hyperplasia and the colloid is homogeneous.

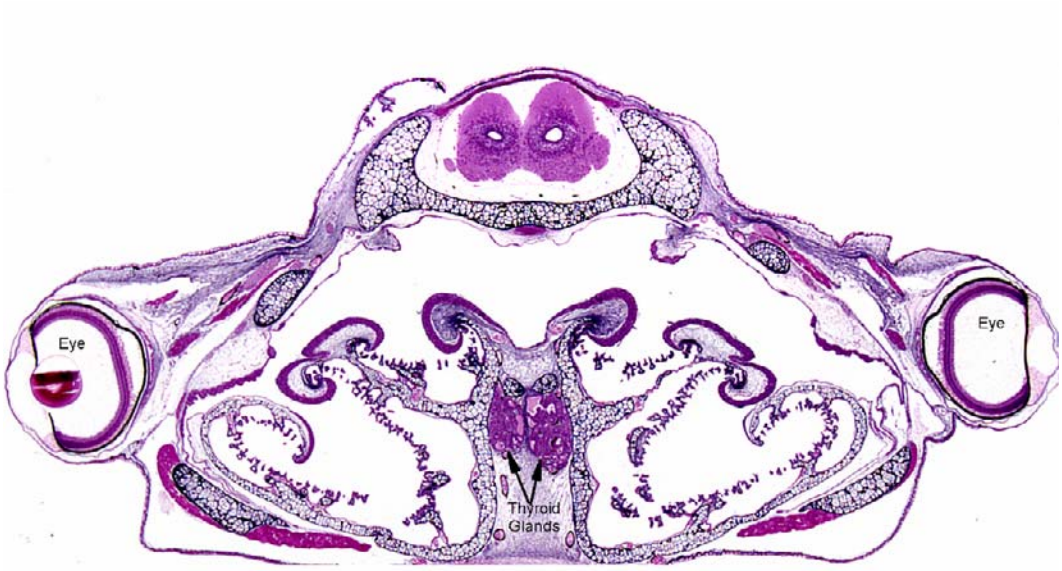


Z10450 *Xenopus laevis* 54-325 Group 033C
BATT02-00051 10.0 mg/L PTU H&E 1.25x

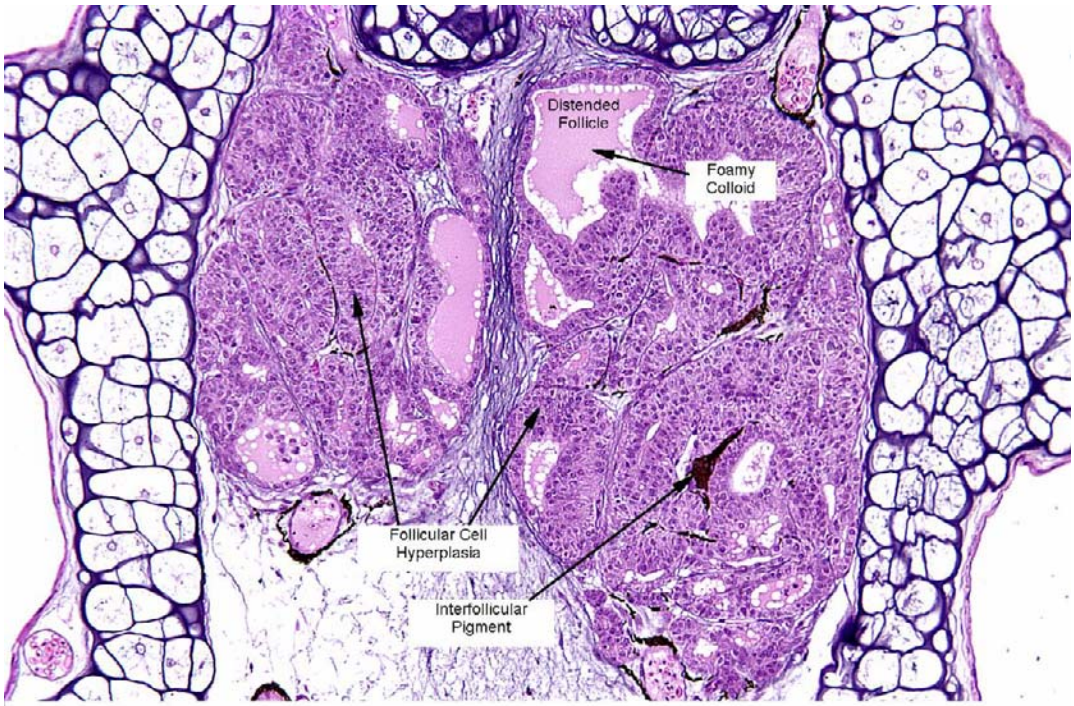


Z10461 *Xenopus laevis* 54-325 Group 033C
BATT02-00051 10.0 mg/L PTU H&E 10x

Z10450 and Z10461. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* exposed to 10.0 mg/L PTU for 14 days. There is moderate follicular cell hyperplasia and there is mild diffuse enlargement of the thyroid glands. There is moderate distension of thyroid follicles and the colloid has a foamy appearance.



Z10451 *Xenopus laevis* 54-389 Group 032D
BATT02-00051 20.0 mg/L PTU H&E 1.25x



Z10462 *Xenopus laevis* 54-389 Group 032D
BATT02-00051 20.0 mg/L PTU H&E 10x

Z10451 and Z10462. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* exposed to 20.0 mg/L PTU for 14 days. There is moderate diffuse enlargement of the thyroid glands and there is moderately severe hyperplasia of follicular cells. Some follicles are mildly distended and the colloid has a foamy appearance.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

1 of 2

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	John Young
P.O. #:	

Lab Use Only
Client/Project # - WO #
BATT02-0005 1

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only ID#	Lab Use Only Temp	Lab Use Only pH
026	Set 1: Specimen #s- 041,	3/26/04 1300	10	G	Bouin's Solution	Histological examination of Thyroid gland as			
	044, 051, 053, 057, 067					per protocol # BATT02-1			
↓	072, 073, 075, 076								
027	Set 2: Specimen #s - 168,		10						
	169, 170, 172, 178, 181, 194,								
↓	195, 196, 199								
028	Set 3: Specimen #s-086, 087,		10						
	089, 095, 099, 105, 111,								
↓	116, 117, 118								
029	Set 4: Specimen #s - 002,		10						
	003, 006, 007, 009, 023,								
↓	024, 026, 027, 038								
030	Set 5: Specimen #s - 122,		10						
	125, 129, 132, 137, 145,								
↓	146, 149, 156, 157	↓ ↓		↓	↓	↓			

SPECIAL INSTRUCTIONS: Organisms exposed to PTU (6-Propyl-2-thiouracil)

Lab Use Only
DATE:
INITIALS:

Sample Relinquished By	Date-Time	Sample Received By	Date-Time
John Young	4/1/04 1700	[signature]	4/5/04 0900

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

2 of 2

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	
P.O. #:	

Lab Use Only
Client/Project # - WO #
BATT02-00051

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only ID#	Temp	pH
031	Set6: Specimen #s: 240, 252,	3/26/04 1300	10	G	Bouin's Solution	Histological examination of Thyroid gland as			
	253, 254, 257, 264, 267,					per protocol # BATT02-1			
↓	272, 277, 278								
032	Set7: Specimen #s-362,		10						
	364, 368, 373, 377, 382,								
↓	385, 386, 387, 389								
033	Set8: Specimen #s-321, 322,		10						
	325, 332, 337, 341, 343,								
↓	344, 347, 349								
034	Set9: Specimen #s-207		10						
	209, 214, 216, 219, 221,								
↓	222, 223, 227, 232								
035	Set10: Specimen #s-281,		10						
	283, 293, 294, 295, 303,								
↓	305, 309, 310, 313	↓		↓	↓	↓			

DATE:
INITIALS:

SPECIAL INSTRUCTIONS: Organisms exposed to PTU (6-Propyl-2-thiouracil)

Sample Relinquished By	Date-Time	Sample Received By	Date-Time
	4/1/04 1700		4/5/04 0900

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

1 of 2

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	John Young
P.O. #:	

Lab Use Only
Client/Project # - WO #
BATT02-0005 1

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only ID#	Lab Use Only Temp	Lab Use Only pH
026	Set 1: Specimen #s- 041,	3/26/04 1300	10	G	Bouin's Solution	Histological examination of Thyroid gland as			
	044, 051, 053, 057, 067					per protocol # BATT02-1			
↓	072, 073, 075, 076								
027	Set 2: Specimen #s - 168,		10						
	169, 170, 172, 178, 181, 194,								
↓	195, 196, 199								
028	Set 3: Specimen #s-086, 087,		10						
	089, 095, 099, 105, 111,								
↓	116, 117, 118								
029	Set 4: Specimen #s - 002,		10						
	003, 006, 007, 009, 023,								
↓	024, 026, 027, 038								
030	Set 5: Specimen #s - 122,		10						
	125, 129, 132, 137, 145,								
↓	146, 149, 156, 157	↓ ↓		↓	↓	↓			

SPECIAL INSTRUCTIONS: Organisms exposed to PTU (6-Propyl-2-thiouracil)

Lab Use Only
DATE:
INITIALS:

Sample Relinquished By	Date-Time	Sample Received By	Date-Time
John Young	4/1/04 1700	[signature]	4/5/04 0900

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

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2 of 2

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	
P.O. #:	

Lab Use Only
Client/Project # - WO #
BATT02-00051

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only ID#	Temp	pH
031	Set6: Specimen #s: 240, 252,	3/26/04 1300	10	G	Bouin's Solution	Histological examination of Thyroid gland as			
	253, 254, 257, 264, 267,					per protocol # BATT02-1			
↓	272, 277, 278								
032	Set7: Specimen #s-362,		10						
	364, 368, 373, 377, 382,								
↓	385, 386, 387, 389								
033	Set8: Specimen #s-321, 322,		10						
	325, 332, 337, 341, 343,								
↓	344, 347, 349								
034	Set9: Specimen #s-207		10						
	209, 214, 216, 219, 221,								
↓	222, 223, 227, 232								
035	Set10: Specimen #s-281,		10						
	283, 293, 294, 295, 303,								
↓	305, 309, 310, 313	↓		↓	↓	↓			

DATE:
INITIALS:

SPECIAL INSTRUCTIONS: Organisms exposed to PTU (6-Propyl-2-thiouracil)

Sample Relinquished By	Date-Time	Sample Received By	Date-Time
	4/1/04 1700		4/5/04 0900

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

Appendix D
Methimazole

Metamorphosis Assay – Raw Data:

Daily Operational Data
Test Termination Data – Stage and Growth
Statistical Data – Stage and Growth

Test Chemical Analytical Data from Battelle:

Purity / Stability Report
In-Life Test Chemical Analysis Report

Thyroid Histology Data from EPL:

Sample Identification Key
Thyroid Pathology Report

Methimazole Metamorphosis Assay: Daily Operational Data

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00054 **Test No: 1**
Test Chemical: Methimazole (MTMZ) **Target Conc: 0.0 mg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc[1] (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	04/08/04			100.0	21.0	8.2	10.1	1400	25			100.0	20.0	8.2	9.9	1460	25	100.0	20.5	8.2	10.0	1430	25	0.02
1	04/09/04		20	100.0	25.1	7.4	7.5	1489	25		20	100.0	22.0	7.6	9.1	1506	25	100.0	23.6	7.5	8.3	1498	25	
2	04/10/04		20	100.0	20.8	7.5	9.1	1185	25		20	100.0	20.0	7.4	9.0	1290	25	100.0	20.4	7.5	9.1	1238	25	
3	04/11/04		20	100.0	20.4	7.1	9.0	1210	24		20	100.0	20.2	7.4	9.3	1230	24	100.0	20.3	7.3	9.2	1220	24	
4	04/12/04		20	100.0	21.2	7.2	8.8	1537	25		20	100.0	20.4	7.2	8.7	1538	25	100.0	20.8	7.2	8.8	1538	25	
5	04/13/04		20	100.0	21.2	7.2	8.3	1454	24		20	100.0	20.4	7.2	8.3	1629	24	100.0	20.8	7.2	8.3	1542	24	
6	04/14/04		20	100.0	21.1	6.9	8.1	1140	24		20	100.0	20.1	7.1	7.7	980	24	100.0	20.6	7.0	7.9	1060	24	
7	04/15/04		20	100.0	21.3	6.9	7.3	1180	24		20	100.0	19.9	6.9	7.4	1260	24	100.0	20.6	6.9	7.4	1220	24	
8	04/16/04		20	100.0	22.0	7.0	8.6	1390	24		20	100.0	20.0	7.0	8.1	1515	24	100.0	21.0	7.0	8.4	1453	24	0.02
9	04/17/04		20	100.0	21.3	6.1	8.5	1485	24		20	100.0	20.7	6.8	6.8	1733	24	100.0	21.0	6.5	7.7	1609	24	
10	04/18/04		20	100.0	21.3	7.2	8.5	1623	24		20	100.0	20.8	7.2	6.7	1720	24	100.0	21.1	7.2	7.6	1672	24	
11	04/19/04		20	100.0	21.7	6.6	8.2	1546	25		20	100.0	20.8	6.7	8.0	1485	25	100.0	21.3	6.7	8.1	1516	25	
12	04/20/04		20	100.0	22.0	6.5	7.6	1425	25		20	100.0	21.6	7.0	7.2	1549	25	100.0	21.8	6.8	7.4	1487	25	
13	04/21/04		20	100.0	22.1	6.6	7.2	1195	26		20	100.0	21.5	6.9	7.5	1110	26	100.0	21.8	6.8	7.4	1153	26	
14	04/22/04		20	100.0	21.7	6.9	7.5	1245	25		20	100.0	21.4	6.8	7.4	1115	25	100.0	21.6	6.9	7.5	1180	25	
15	04/23/04		20	100.0	21.8	7.0	7.5	1545	26		20	100.0	21.5	7.0	7.5	1685	26	100.0	21.7	7.0	7.5	1615	26	
16	04/24/04		20	100.0	21.1	7.1	8.0	1486	26		20	100.0	21.3	7.3	7.7	1698	26	100.0	21.2	7.2	7.9	1592	26	
17	04/25/04		20	100.0	21.5	7.1	8.3	1685	26		20	100.0	20.4	7.2	8.0	1796	26	100.0	21.0	7.2	8.2	1741	26	
18	04/26/04		20	100.0	21.5	7.1	8.1	1170	27		20	100.0	21.1	7.2	7.7	1345	27	100.0	21.3	7.2	7.9	1258	27	
19	04/27/04		20	100.0	21.9	7.0	6.9	1419	27		20	100.0	21.0	7.2	6.9	1637	27	100.0	21.5	7.1	6.9	1528	27	
20	04/28/04		20	100.0	21.8	7.1	6.7	1673	26		20	100.0	21.0	7.2	6.5	1731	26	100.0	21.4	7.2	6.6	1702	26	
21	04/29/04		20	100.0							20	100.0						100.0						0.02
	Median:				**21.5**	**7.1**	**8.1**	**1425**	**25**				**20.8**	**7.2**	**7.7**	**1515**	**25**		**21.1**	**7.2**	**7.9**	**1498**	**25**	
	Mode:				**21.3**	**7.1**	**7.5**	**#N/A**	**25**				**20.0**	**7.2**	**7.7**	**#N/A**	**25**		**20.8**	**7.2**	**8.3**	**1220**	**25**	
	Mean:			**100.0**	**21.6**	**7.0**	**8.1**	**1404**	**25.1**			**100.0**	**20.8**	**7.2**	**7.9**	**1477**	**25.1**	**100.0**	**21.2**	**7.1**	**8.0**	**1440**	**25.1**	**0.020**
	SEM:			**0.00**	**0.20**	**0.09**	**0.18**	**38.11**	**0.22**			**0.00**	**0.14**	**0.07**	**0.20**	**51.56**	**0.22**	**0.00**	**0.15**	**0.08**	**0.17**	**43.63**	**0.22**	**0.00**
	% CV:			**0.00**	**19.57**	**15.56**	**28.51**	**466.14**	**19.87**			**0.00**	**13.78**	**12.12**	**32.36**	**614.82**	**19.87**	**0.00**	**15.23**	**13.44**	**28.06**	**526.86**	**19.87**	**0.00**

[1]All measured test concentrations reported by Battelle as less than method detection limit (MDL) [0.0338 mg/L] or none detected (ND) are expressed as 0.02 mg/L (1/2 of MDL).

DAILY DATA

Workorder No: 00054 **Test No: 1**
Test Chemical: Methimazole (MTMZ) **Target Conc: 6.25 mg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	04/08/04			100.0	20.6	8.2	10.6	970	26			100.0	20.0	8.2	10.0	1130	26	100.0	20.3	8.2	10.3	1050	26	6.84
1	04/09/04		20	100.0	24.2	7.5	8.8	1510	23		20	100.0	22.5	7.6	8.7	1090	23	100.0	23.4	7.6	8.8	1300	23	
2	04/10/04		20	100.0	20.8	7.4	7.4	1270	24		20	100.0	19.2	7.6	9.1	950	24	100.0	20.0	7.5	8.3	1110	24	
3	04/11/04		20	100.0	20.5	7.6	9.2	1380	25		20	100.0	20.1	7.6	8.8	1190	25	100.0	20.3	7.6	9.0	1285	25	
4	04/12/04		20	100.0	21.0	7.2	9.1	1531	26		20	100.0	20.0	7.2	9.1	1010	26	100.0	20.5	7.2	9.1	1271	26	
5	04/13/04		20	100.0	20.3	7.2	8.5	1362	23		20	100.0	20.2	7.3	8.5	1321	23	100.0	20.3	7.3	8.5	1342	23	
6	04/14/04		20	100.0	21.2	7.0	7.8	1120	24		20	100.0	20.1	7.0	7.8	1220	24	100.0	20.7	7.0	7.8	1170	24	
7	04/15/04		20	100.0	21.3	6.8	7.6	1380	24		20	100.0	19.9	6.9	7.2	1295	24	100.0	20.6	6.9	7.4	1338	24	
8	04/16/04		20	100.0	21.0	7.1	8.9	1489	22		20	100.0	20.0	7.0	7.8	1203	22	100.0	20.5	7.1	8.4	1346	22	10.05
9	04/17/04		20	100.0	21.9	6.9	8.4	1663	24		20	100.0	20.7	6.8	6.1	1431	24	100.0	21.3	6.9	7.3	1547	24	
10	04/18/04		20	100.0	21.2	7.4	8.6	1855	25		20	100.0	20.4	7.3	7.5	1242	25	100.0	20.8	7.4	8.1	1549	25	
11	04/19/04		20	100.0	20.4	6.7	8.3	1599	26		20	100.0	20.1	6.8	8.4	1403	26	100.0	20.3	6.8	8.4	1501	26	
12	04/20/04		20	100.0	21.2	6.7	8.1	1528	26		20	100.0	21.2	6.7	7.9	1333	26	100.0	21.2	6.7	8.0	1431	26	
13	04/21/04		20	100.0	22.3	6.9	7.6	1160	25		20	100.0	21.2	7.0	7.4	1225	25	100.0	21.8	7.0	7.5	1193	25	
14	04/22/04		20	100.0	21.9	6.8	7.6	1190	25		20	100.0	21.0	6.7	7.2	1220	25	100.0	21.5	6.8	7.4	1205	25	6.44
15	04/23/04		20	100.0	21.3	7.1	7.7	1821	26		20	100.0	20.7	7.0	7.3	1346	26	100.0	21.0	7.1	7.5	1584	26	
16	04/24/04		20	100.0	21.4	7.4	8.1	1893	26		20	100.0	21.1	7.3	7.6	1327	26	100.0	21.3	7.4	7.9	1610	26	
17	04/25/04		20	100.0	21.7	7.3	8.4	1953	27		20	100.0	20.3	7.2	8.0	1407	27	100.0	21.0	7.3	8.2	1680	27	
18	04/26/04		20	100.0	21.4	7.2	7.5	1180	26		20	100.0	21.0	7.2	7.4	1620	26	100.0	21.2	7.2	7.5	1400	26	
19	04/27/04		20	100.0	21.7	7.2	7.0	1523	27		20	100.0	20.9	7.2	6.5	1322	27	100.0	21.3	7.2	6.8	1423	27	
20	04/28/04		20	100.0	21.7	7.2	6.3	1903	26		20	100.0	20.9	7.2	6.7	1348	26	100.0	21.3	7.2	6.5	1626	26	
21	04/29/04		20	100.0							20	100.0						100.0						5.89
	Median:				**21.3**	**7.2**	**8.1**	**1510**	**25**				**20.4**	**7.2**	**7.8**	**1295**	**25**		**21.0**	**7.2**	**8.0**	**1346**	**25**	
	Mode:				**21.2**	**7.2**	**7.6**	**1380**	**26**				**20.0**	**7.2**	**9.1**	**1220**	**26**		**21.3**	**7.2**	**7.4**	**#N/A**	**26**	
	Mean:			**100.0**	**21.4**	**7.2**	**8.2**	**1490**	**25.0**			**100.0**	**20.5**	**7.2**	**7.9**	**1268**	**25.0**	**100.0**	**21.0**	**7.2**	**8.0**	**1379**	**25.0**	**7.305**
	SEM:			**0.00**	**0.18**	**0.08**	**0.20**	**62.26**	**0.30**			**0.00**	**0.15**	**0.08**	**0.21**	**32.75**	**0.30**	**0.00**	**0.16**	**0.08**	**0.19**	**39.15**	**0.30**	**0.94**
	% CV:			**0.00**	**18.02**	**13.01**	**31.71**	**739.21**	**27.16**			**0.00**	**15.29**	**13.38**	**33.91**	**421.45**	**27.16**	**0.00**	**15.90**	**13.09**	**30.21**	**483.16**	**27.16**	**69.33**

DAILY DATA

Workorder No: 00054 **Test No: 1**
Test Chemical: Methimazole (MTMZ) **Target Conc: 12.5 mg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	04/08/04			100.0	20.3	8.2	10.4	1390	24			100.0	20.0	8.2	10.3	1590	24	100.0	20.2	8.2	10.4	1490	24	11.73
1	04/09/04		20	100.0	23.4	7.6	9.2	1624	24		20	100.0	23.0	7.4	7.0	1583	24	100.0	23.2	7.5	8.1	1604	24	
2	04/10/04		20	100.0	20.4	7.6	9.0	1220	25		20	100.0	20.0	7.5	8.9	1260	25	100.0	20.2	7.6	9.0	1240	25	
3	04/11/04		20	100.0	21.0	7.2	9.0	1205	25		20	100.0	20.6	7.3	9.0	1215	25	100.0	20.8	7.3	9.0	1210	25	
4	04/12/04		20	100.0	20.7	7.2	9.2	1741	24		20	100.0	20.0	7.3	9.4	1792	24	100.0	20.4	7.3	9.3	1767	24	
5	04/13/04		20	100.0	20.1	7.3	8.5	1482	26		20	100.0	20.0	7.3	8.8	1780	26	100.0	20.1	7.3	8.7	1631	26	
6	04/14/04		20	100.0	20.9	7.1	8.0	1220	22		20	100.0	20.1	7.1	7.8	1360	22	100.0	20.5	7.1	7.9	1290	22	
7	04/15/04		20	100.0	21.1	7.0	7.6	1305	25		20	100.0	20.1	7.0	7.7	1150	25	100.0	20.6	7.0	7.7	1228	25	
8	04/16/04		20	100.0	21.2	7.1	8.7	1660	26		20	100.0	20.5	7.1	8.9	1790	26	100.0	20.9	7.1	8.8	1725	26	17.07
9	04/17/04		20	100.0	21.1	7.0	8.4	1642	26		20	100.0	20.1	7.0	8.8	1762	26	100.0	20.6	7.0	8.6	1702	26	
10	04/18/04		20	100.0	21.1	7.4	8.5	1915	26		20	100.0	20.3	7.5	8.9	1995	26	100.0	20.7	7.5	8.7	1955	26	
11	04/19/04		20	100.0	21.3	6.8	8.2	1601	27		20	100.0	21.6	6.9	8.7	1665	27	100.0	21.5	6.9	8.5	1633	27	
12	04/20/04		20	100.0	21.1	6.8	7.8	1672	27		20	100.0	21.2	6.8	8.2	1792	27	100.0	21.2	6.8	8.0	1732	27	
13	04/21/04		20	100.0	21.9	7.0	7.6	1255	27		20	100.0	21.1	7.1	7.8	1160	27	100.0	21.5	7.1	7.7	1208	27	
14	04/22/04		20	100.0	21.8	7.0	7.3	1260	27		20	100.0	21.3	7.1	7.8	1285	27	100.0	21.6	7.1	7.6	1273	27	14.14
15	04/23/04		20	100.0	21.6	7.1	7.6	1817	27		20	100.0	21.8	7.1	7.9	1812	27	100.0	21.7	7.1	7.8	1815	27	
16	04/24/04		20	100.0	20.8	7.4	7.9	1913	27		20	100.0	21.7	7.5	8.2	1884	27	100.0	21.3	7.5	8.1	1899	27	
17	04/25/04		20	100.0	21.5	7.4	8.2	1850	25		20	100.0	20.6	7.3	8.1	1974	25	100.0	21.1	7.4	8.2	1912	25	
18	04/26/04		20	100.0	21.7	7.3	7.3	1670	25		20	100.0	21.4	7.3	7.4	1810	25	100.0	21.6	7.3	7.4	1740	25	
19	04/27/04		20	100.0	21.4	7.2	7.1	1617	26		20	100.0	21.2	7.2	6.9	1856	26	100.0	21.3	7.2	7.0	1737	26	
20	04/28/04		20	100.0	21.4	7.3	6.6	1915	26		20	100.0	21.2	7.3	6.9	1919	26	100.0	21.3	7.3	6.8	1917	26	
21	04/29/04		20	100.0							20	100.0						100.0						12.48
	Median:				**21.1**	**7.2**	**8.2**	**1624**	**26**				**20.6**	**7.3**	**8.2**	**1780**	**26**		**21.1**	**7.3**	**8.1**	**1702**	**26**	
	Mode:				**21.1**	**7.0**	**7.6**	**1220**	**27**				**20.0**	**7.3**	**8.9**	**1792**	**27**		**20.6**	**7.3**	**#N/A**	**#N/A**	**27**	
	Mean:			**100.0**	**21.2**	**7.2**	**8.2**	**1570**	**25.5**			**100.0**	**20.8**	**7.3**	**8.3**	**1640**	**25.5**	**100.0**	**21.0**	**7.2**	**8.2**	**1605**	**25.5**	**13.857**
	SEM:			**0.00**	**0.15**	**0.07**	**0.19**	**54.51**	**0.29**			**0.00**	**0.18**	**0.06**	**0.19**	**61.52**	**0.29**	**0.00**	**0.15**	**0.07**	**0.18**	**56.90**	**0.29**	**1.18**
	% CV:			**0.00**	**14.96**	**11.60**	**30.60**	**630.41**	**26.15**			**0.00**	**17.57**	**10.80**	**30.29**	**696.22**	**26.15**	**0.00**	**15.44**	**11.11**	**28.82**	**650.89**	**26.15**	**63.63**

DAILY DATA

Workorder No: 00054 **Test No: 1**
Test Chemical: Methimazole (MTMZ) **Target Conc: 25.0 mg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	04/08/04			100.0	20.7	8.2	10.6	1300	25			100.0	20.0	8.2	10.1	1245	25	100.0	20.4	8.2	10.4	1273	25	26.49
1	04/09/04		20	100.0	24.0	7.6	8.9	1572	23		20	100.0	22.8	7.6	8.9	1314	23	100.0	23.4	7.6	8.9	1443	23	
2	04/10/04		20	100.0	20.6	7.2	9.0	1150	24		20	100.0	20.2	7.5	9.0	500	24	100.0	20.4	7.4	9.0	825	24	
3	04/11/04		20	100.0	21.1	7.2	9.1	1245	23		20	100.0	20.1	7.3	9.0	1195	23	100.0	20.6	7.3	9.1	1220	23	
4	04/12/04		20	100.0	21.0	7.2	9.1	1570	25		20	100.0	20.6	7.2	8.9	1358	25	100.0	20.8	7.2	9.0	1464	25	
5	04/13/04		20	100.0	20.6	7.3	8.6	1445	23		20	100.0	20.7	7.3	8.4	1293	23	100.0	20.7	7.3	8.5	1369	23	
6	04/14/04		20	100.0	21.0	7.1	8.1	725	22		20	100.0	20.6	7.0	7.7	1005	22	100.0	20.8	7.1	7.9	865	22	
7	04/15/04		20	100.0	21.0	6.9	7.8	1060	23		20	100.0	20.8	7.0	7.5	1075	23	100.0	20.9	7.0	7.7	1068	23	
8	04/16/04		20	100.0	21.5	7.1	8.0	1666	22		20	100.0	21.1	7.1	8.4	1461	22	100.0	21.3	7.1	8.2	1564	22	27.86
9	04/17/04		20	100.0	21.1	7.0	8.5	1611	22		20	100.0	20.4	7.0	8.2	1570	22	100.0	20.8	7.0	8.4	1591	22	
10	04/18/04		20	100.0	21.1	7.5	8.7	1878	25		20	100.0	20.8	7.5	8.4	1670	25	100.0	21.0	7.5	8.6	1774	25	
11	04/19/04		20	100.0	21.2	6.9	8.5	1471	26		20	100.0	21.4	7.0	8.2	1434	26	100.0	21.3	7.0	8.4	1453	26	
12	04/20/04		20	100.0	21.2	6.8	8.6	1577	27		20	100.0	21.2	6.9	7.4	1519	27	100.0	21.2	6.9	8.0	1548	27	
13	04/21/04		20	100.0	21.8	7.1	7.9	1230	25		20	100.0	21.6	7.2	7.4	1050	25	100.0	21.7	7.2	7.7	1140	25	
14	04/22/04		20	100.0	21.8	6.9	7.9	1310	26		20	100.0	21.7	7.1	7.4	1195	26	100.0	21.8	7.0	7.7	1253	26	24.58
15	04/23/04		20	100.0	21.1	7.2	8.1	1709	27		20	100.0	21.5	7.1	7.7	1528	27	100.0	21.3	7.2	7.9	1619	27	
16	04/24/04		20	100.0	21.5	7.5	8.3	1704	27		20	100.0	20.9	7.4	7.5	1633	27	100.0	21.2	7.5	7.9	1669	27	
17	04/25/04		20	100.0	21.5	7.4	8.5	1769	27		20	100.0	21.1	7.4	8.0	1625	27	100.0	21.3	7.4	8.3	1697	27	
18	04/26/04		20	100.0	21.7	7.3	7.6	1305	27		20	100.0	21.5	7.3	7.7	1290	27	100.0	21.6	7.3	7.7	1298	27	
19	04/27/04		20	100.0	21.4	7.3	7.5	1587	28		20	100.0	21.6	7.2	6.7	1484	28	100.0	21.5	7.3	7.1	1536	28	
20	04/28/04		20	100.0	21.5	7.3	7.3	1878	27		20	100.0	21.6	7.3	6.5	1606	27	100.0	21.6	7.3	6.9	1742	27	
21	04/29/04		20	100.0							20	100.0						100.0						25.84
	Median:				**21.2**	**7.2**	**8.5**	**1570**	**25**				**21.1**	**7.2**	**8.0**	**1358**	**25**		**21.2**	**7.3**	**8.2**	**1453**	**25**	
	Mode:				**21.1**	**7.2**	**8.5**	**1878**	**27**				**21.6**	**7.3**	**8.4**	**1195**	**27**		**21.3**	**7.3**	**7.7**	**#N/A**	**27**	
	Mean:			**100.0**	**21.4**	**7.2**	**8.4**	**1465**	**25.0**			**100.0**	**21.1**	**7.3**	**8.0**	**1336**	**25.0**	**100.0**	**21.2**	**7.3**	**8.2**	**1400**	**25.0**	**26.191**
	SEM:			**0.00**	**0.15**	**0.07**	**0.16**	**62.75**	**0.43**			**0.00**	**0.14**	**0.06**	**0.18**	**60.55**	**0.43**	**0.00**	**0.14**	**0.06**	**0.17**	**58.83**	**0.43**	**0.68**
	% CV:			**0.00**	**15.19**	**11.42**	**24.98**	**751.28**	**39.78**			**0.00**	**14.43**	**10.64**	**29.84**	**759.20**	**39.78**	**0.00**	**14.16**	**10.88**	**26.75**	**720.48**	**39.78**	**26.73**

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

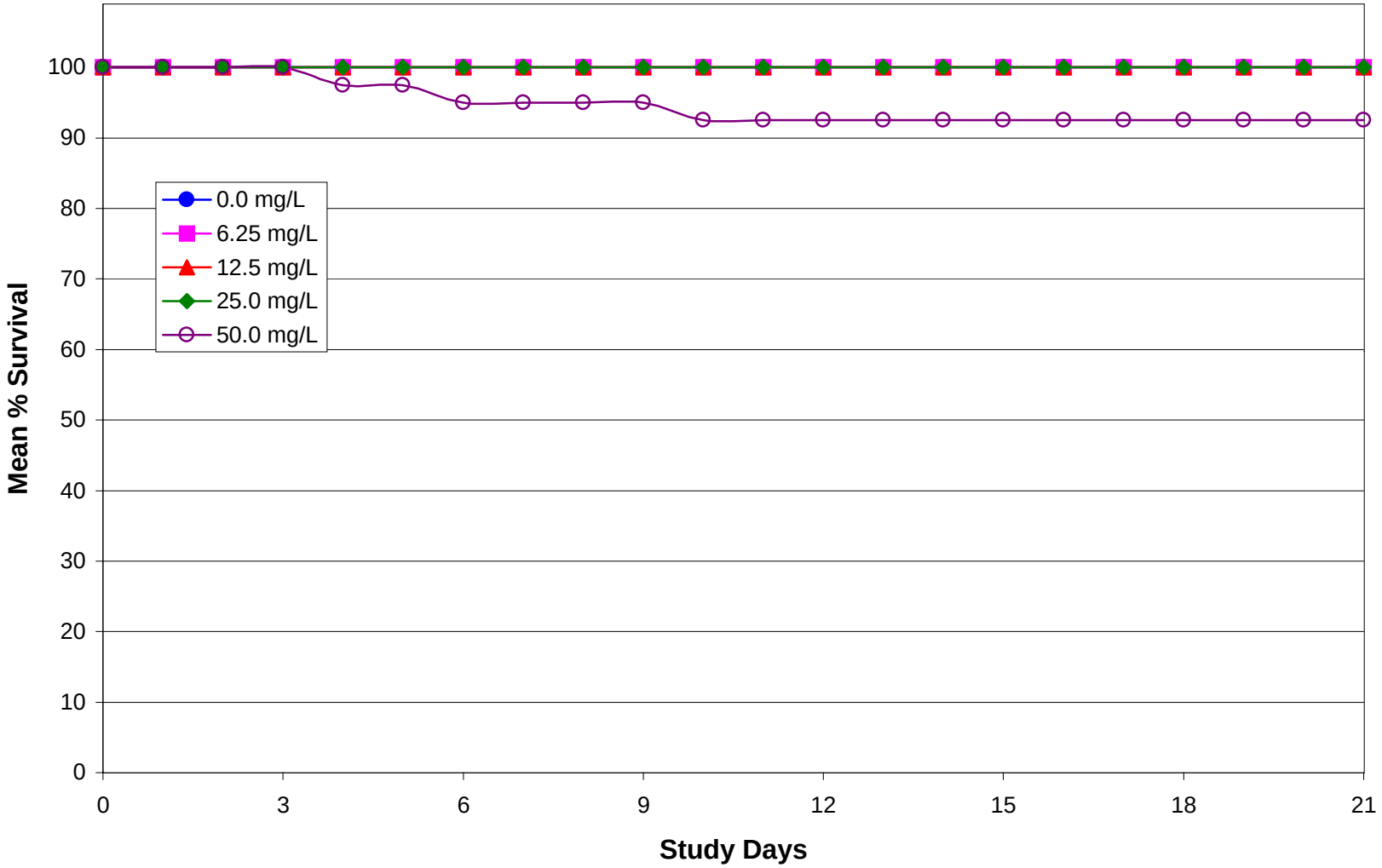
FEL

DAILY DATA

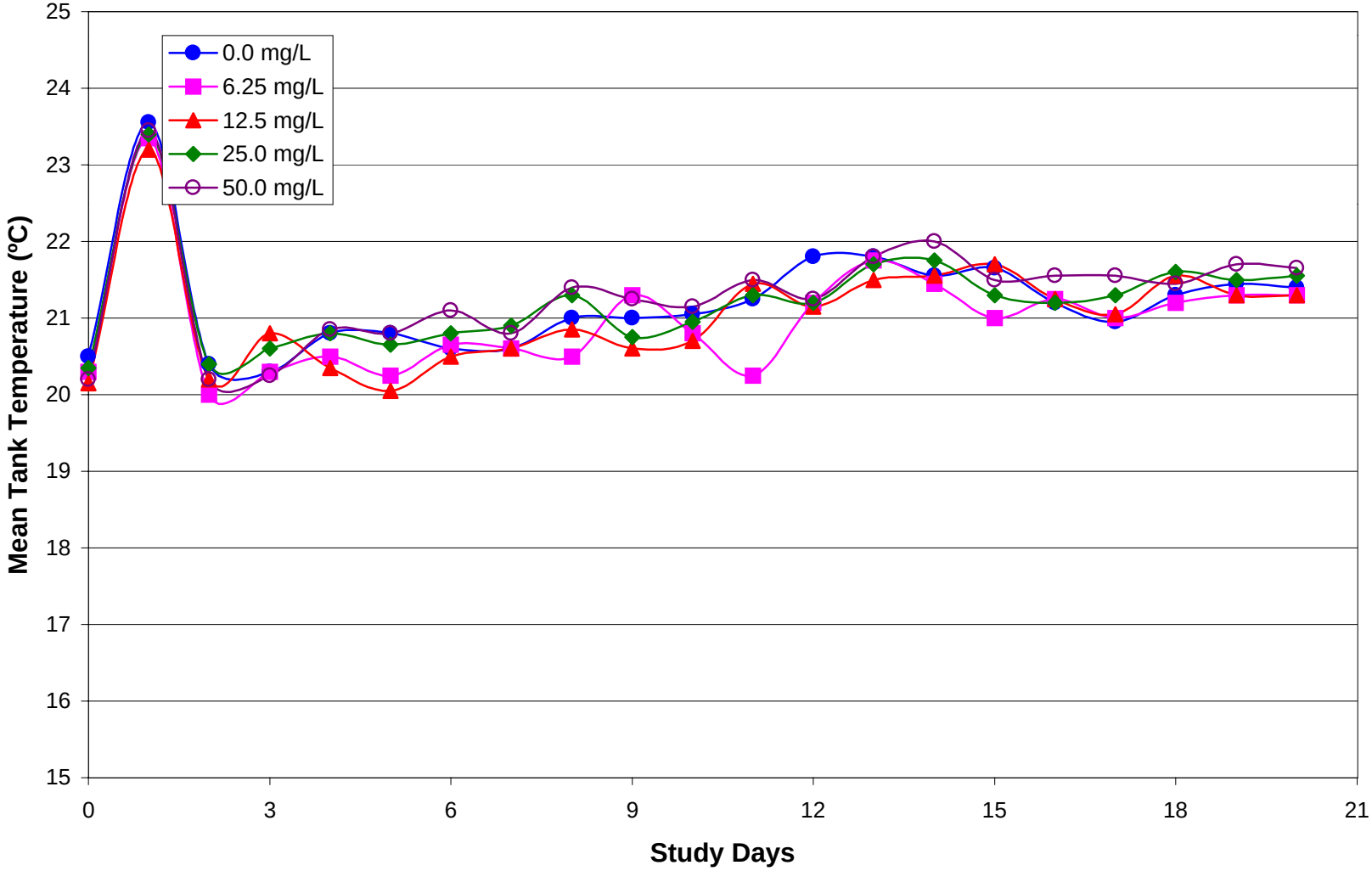
Workorder No: 00054 **Test No: 1**
Test Chemical: Methimazole **Target Conc: 50.0 mg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	04/08/04			100.0	20.4	8.2	10.3	1170	25			100.0	20.0	8.2	10.3	1460	25	100.0	20.2	8.2	10.3	1315	25	53.01
1	04/09/04		20	100.0	23.9	7.5	8.6	1532	24		20	100.0	23.0	7.6	8.9	1580	24	100.0	23.5	7.6	8.8	1556	24	
2	04/10/04		20	100.0	20.3	7.7	8.9	1090	23		20	100.0	20.1	7.5	9.1	1260	23	100.0	20.2	7.6	9.0	1175	23	
3	04/11/04		20	100.0	20.3	7.5	9.1	1180	23		20	100.0	20.2	7.4	9.1	1235	23	100.0	20.3	7.5	9.1	1208	23	
4	04/12/04		20	100.0	20.9	7.2	8.7	1520	25	1	19	95.0	20.8	7.2	9.0	1706	25	97.5	20.9	7.2	8.9	1613	25	
5	04/13/04		20	100.0	20.8	7.2	8.3	1466	21		19	95.0	20.8	7.3	8.4	1692	21	97.5	20.8	7.3	8.4	1579	21	
6	04/14/04		20	100.0	21.2	7.0	8.2	980	20	1	18	90.0	21.0	7.1	7.9	1010	20	95.0	21.1	7.1	8.1	995	20	
7	04/15/04		20	100.0	20.9	6.9	6.9	1210	23		18	90.0	20.7	6.9	7.5	1205	23	95.0	20.8	6.9	7.2	1208	23	
8	04/16/04		20	100.0	21.6	7.1	8.0	1469	20		18	90.0	21.2	7.1	8.6	1768	20	95.0	21.4	7.1	8.3	1619	20	55.73
9	04/17/04		20	100.0	21.3	7.0	8.2	1508	22		18	90.0	21.2	7.0	8.5	1726	22	95.0	21.3	7.0	8.4	1617	22	
10	04/18/04		20	100.0	21.2	7.5	8.3	1720	22	1	17	85.0	21.1	7.5	8.7	1975	22	92.5	21.2	7.5	8.5	1848	22	
11	04/19/04		20	100.0	21.8	7.0	8.0	1432	23		17	85.0	21.2	7.0	8.5	1654	23	92.5	21.5	7.0	8.3	1543	23	
12	04/20/04		20	100.0	21.2	6.9	7.5	1559	24		17	85.0	21.3	6.9	7.9	1777	24	92.5	21.3	6.9	7.7	1668	24	
13	04/21/04		20	100.0	21.9	7.1	7.1	1055	24		17	85.0	21.7	7.1	7.6	1160	24	92.5	21.8	7.1	7.4	1108	24	
14	04/22/04		20	100.0	22.0	7.2	7.0	1170	24		17	85.0	22.0	7.1	7.4	1210	24	92.5	22.0	7.2	7.2	1190	24	50.27
15	04/23/04		20	100.0	21.4	7.2	6.7	1565	23		17	85.0	21.6	7.1	7.5	1733	23	92.5	21.5	7.2	7.1	1649	23	
16	04/24/04		20	100.0	21.7	7.5	7.5	1805	24		17	85.0	21.4	7.5	7.8	1805	24	92.5	21.6	7.5	7.7	1805	24	
17	04/25/04		20	100.0	21.7	7.4	7.7	1830	23		17	85.0	21.4	7.4	8.0	1890	23	92.5	21.6	7.4	7.9	1860	23	
18	04/26/04		20	100.0	21.5	7.2	7.4	1335	23		17	85.0	21.4	7.2	7.5	1430	23	92.5	21.5	7.2	7.5	1383	23	
19	04/27/04		20	100.0	21.7	7.2	6.0	1477	23		17	85.0	21.7	7.2	7.0	1838	23	92.5	21.7	7.2	6.5	1658	23	
20	04/28/04		20	100.0	21.6	7.2	5.8	1714	23		17	85.0	21.7	7.2	6.6	1892	23	92.5	21.7	7.2	6.2	1803	23	
21	04/29/04		20	100.0							17	85.0						92.5						52.17
	Median:				**21.4**	**7.2**	**8.0**	**1469**	**23**				**21.2**	**7.2**	**8.0**	**1692**	**23**		**21.4**	**7.2**	**8.1**	**1579**	**23**	
	Mode:				**21.2**	**7.2**	**8.3**	**1170**	**23**				**21.2**	**7.1**	**7.5**	**#N/A**	**23**		**21.3**	**7.2**	**7.2**	**1208**	**23**	
	Mean:			**100.0**	**21.4**	**7.3**	**7.8**	**1418**	**23.0**			**89.5**	**21.2**	**7.3**	**8.2**	**1572**	**23.0**	**94.8**	**21.3**	**7.3**	**8.0**	**1495**	**23.0**	**52.794**
	SEM:			**0.00**	**0.17**	**0.07**	**0.23**	**54.36**	**0.30**			**1.27**	**0.15**	**0.06**	**0.19**	**62.78**	**0.30**	**0.64**	**0.16**	**0.07**	**0.21**	**57.56**	**0.30**	**1.13**
	% CV:			**0.00**	**16.60**	**11.32**	**37.71**	**661.38**	**28.37**			**62.96**	**14.71**	**10.98**	**29.90**	**725.63**	**28.37**	**30.60**	**15.45**	**11.07**	**33.52**	**682.20**	**28.37**	**31.24**

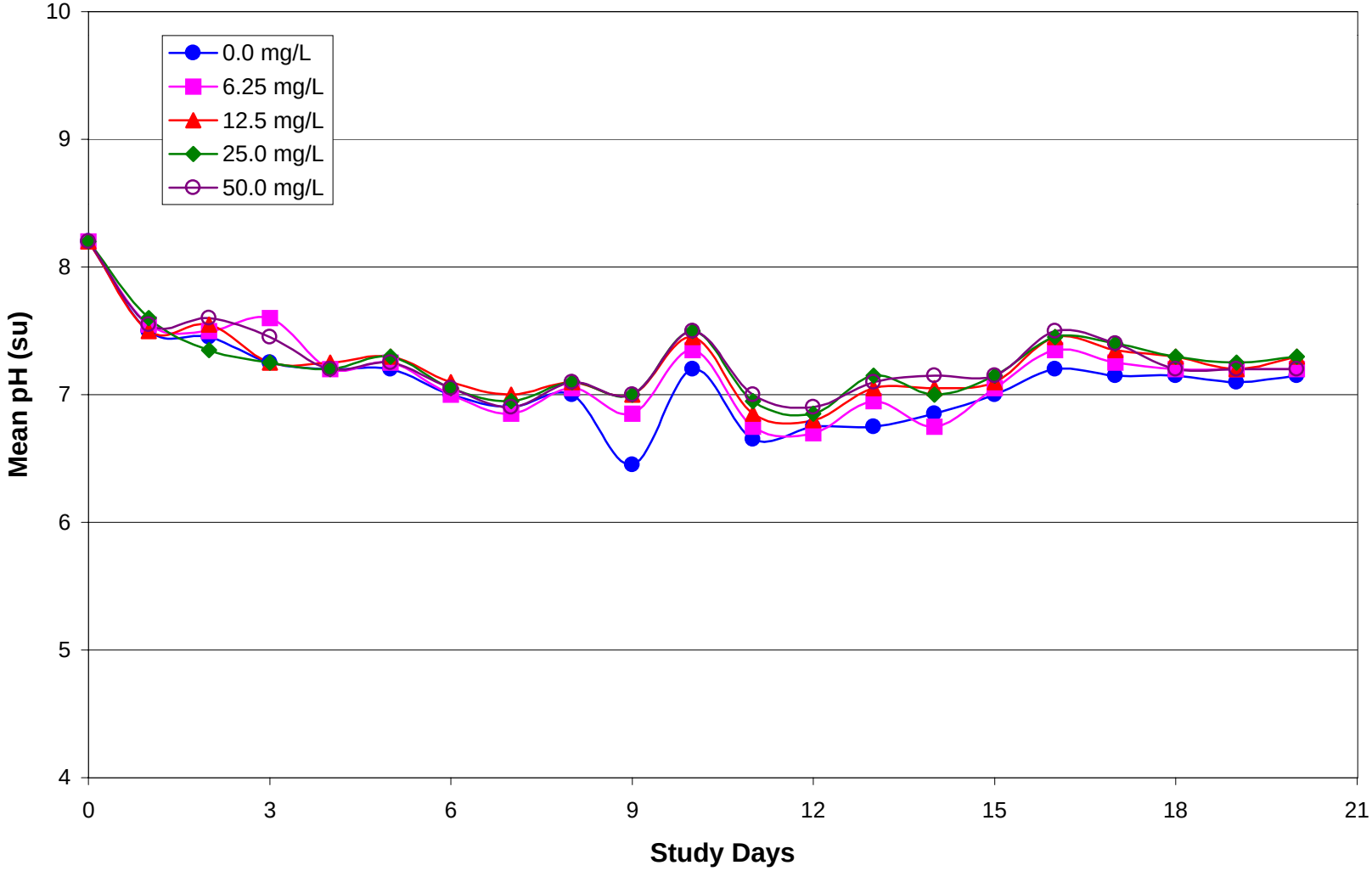
BATT02-00054
Methimazole
Daily Average of Larval Survival for Initial Stage 51 Test



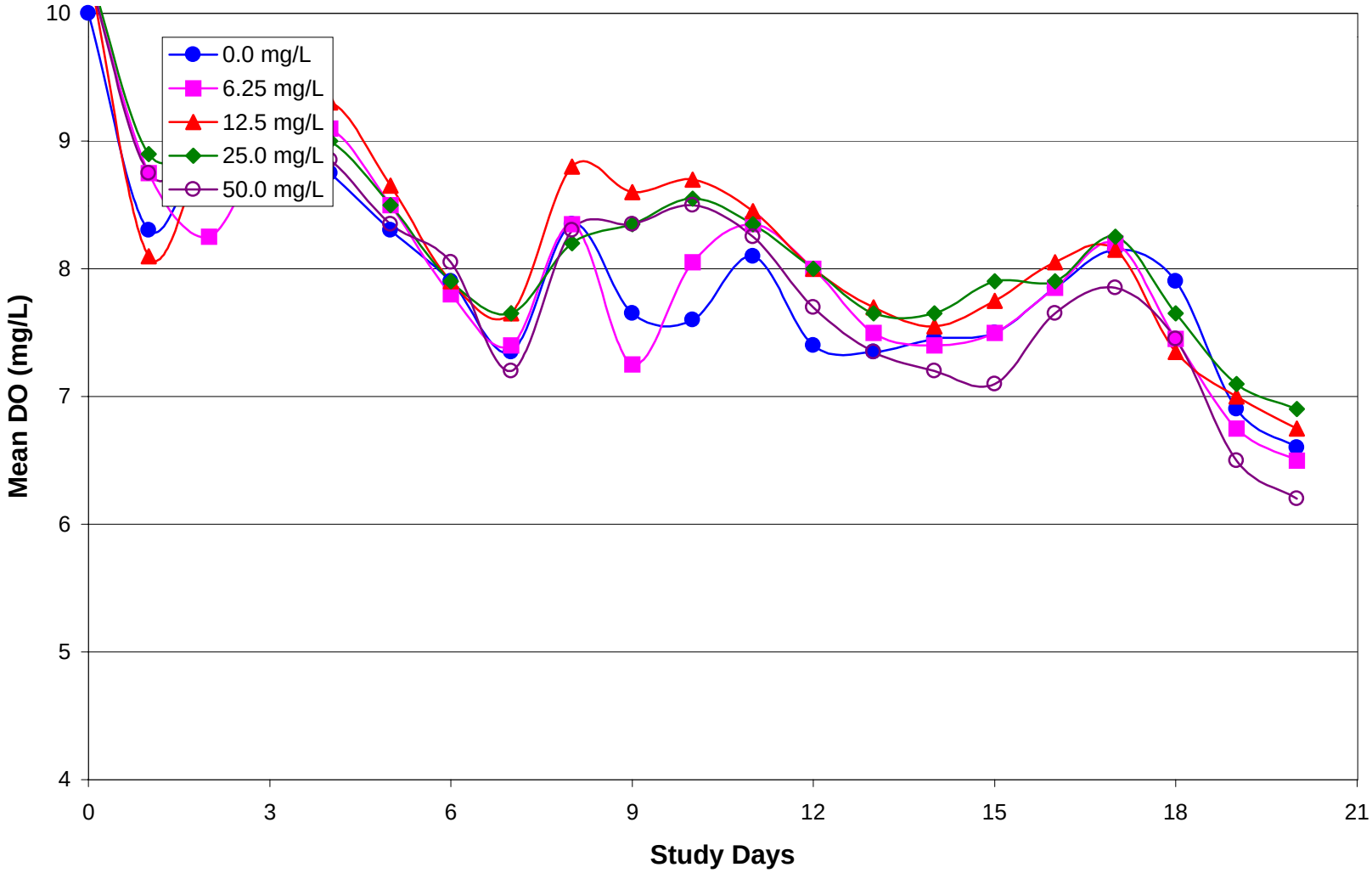
BATT02-00054
Methimazole
Daily Average Tank Temperatures for Initial Stage 51 Test



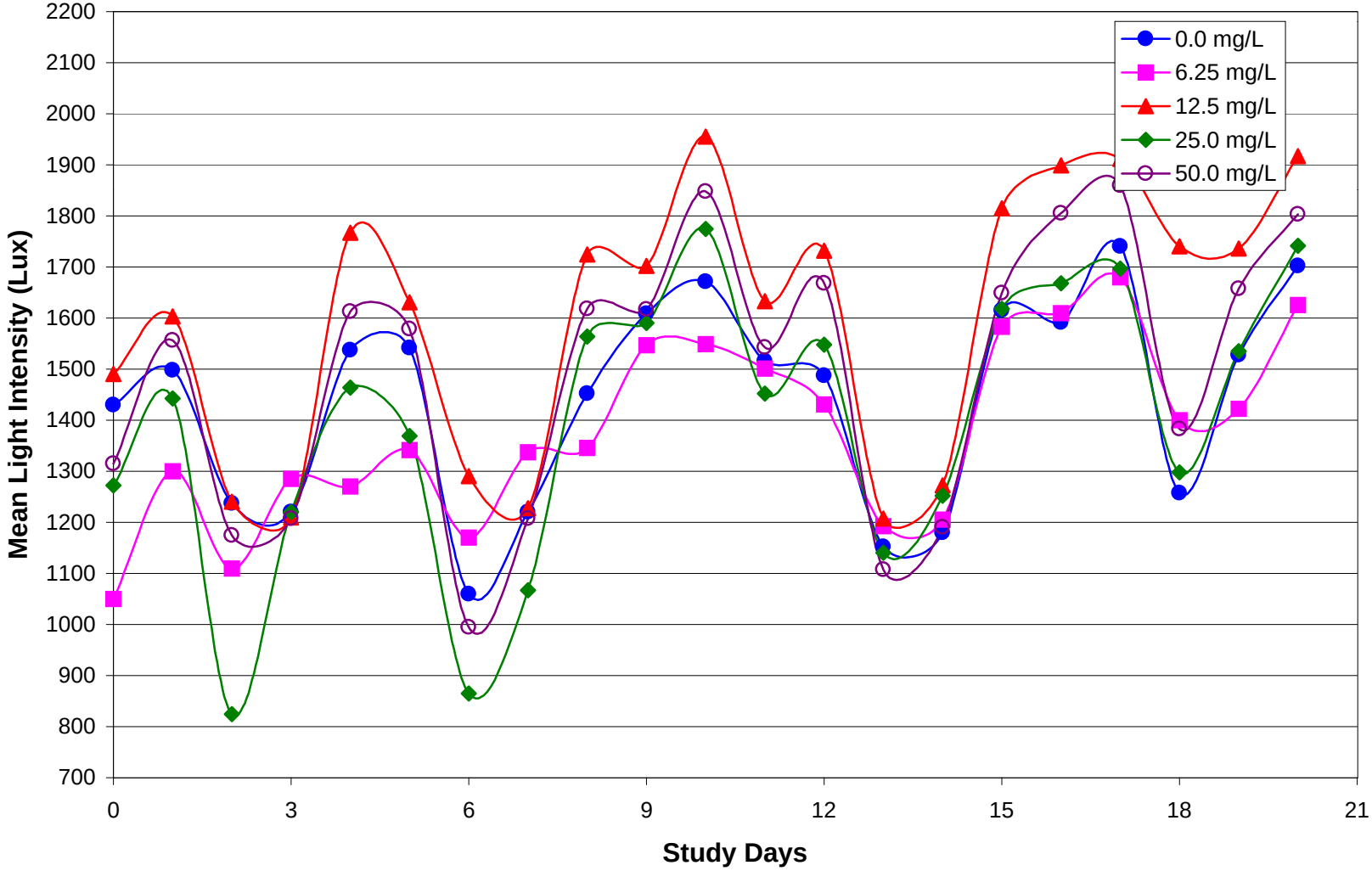
BATT02-00054
Methimazole
Daily Average pH for Initial Stage 51 Test



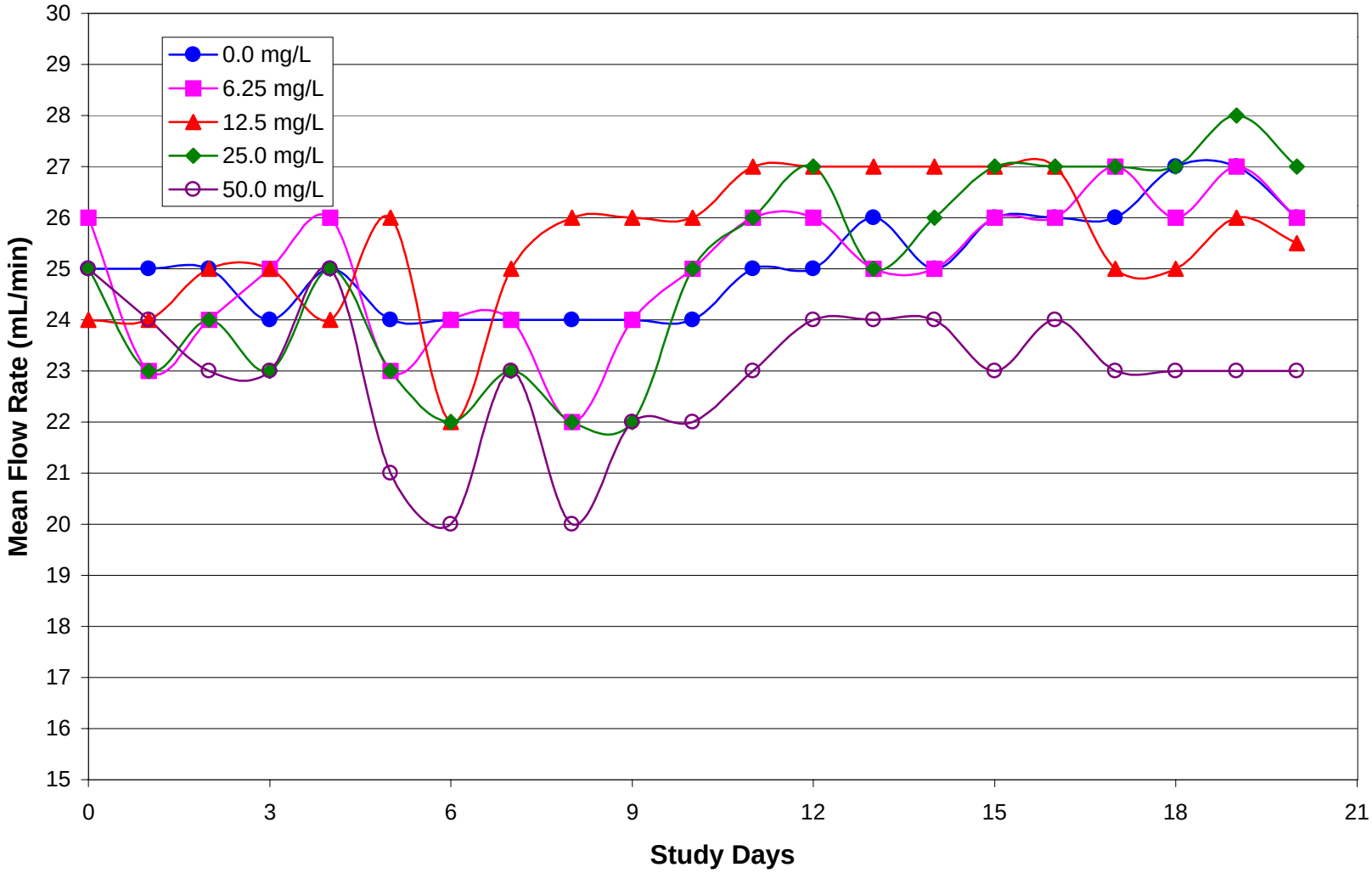
BATT02-00054
Methimazole
Daily Average Dissolved Oxygen (DO) for Initial Stage 51 Test



BATT02-00054
Methimazole
Daily Average Light Intensity for Initial Stage 51 Test



BATT02-00054
Methimazole
Daily Average Flow Rates for Initial Stage 51 Test



WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00054 **Test No: 1**
Test Chemical: Methimazole (MTZM) **Target Conc: 0.0 mg/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Conc[1] (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	04/16/04			100.0	21.7	7.3	10.4	1435	24			100.0	20.5	7.3	10.1	1309	24	100.0	21.1	7.3	10.3	1372	24	0.02
1	04/17/04		20	100.0	22.0	6.9	6.3	1595	24		20	100.0	20.4	7.1	9.1	1380	24	100.0	21.2	7.0	7.7	1488	24	
2	04/18/04		20	100.0	21.9	7.4	7.4	1780	24		20	100.0	20.4	7.6	9.2	1579	24	100.0	21.2	7.5	8.3	1680	24	
3	04/19/04		20	100.0	21.7	7.0	8.1	1410	25		20	100.0	21.3	7.0	8.3	1327	25	100.0	21.5	7.0	8.2	1369	25	
4	04/20/04		20	100.0	21.6	6.9	8.0	1452	25		20	100.0	21.1	7.0	8.3	1374	25	100.0	21.4	7.0	8.2	1413	25	
5	04/21/04		20	100.0	21.8	6.9	7.9	1520	26		20	100.0	21.5	7.1	8.1	1230	26	100.0	21.7	7.0	8.0	1375	26	
6	04/22/04		20	100.0	21.6	7.0	7.6	1205	25		20	100.0	21.2	7.0	7.8	1185	25	100.0	21.4	7.0	7.7	1195	25	
7	04/23/04		20	100.0	20.9	7.1	7.7	1506	26		20	100.0	21.3	7.2	7.6	1441	26	100.0	21.1	7.2	7.7	1474	26	
8	04/24/04		20	100.0	21.8	7.4	8.0	1733	26		20	100.0	20.9	7.4	7.9	1628	26	100.0	21.4	7.4	8.0	1681	26	
9	04/25/04		20	100.0	20.9	7.4	8.4	1711	26		20	100.0	20.5	7.3	8.2	1591	26	100.0	20.7	7.4	8.3	1651	26	
10	04/26/04		20	100.0	21.7	7.1	7.8	1295	27		20	100.0	21.4	7.2	7.7	1425	27	100.0	21.6	7.2	7.8	1360	27	
11	04/27/04		20	100.0	22.0	7.1	7.0	1507	27		20	100.0	21.9	7.2	7.2	1412	27	100.0	22.0	7.2	7.1	1460	27	
12	04/28/04		20	100.0	21.9	7.1	6.8	1720	26		20	100.0	21.8	7.2	6.8	1710	26	100.0	21.9	7.2	6.8	1715	26	
13	04/29/04		20	100.0	22.0	7.1	6.4	1575	26		20	100.0	21.6	7.2	6.6	1525	26	100.0	21.8	7.2	6.5	1550	26	0.02
14	04/30/04		20	100.0							20	100.0						100.0						
	Median:				**21.8**	**7.1**	**7.8**	**1514**	**26**				**21.3**	**7.2**	**8.0**	**1419**	**26**		**21.4**	**7.2**	**7.9**	**1467**	**26**	
	Mode:				**21.7**	**7.1**	**8.0**	**#N/A**	**26**				**20.5**	**7.2**	**8.3**	**#N/A**	**26**		**21.1**	**7.2**	**7.7**	**#N/A**	**26**	
	Mean:			**100.0**	**21.7**	**7.1**	**7.7**	**1532**	**25.5**			**100.0**	**21.1**	**7.2**	**8.1**	**1437**	**25.5**	**100.0**	**21.4**	**7.2**	**7.9**	**1484**	**25.5**	**0.020**
	SEM:			**0.00**	**0.10**	**0.05**	**0.27**	**45.11**	**0.27**			**0.00**	**0.14**	**0.04**	**0.25**	**40.97**	**0.27**	**0.00**	**0.09**	**0.05**	**0.23**	**40.93**	**0.27**	**0.00**
	% CV:			**0.00**	**7.68**	**6.92**	**36.34**	**431.26**	**20.18**			**0.00**	**11.21**	**6.20**	**33.22**	**404.40**	**20.18**	**0.00**	**7.43**	**6.35**	**31.21**	**397.49**	**20.18**	**0.00**

[1]All measured test concentrations reported by Battelle as less than method detection limit (MDL) [0.0338 mg/L] or none detected (ND) are expressed as 0.02 mg/L (1/2 of MDL).

DAILY DATA

Workorder No: 00054 **Test No: 1**
Test Chemical: Methimazole (MTZM) **Target Conc: 6.25 mg/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	04/16/04			100.0	20.2	7.4	10.3	1280	22			100.0	21.1	7.4	10.1	1541	22	100.0	20.7	7.4	10.2	1411	22	10.05
1	04/17/04		20	100.0	20.1	7.1	9.1	1557	24		20	100.0	21.2	7.1	8.7	1641	24	100.0	20.7	7.1	8.9	1599	24	
2	04/18/04		20	100.0	21.3	7.6	8.9	1600	25		20	100.0	20.0	7.6	9.1	1923	25	100.0	20.7	7.6	9.0	1762	25	
3	04/19/04		20	100.0	21.2	7.0	8.3	1471	26		20	100.0	21.1	7.1	8.5	1540	26	100.0	21.2	7.1	8.4	1506	26	
4	04/20/04		20	100.0	22.0	7.0	7.9	1485	26		20	100.0	21.3	7.0	8.1	1588	26	100.0	21.7	7.0	8.0	1537	26	
5	04/21/04		20	100.0	21.8	7.1	7.8	1480	25		20	100.0	21.5	7.1	7.8	1660	25	100.0	21.7	7.1	7.8	1570	25	
6	04/22/04		20	100.0	21.7	7.2	7.7	1170	25		20	100.0	21.0	7.1	7.7	1240	25	100.0	21.4	7.2	7.7	1205	25	6.44
7	04/23/04		20	100.0	22.0	7.2	7.3	1563	26		20	100.0	21.5	7.2	7.9	1757	26	100.0	21.8	7.2	7.6	1660	26	
8	04/24/04		20	100.0	21.8	7.5	7.8	1530	26		20	100.0	20.8	7.5	8.1	1881	26	100.0	21.3	7.5	8.0	1706	26	
9	04/25/04		20	100.0	21.5	7.4	8.3	1594	27		20	100.0	20.7	7.4	8.3	1907	27	100.0	21.1	7.4	8.3	1751	27	
10	04/26/04		20	100.0	21.6	7.3	7.9	1510	26		20	100.0	21.3	7.3	7.5	1190	26	100.0	21.5	7.3	7.7	1350	26	
11	04/27/04		20	100.0	21.3	7.2	6.8	1494	27		20	100.0	21.4	7.2	7.5	1644	27	100.0	21.4	7.2	7.2	1569	27	
12	04/28/04		20	100.0	21.3	7.2	6.4	1587	26		20	100.0	21.4	7.2	6.9	1996	26	100.0	21.4	7.2	6.7	1792	26	
13	04/29/04		20	100.0	21.6	7.2	6.2	1655	26		20	100.0	21.8	7.2	7.0	1805	26	100.0	21.7	7.2	6.6	1730	26	5.89
14	04/30/04		20	100.0							20	100.0						100.0						
	Median:				**21.6**	**7.2**	**7.9**	**1520**	**26**				**21.3**	**7.2**	**8.0**	**1652**	**26**		**21.4**	**7.2**	**7.9**	**1585**	**26**	
	Mode:				**21.3**	**7.2**	**8.3**	**#N/A**	**26**				**21.1**	**7.1**	**8.1**	**#N/A**	**26**		**20.7**	**7.2**	**7.7**	**#N/A**	**26**	
	Mean:			**100.0**	**21.4**	**7.2**	**7.9**	**1498**	**25.5**			**100.0**	**21.2**	**7.2**	**8.1**	**1665**	**25.5**	**100.0**	**21.3**	**7.2**	**8.0**	**1582**	**25.5**	**7.460**
	SEM:			**0.00**	**0.16**	**0.05**	**0.29**	**34.56**	**0.34**			**0.00**	**0.12**	**0.05**	**0.23**	**64.35**	**0.34**	**0.00**	**0.10**	**0.05**	**0.25**	**45.79**	**0.34**	**1.31**
	% CV:			**0.00**	**12.60**	**6.63**	**38.57**	**334.09**	**25.47**			**0.00**	**9.56**	**6.47**	**29.61**	**590.03**	**25.47**	**0.00**	**8.40**	**6.51**	**33.67**	**430.81**	**25.47**	**82.86**

DAILY DATA

Workorder No: 00054 **Test No: 1**
Test Chemical: Methimazole (MTZM) **Target Conc: 12.5 mg/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	04/16/04			100.0	21.7	7.4	9.7	1234	26			100.0	20.3	7.4	9.9	1085	26	100.0	21.0	7.4	9.8	1160	26	17.07
1	04/17/04		20	100.0	21.5	7.1	8.8	1449	26		20	100.0	20.5	7.1	8.5	1184	26	100.0	21.0	7.1	8.7	1317	26	
2	04/18/04		20	100.0	21.7	7.6	9.0	1224	26		20	100.0	20.5	7.5	7.5	1470	26	100.0	21.1	7.6	8.3	1347	26	
3	04/19/04		20	100.0	21.5	7.1	8.2	1407	27		20	100.0	20.4	7.1	8.3	1190	27	100.0	21.0	7.1	8.3	1299	27	
4	04/20/04		20	100.0	21.4	7.0	8.0	1463	27		20	100.0	21.1	7.0	8.0	1100	27	100.0	21.3	7.0	8.0	1282	27	
5	04/21/04		20	100.0	22.1	7.1	7.9	965	27		20	100.0	21.1	7.2	7.8	990	27	100.0	21.6	7.2	7.9	978	27	
6	04/22/04		20	100.0	21.8	6.9	7.2	1265	27		20	100.0	21.4	7.0	7.5	1305	27	100.0	21.6	7.0	7.4	1285	27	14.14
7	04/23/04		20	100.0	21.5	7.2	7.5	1300	27		20	100.0	21.5	7.2	7.3	1207	27	100.0	21.5	7.2	7.4	1254	27	
8	04/24/04		20	100.0	21.8	7.5	7.9	1230	27		20	100.0	21.5	7.5	7.7	1404	27	100.0	21.7	7.5	7.8	1317	27	
9	04/25/04		20	100.0	21.6	7.4	8.3	1250	25		20	100.0	20.4	7.4	8.1	1440	25	100.0	21.0	7.4	8.2	1345	25	
10	04/26/04		20	100.0	21.4	7.3	7.4	1245	25		20	100.0	21.2	7.3	7.6	1365	25	100.0	21.3	7.3	7.5	1305	25	
11	04/27/04		20	100.0	21.5	7.2	7.1	1251	26		20	100.0	21.2	7.2	6.8	1179	26	100.0	21.4	7.2	7.0	1215	26	
12	04/28/04		20	100.0	21.4	7.2	6.6	1266	26		20	100.0	21.3	7.2	6.3	1440	26	100.0	21.4	7.2	6.5	1353	26	
13	04/29/04		20	100.0	22.0	7.2	6.2	1050	25		20	100.0	20.9	7.1	6.2	1120	25	100.0	21.5	7.2	6.2	1085	25	12.48
14	04/30/04		20	100.0							20	100.0						100.0						
	Median:				**21.6**	**7.2**	**7.9**	**1251**	**26**				**21.1**	**7.2**	**7.7**	**1199**	**26**		**21.3**	**7.2**	**7.8**	**1292**	**26**	
	Mode:				**21.5**	**7.2**	**7.9**	**#N/A**	**27**				**20.5**	**7.2**	**7.5**	**1440**	**27**		**21.0**	**7.2**	**8.3**	**#N/A**	**27**	
	Mean:			**100.0**	**21.6**	**7.2**	**7.8**	**1257**	**26.2**			**100.0**	**21.0**	**7.2**	**7.7**	**1249**	**26.2**	**100.0**	**21.3**	**7.2**	**7.8**	**1253**	**26.2**	**14.566**
	SEM:			**0.00**	**0.06**	**0.05**	**0.25**	**35.89**	**0.22**			**0.00**	**0.12**	**0.05**	**0.25**	**41.24**	**0.22**	**0.00**	**0.07**	**0.05**	**0.24**	**29.31**	**0.22**	**1.34**
	% CV:			**0.00**	**4.82**	**7.21**	**33.72**	**378.79**	**16.08**			**0.00**	**9.65**	**6.26**	**33.74**	**436.76**	**16.08**	**0.00**	**5.39**	**6.67**	**32.82**	**309.84**	**16.08**	**60.89**

DAILY DATA

Workorder No: 00054 **Test No: 1**
Test Chemical: Methimazole (MTZM) **Target Conc: 25.0 mg/L**

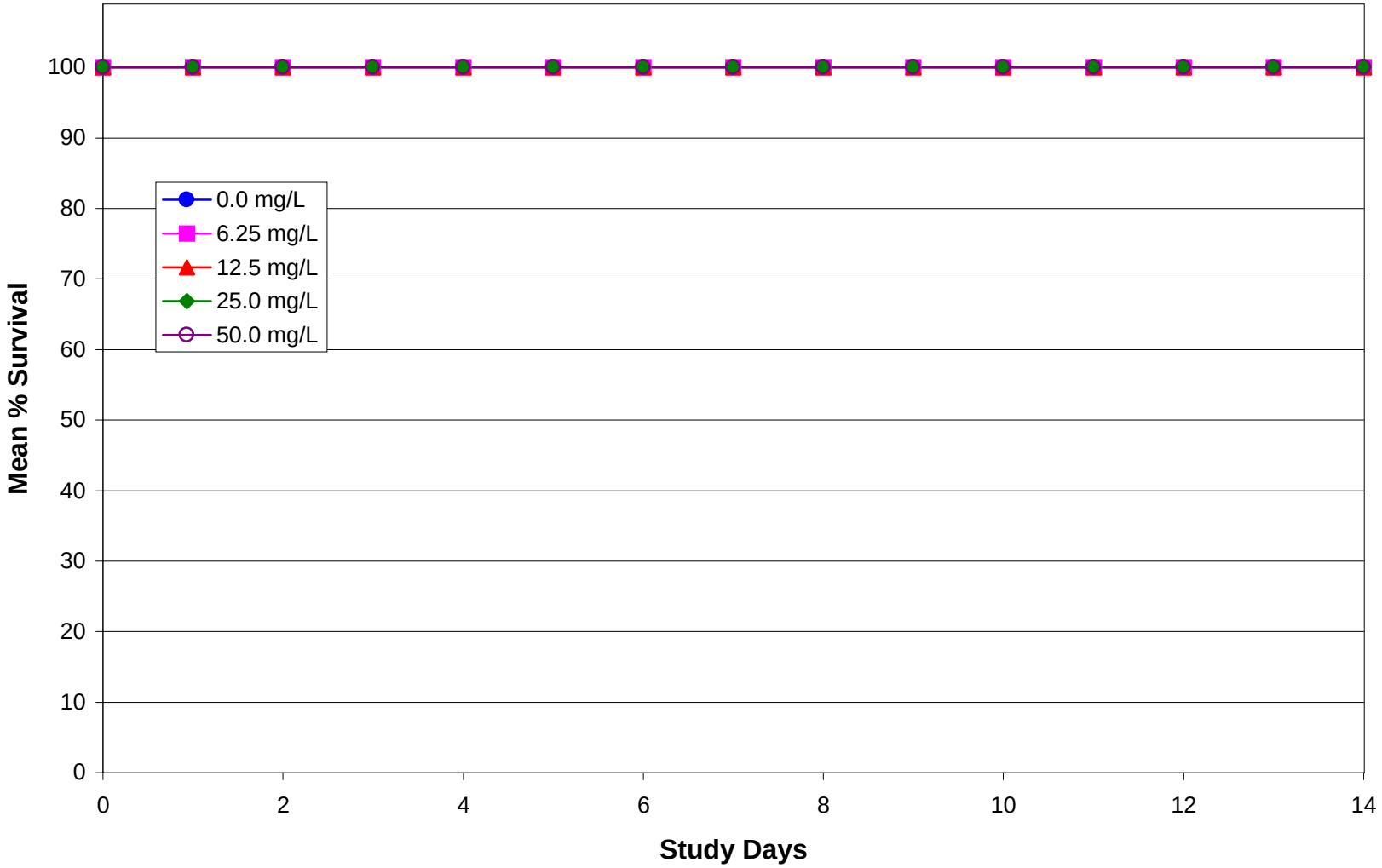
		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	04/16/04			100.0	20.3	7.4	10.3	1544	22			100.0	20.3	7.4	10.2	1529	22	100.0	20.3	7.4	10.3	1537	22	27.86
1	04/17/04		20	100.0	20.2	7.1	8.6	1731	22		20	100.0	20.1	7.1	8.7	1737	22	100.0	20.2	7.1	8.7	1734	22	
2	04/18/04		20	100.0	20.3	7.6	8.8	1907	25		20	100.0	20.2	7.6	8.9	1751	25	100.0	20.3	7.6	8.9	1829	25	
3	04/19/04		20	100.0	20.5	7.1	8.4	1598	26		20	100.0	20.8	7.1	8.5	1567	26	100.0	20.7	7.1	8.5	1583	26	
4	04/20/04		20	100.0	21.0	7.0	8.2	1607	27		20	100.0	21.0	7.1	8.2	1681	27	100.0	21.0	7.1	8.2	1644	27	
5	04/21/04		20	100.0	21.6	7.1	8.0	1625	25		20	100.0	21.4	7.0	7.9	1280	25	100.0	21.5	7.1	8.0	1453	25	
6	04/22/04		20	100.0	21.9	7.2	7.6	1275	26		20	100.0	21.4	7.1	7.5	1130	26	100.0	21.7	7.2	7.6	1203	26	24.58
7	04/23/04		20	100.0	20.9	7.3	7.8	1673	27		20	100.0	21.0	7.3	8.0	1785	27	100.0	21.0	7.3	7.9	1729	27	
8	04/24/04		20	100.0	20.9	7.5	8.2	1810	27		20	100.0	20.7	7.5	8.2	1725	27	100.0	20.8	7.5	8.2	1768	27	
9	04/25/04		20	100.0	20.7	7.4	8.4	1858	27		20	100.0	20.5	7.4	8.5	1812	27	100.0	20.6	7.4	8.5	1835	27	
10	04/26/04		20	100.0	21.6	7.4	7.6	1425	27		20	100.0	21.3	7.4	7.5	1190	27	100.0	21.5	7.4	7.6	1308	27	
11	04/27/04		20	100.0	21.0	7.2	7.2	1678	28		20	100.0	21.0	7.3	7.5	1725	28	100.0	21.0	7.3	7.4	1702	28	
12	04/28/04		20	100.0	21.1	7.3	7.1	1868	27		20	100.0	21.1	7.3	7.1	1873	27	100.0	21.1	7.3	7.1	1871	27	
13	04/29/04		20	100.0	20.9	7.2	6.9	1630	27		20	100.0	21.2	7.2	7.0	1240	27	100.0	21.1	7.2	7.0	1435	27	25.84
14	04/30/04		20	100.0							20	100.0						100.0						
	Median:				**20.9**	**7.3**	**8.1**	**1652**	**27**				**21.0**	**7.3**	**8.1**	**1703**	**27**		**21.0**	**7.3**	**8.1**	**1673**	**27**	
	Mode:				**20.9**	**7.4**	**8.4**	**#N/A**	**27**				**21.0**	**7.1**	**7.5**	**1725**	**27**		**21.0**	**7.4**	**8.5**	**#N/A**	**27**	
	Mean:			**100.0**	**20.9**	**7.3**	**8.1**	**1659**	**25.9**			**100.0**	**20.9**	**7.3**	**8.1**	**1573**	**25.9**	**100.0**	**20.9**	**7.3**	**8.1**	**1616**	**25.9**	**26.091**
	SEM:			**0.00**	**0.14**	**0.05**	**0.23**	**46.72**	**0.50**			**0.00**	**0.12**	**0.05**	**0.22**	**68.35**	**0.50**	**0.00**	**0.12**	**0.05**	**0.23**	**54.92**	**0.50**	**0.96**
	% CV:			**0.00**	**11.25**	**6.41**	**30.27**	**429.12**	**36.51**			**0.00**	**9.59**	**6.57**	**29.33**	**644.74**	**36.51**	**0.00**	**10.18**	**6.41**	**29.73**	**511.14**	**36.51**	**32.44**

DAILY DATA

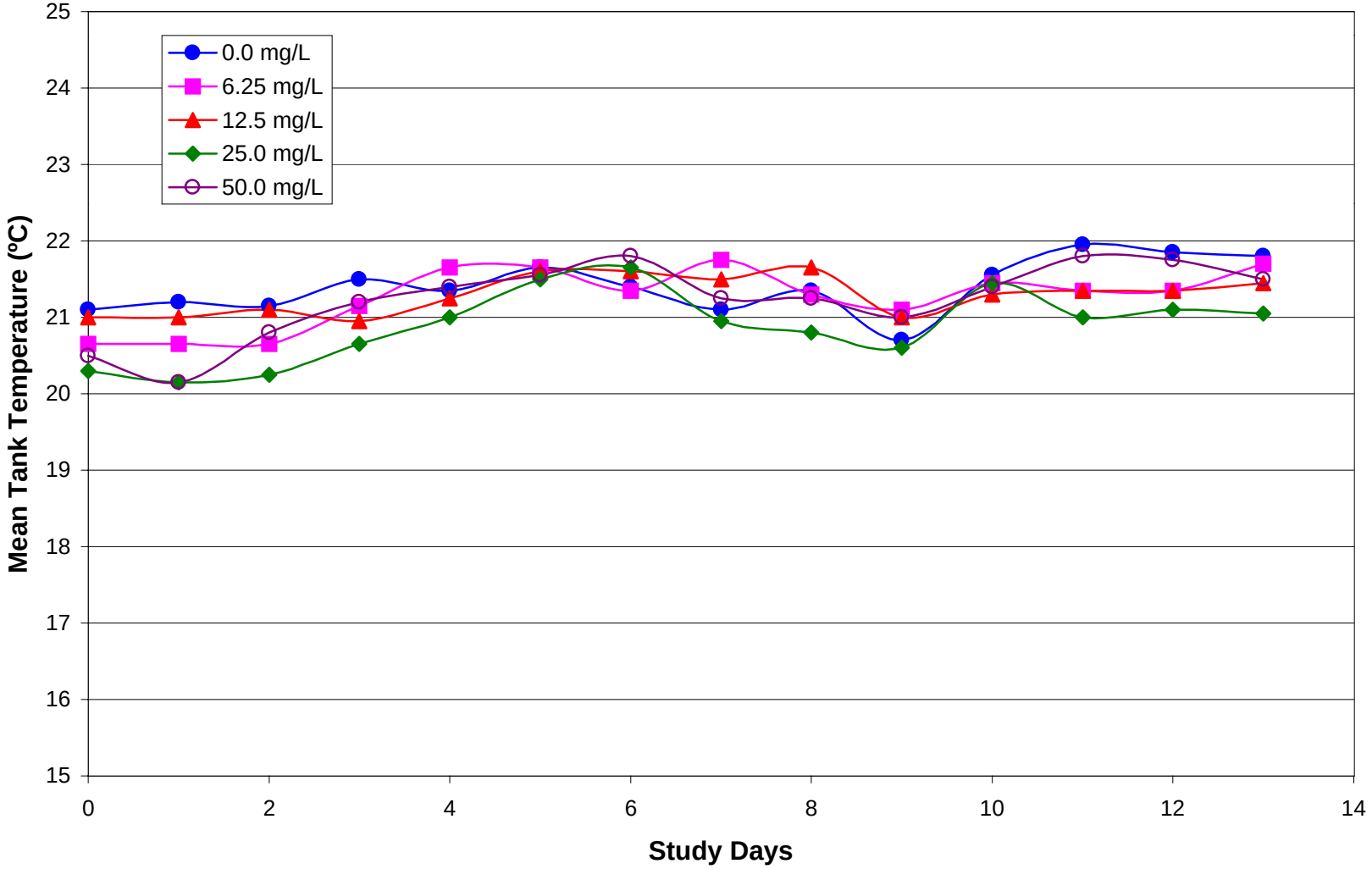
Workorder No: 00054 **Test No: 1**
Test Chemical: Methimazole **Target Conc: 50.0 mg/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**														
0	04/16/04			100.0	20.1	7.4	10.3	1749	20			100.0	20.9	7.4	9.9	1378	20	100.0	20.5	7.4	10.1	1564	20	55.73
1	04/17/04		20	100.0	20.1	7.2	8.8	1884	22		20	100.0	20.2	7.1	8.6	1455	22	100.0	20.2	7.2	8.7	1670	22	
2	04/18/04		20	100.0	21.6	7.6	8.9	1998	22		20	100.0	20.0	7.6	8.8	1789	22	100.0	20.8	7.6	8.9	1894	22	
3	04/19/04		20	100.0	21.2	7.1	8.5	1773	23		20	100.0	21.2	7.2	8.3	1351	23	100.0	21.2	7.2	8.4	1562	23	
4	04/20/04		20	100.0	21.5	7.1	8.0	1773	24		20	100.0	21.3	7.1	8.1	1364	24	100.0	21.4	7.1	8.1	1569	24	
5	04/21/04		20	100.0	21.9	7.2	7.9	1470	24		20	100.0	21.2	7.1	7.7	1060	24	100.0	21.6	7.2	7.8	1265	24	
6	04/22/04		20	100.0	22.0	7.2	7.1	1190	24		20	100.0	21.6	7.2	7.6	1210	24	100.0	21.8	7.2	7.4	1200	24	50.27
7	04/23/04		20	100.0	21.3	7.2	7.8	1768	23		20	100.0	21.2	7.3	8.9	1501	23	100.0	21.3	7.3	8.4	1635	23	
8	04/24/04		20	100.0	21.8	7.5	8.1	1790	24		20	100.0	20.7	7.5	8.1	1818	24	100.0	21.3	7.5	8.1	1804	24	
9	04/25/04		20	100.0	21.6	7.4	8.4	1771	23		20	100.0	20.4	7.4	8.4	1870	23	100.0	21.0	7.4	8.4	1821	23	
10	04/26/04		20	100.0	21.4	7.4	7.4	1260	23		20	100.0	21.4	7.4	7.7	1540	23	100.0	21.4	7.4	7.6	1400	23	
11	04/27/04		20	100.0	21.9	7.3	7.1	1711	23		20	100.0	21.7	7.3	7.2	1677	23	100.0	21.8	7.3	7.2	1694	23	
12	04/28/04		20	100.0	21.8	7.3	6.6	1823	23		20	100.0	21.7	7.3	6.6	1829	23	100.0	21.8	7.3	6.6	1826	23	
13	04/29/04		20	100.0	21.7	7.2	6.2	1750	22		20	100.0	21.3	7.2	6.6	1360	22	100.0	21.5	7.2	6.4	1555	22	52.17
14	04/30/04		20	100.0							20	100.0						100.0						
	Median:				**21.6**	**7.3**	**8.0**	**1770**	**23**				**21.2**	**7.3**	**8.1**	**1478**	**23**		**21.3**	**7.3**	**8.1**	**1602**	**23**	
	Mode:				**20.1**	**7.2**	**7.1**	**1773**	**23**				**21.2**	**7.4**	**8.1**	**#N/A**	**23**		**21.4**	**7.4**	**8.4**	**#N/A**	**23**	
	Mean:			**100.0**	**21.4**	**7.3**	**7.9**	**1694**	**22.8**			**100.0**	**21.1**	**7.3**	**8.0**	**1514**	**22.8**	**100.0**	**21.2**	**7.3**	**8.0**	**1604**	**22.8**	**52.722**
	SEM:			**0.00**	**0.16**	**0.04**	**0.28**	**61.00**	**0.30**			**0.00**	**0.15**	**0.04**	**0.24**	**66.91**	**0.30**	**0.00**	**0.13**	**0.04**	**0.26**	**55.48**	**0.30**	**1.60**
	% CV:			**0.00**	**13.11**	**5.52**	**37.27**	**554.63**	**23.80**			**0.00**	**11.87**	**5.71**	**31.81**	**643.29**	**23.80**	**0.00**	**10.58**	**5.52**	**33.97**	**518.28**	**23.80**	**38.21**

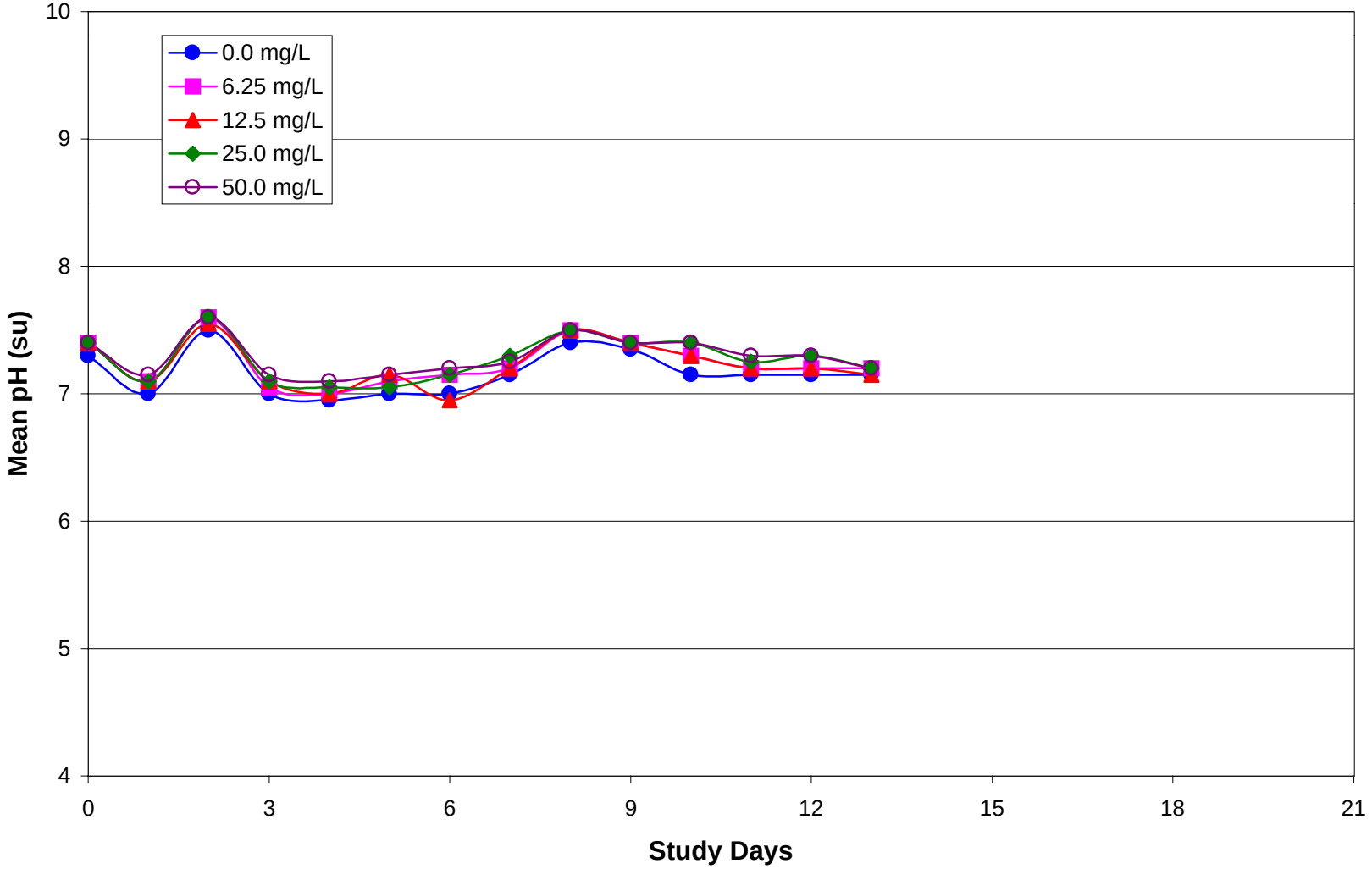
BATT02-00054
Methimazole
Daily Average of Larval Survival for Initial Stage 54 Test



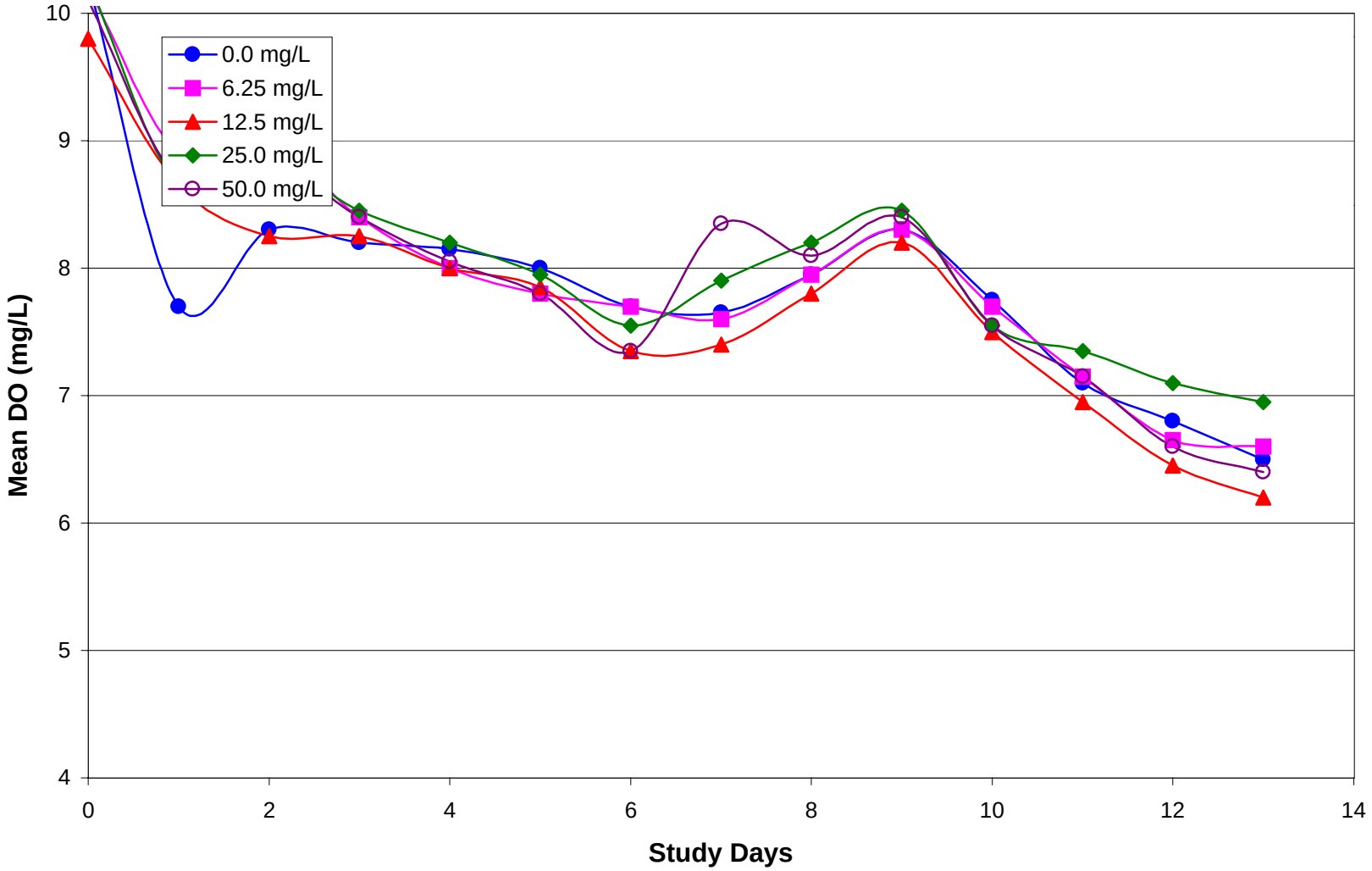
BATT02-00054
Methimazole
Daily Average Tank Temperatures for Initial Stage 54 Test



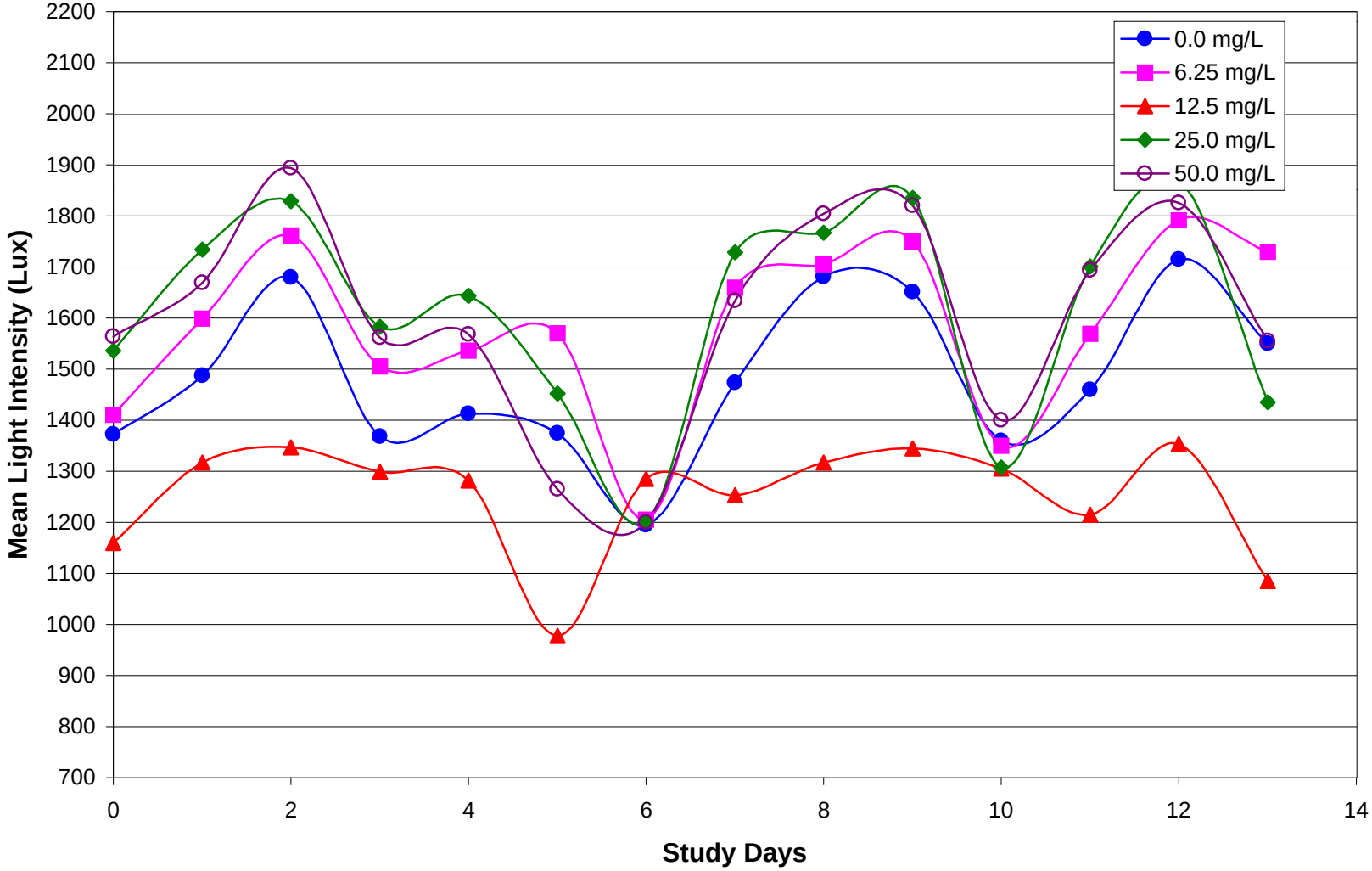
BATT02-00054
Methimazole
Daily Average pH for Initial Stage 54 Test



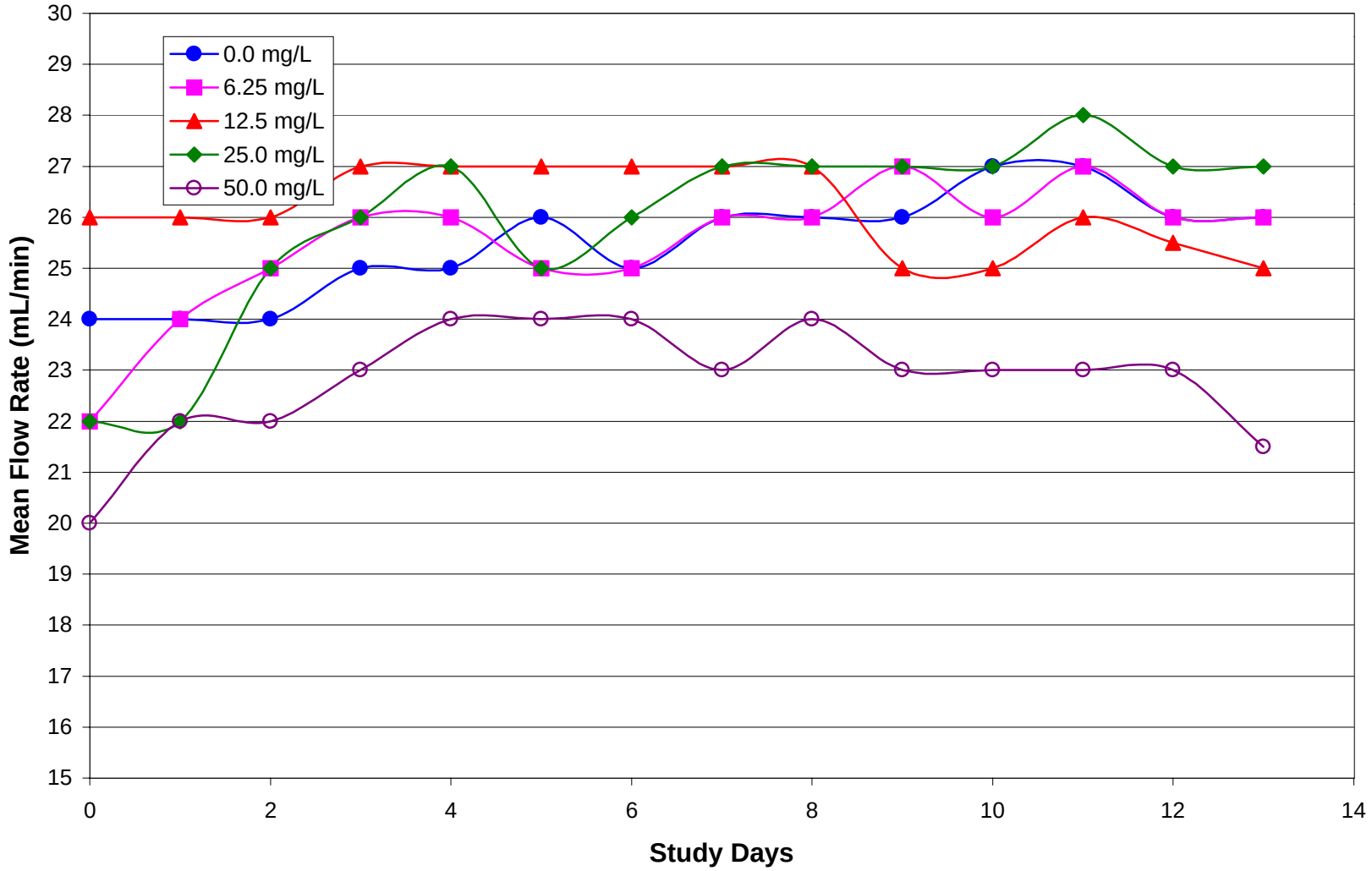
BATT02-00054
Methimazole
Daily Average Dissolved Oxygen (DO) for Initial Stage 54 Test



BATT02-00054
Methimazole
Daily Average Light Intensity for Initial Stage 54 Test



BATT02-00054
Methimazole
Daily Average Flow Rates for Initial Stage 54 Test



Methimazole
Metamorphosis Assay:
Test Termination Data – Stage and Growth

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00054 **Test No: 1**

Test Chemical: Methimazole (MTZM) **Target Conc: 0.0 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	001	0.5962	57	
	002	0.5772	57	
	003	0.4578	56	Kinked Tail
	004	0.3345	56	
	005	0.3557	56	
	006	0.4870	57	
	007	0.5650	57	
	008	0.7802	59	
	009	0.4847	56	
	010	0.4374	56	
	011	0.5721	57	
	012	0.4745	56	
	013	0.3990	56	
	014	0.6376	57	Kinked Tail
	015	0.5999	57	Kinked Tail
	016	0.6940	59	Kinked Tail
	017	0.5047	57	
	018	0.3942	56	Kinked Tail
	019	0.3773	56	
	020	0.6030	59	
	Median:	**0.496**	**57**	
	Mode:		**56**	
	Mean:	**0.517**	**56.850**	
	SEM:	**0.026**	**0.233**	
	%CV:	**16.469**	**13.793**	
B	021	0.8200	57	
	022	0.4030	56	
	023	0.7341	57	
	024	0.4693	56	Kinked Tail
	025	0.6868	57	
	026	0.5940	56	
	027	0.7003	57	
	028	0.3541	55	
	029	0.5543	57	
	030	0.3793	56	
	031	0.4042	56	
	032	0.6392	57	Kinked Tail
	033	0.5631	56	
	034	0.4961	56	
	035	0.6229	57	
	036	0.4059	55	
	037	0.6370	56	Kinked Tail
	038	0.5625	57	
	039	0.6176	57	
	040	0.5810	57	
	Median:	**0.572**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.561**	**56.400**	
	SEM:	**0.029**	**0.152**	
	%CV:	**17.182**	**9.062**	
	Median:	**0.563**	**57**	
	Mode:		**57**	
	Total Mean:	**0.539**	**56.625**	
	SEM:	**0.020**	**0.142**	
	%CV:	**16.909**	**11.919**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00054 **Test No: 1**

Test Chemical: Methimazole (MTZM) **Target Conc: 6.25 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	041	0.8730	57	
	042	0.9673	59	
	043	0.7969	59	
	044	0.6981	56	Kinked Tail
	045	0.7666	57	
	046	0.8508	54	
	047	0.4371	55	
	048	0.4902	57	
	049	0.5589	57	
	050	0.4242	56	
	051	0.3609	55	Kinked Tail
	052	0.5973	56	
	053	0.3632	55	
	054	0.4806	56	
	055	0.4760	56	Kinked Tail
	056	0.7566	57	
	057	0.4251	56	
	058	0.5156	57	Kinked Tail
	059	0.4298	56	
	060	0.5926	56	Kinked Tail
	Median:	**0.537**	**56**	
	Mode:		**56**	
	Mean:	**0.593**	**56.350**	
	SEM:	**0.041**	**0.274**	
	%CV:	**24.071**	**16.330**	
B	061	0.8280	57	Kinked Tail
	062	0.7107	57	Kinked Tail
	063	0.6986	57	Kinked Tail
	064	0.3988	55	
	065	0.8096	57	
	066	0.6888	57	
	067	0.7906	57	
	068	0.6172	57	
	069	0.2466	54	
	070	0.6646	57	
	071	0.3930	56	
	072	0.8563	57	
	073	0.4173	55	
	074	0.4719	56	
	075	0.5216	56	Kinked Tail
	076	0.5302	56	
	077	0.5842	56	
	078	0.5191	56	Kinked Tail
	079	0.5816	56	Kinked Tail
	080	0.5104	56	
	Median:	**0.583**	**56**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.592**	**56.250**	
	SEM:	**0.037**	**0.190**	
	%CV:	**21.402**	**11.343**	
	Median:	**0.570**	**56**	
	Mode:		**56**	
	Total Mean:	**0.592**	**56.300**	
	SEM:	**0.027**	**0.165**	
	%CV:	**22.483**	**13.896**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00054 **Test No: 1**

Test Chemical: Methimazole (MTZM) **Target Conc: 12.5 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	081	0.6426	56	Kinked Tail
	082	0.7099	57	
	083	0.4870	55	
	084	0.2906	55	
	085	0.3016	53	
	086	0.4744	53	
	087	0.4299	56	
	088	0.4972	56	
	089	0.3774	54	Kinked Tail
	090	0.6129	56	
	091	0.9591	57	
	092	0.5318	55	
	093	0.2747	52	
	094	0.5049	56	
	095	0.6382	56	Kinked Tail
	096	0.5709	55	
	097	0.3980	55	
	098	0.3384	55	
	099	0.3833	54	
	100	0.4703	55	
	Median:	**0.481**	**55**	
	Mode:		**55**	
	Mean:	**0.495**	**55.050**	
	SEM:	**0.037**	**0.294**	
	%CV:	**23.483**	**17.749**	
B	101	0.3720	54	
	102	0.6905	57	
	103	0.7207	57	
	104	0.3821	55	
	105	0.5467	56	
	106	0.5264	56	
	107	0.5604	57	
	108	0.8555	56	
	109	0.4726	56	
	110	0.4493	55	
	111	0.5781	56	
	112	0.7314	53	
	113	0.4735	55	
	114	0.3285	54	
	115	0.2775	52	
	116	0.6248	56	
	117	0.6037	56	
	118	0.3648	56	
	119	0.3371	54	
	120	0.3268	53	
	Median:	**0.500**	**56**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.511**	**55.200**	
	SEM:	**0.036**	**0.321**	
	%CV:	**22.450**	**19.333**	
	Median:	**0.481**	**55**	
	Mode:		**56**	
	Total Mean:	**0.503**	**55.125**	
	SEM:	**0.025**	**0.215**	
	%CV:	**22.698**	**18.348**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00054 **Test No: 1**

Test Chemical: Methimazole (MTZM) **Target Conc: 25.0 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	121	0.9657	55	Kinked Tail
	122	0.3183	53	
	123	0.3457	55	
	124	0.5176	55	
	125	0.4952	55	
	126	0.6197	55	
	127	0.2647	54	
	128	0.6542	55	
	129	0.4598	55	
	130	0.3869	55	
	131	0.7032	54	Kinked Tail
	132	0.3121	54	
	133	0.3634	54	
	134	0.2737	54	
	135	0.5404	55	Kinked Tail
	136	0.5798	54	
	137	0.6710	53	
	138	0.4465	54	
	139	0.3587	54	
	140	0.2868	54	
	Median:	**0.453**	**54**	
	Mode:		**55**	
	Mean:	**0.478**	**54.350**	
	SEM:	**0.041**	**0.150**	
	%CV:	**26.215**	**9.099**	
B	141	0.5296	54	Kinked Tail
	142	0.7037	55	
	143	1.4578	55	Kinked Tail
	144	0.3911	55	
	145	0.9042	55	
	146	0.6540	54	
	147	0.5481	54	
	148	0.3610	54	
	149	1.0542	55	
	150	0.3297	54	
	151	0.5094	54	
	152	0.5488	55	
	153	1.2291	55	
	154	0.6980	55	
	155	0.4123	54	Kinked Tail
	156	0.3220	54	
	157	0.3984	54	
	158	0.2240	53	
	159	0.6544	54	
	160	0.6478	54	
	Median:	**0.548**	**54**	**Comments:**
	Mode:		**54**	
	Rep Mean:	**0.629**	**54.350**	
	SEM:	**0.071**	**0.131**	
	%CV:	**40.181**	**7.964**	
	Median:	**0.514**	**54**	
	Mode:		**54**	
	Total Mean:	**0.554**	**54.350**	
	SEM:	**0.042**	**0.098**	
	%CV:	**35.890**	**8.440**	

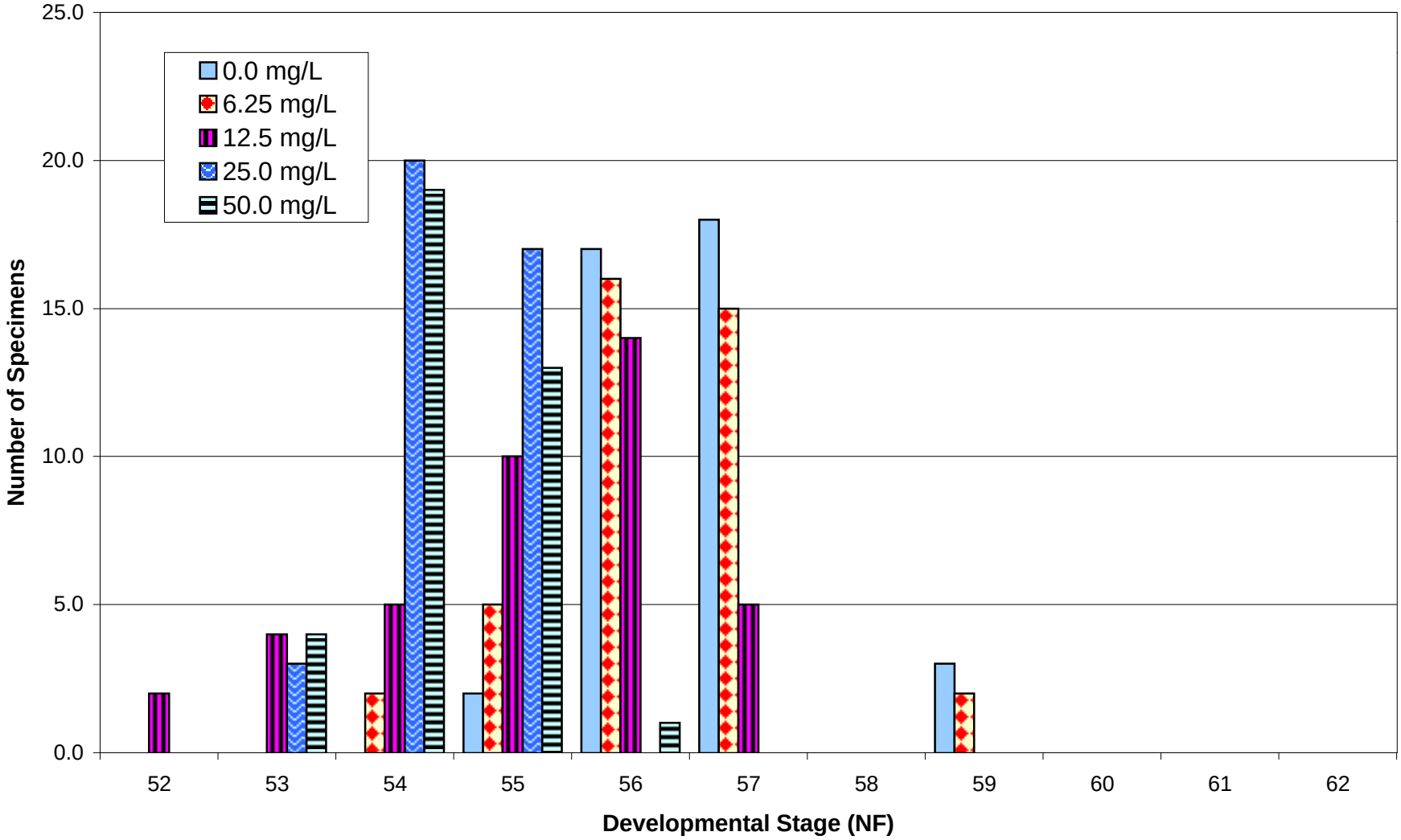
TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00054 **Test No: 1**

Test Chemical: Methimazole (MTZM) **Target Conc: 50.0 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	161	1.4171	55	
	162	0.6574	55	
	163	0.5335	54	
	164	0.4321	53	
	165	0.3775	54	
	166	0.7996	54	
	167	0.4428	53	
	168	0.3543	53	
	169	0.5608	55	Kinked Tail
	170	0.5620	55	
	171	0.5379	54	
	172	0.6190	54	
	173	1.2539	54	
	174	0.5355	55	
	175	0.5072	54	
	176	0.8654	54	Kinked Tail
	177	0.4437	55	
	178	0.2347	54	
	179	0.5336	54	
	180	0.7018	54	
	Median:	**0.537**	**54**	
	Mode:		**54**	
	Mean:	**0.618**	**54.150**	
	SEM:	**0.064**	**0.150**	
	%CV:	**36.456**	**9.116**	
B	181	0.6376	55	
	182	0.7011	55	
	183	0.4229	54	
	184	1.0392	55	
	185	0.5638	54	
	186	0.3418	54	
	187	0.5108	55	
	188	0.8800	54	
	189	0.4453	53	
	190	0.5266	55	
	191	0.6931	54	Kinked Tail
	192	0.5633	55	
	193	0.7403	55	
	194	0.6891	54	
	195	0.6117	56	
	196	0.2913	54	
	197	0.4105	54	
	Median:	**0.564**	**54**	**Comments:**
	Mode:		**54**	
	Rep Mean:	**0.592**	**54.471**	
	SEM:	**0.046**	**0.174**	
	%CV:	**24.900**	**9.721**	
	Median:	**0.561**	**54**	
	Mode:		**54**	
	Total Mean:	**0.606**	**54.297**	
	SEM:	**0.040**	**0.115**	
	%CV:	**31.423**	**9.524**	

BATT02-00054
Methimazole
Stage Distribution for Initial Stage 51 Test



TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00054 **Test No: 1**

Test Chemical: Methimazole (MTZM) **Target Conc: 0.0 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	198	0.9049	59	
	199	0.4066	62	
	200	0.6986	57	
	201	0.5421	57	
	202	0.4438	62	
	203	0.7461	59	
	204	0.8251	59	Kinked Tail
	205	0.7092	58	
	206	0.6545	57	
	207	0.5186	57	
	208	0.3977	62	
	209	0.6169	59	
	210	0.4548	56	
	211	0.5837	57	
	212	0.8272	59	Kinked Tail
	213	0.8391	59	
	214	0.5448	60	
	215	0.7311	57	
	216	0.3458	62	
	217	0.4289	56	
	Median:	**0.600**	**59**	
	Mode:		**59**	
	Mean:	**0.611**	**58.700**	
	SEM:	**0.038**	**0.454**	
	%CV:	**21.656**	**26.479**	
D	218	0.7806	59	
	219	0.7009	59	
	220	0.7538	57	
	221	0.5591	57	
	222	0.4466	57	
	223	0.8496	59	
	224	0.8902	59	Kinked Tail
	225	0.7450	59	
	226	0.5161	57	
	227	0.5490	57	
	228	0.6700	57	
	229	0.7091	59	
	230	0.8408	57	
	231	0.9907	59	
	232	0.7045	57	
	233	0.5104	57	Kinked Tail
	234	0.5332	60	
	235	0.5592	57	
	236	0.5113	60	
	237	0.4191	57	
	Median:	**0.685**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.662**	**58.000**	
	SEM:	**0.036**	**0.262**	
	%CV:	**19.640**	**15.360**	
	Median:	**0.636**	**59**	
	Mode:		**57**	
	Total Mean:	**0.636**	**58.350**	
	SEM:	**0.026**	**0.264**	
	%CV:	**20.622**	**21.896**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00054 **Test No: 1**

Test Chemical: Methimazole (MTZM) **Target Conc: 6.25 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	238	0.7896	59	
	239	0.7996	57	
	240	0.6692	59	Kinked Tail
	241	0.6715	57	
	242	0.6464	57	
	243	0.5242	61	
	244	0.8033	59	
	245	0.8300	57	Kinked Tail
	246	0.5281	57	
	247	0.8531	59	Kinked Tail
	248	0.7075	59	Kinked Tail
	249	0.9969	59	Kinked Tail
	250	0.7596	59	Kinked Tail
	251	0.5929	60	
	252	0.7403	57	Kinked Tail
	253	0.7686	59	
	254	0.5798	60	
	255	0.7250	57	
	256	0.6504	59	Kinked Tail
	257	0.5020	57	
	Median:	**0.716**	**59**	
	Mode:		**59**	
	Mean:	**0.707**	**58.400**	
	SEM:	**0.028**	**0.285**	
	%CV:	**14.930**	**16.661**	
D	258	0.9348	59	
	259	0.7091	59	
	260	0.5602	57	
	261	0.8678	59	
	262	0.9200	59	Kinked Tail
	263	0.7343	59	
	264	0.7234	59	
	265	0.9255	57	
	266	0.8935	59	
	267	0.5619	57	
	268	0.9540	57	Kinked Tail
	269	0.4769	60	
	270	0.5218	60	
	271	0.7806	57	
	272	0.5660	57	
	273	0.5367	57	
	274	1.0045	56	Kinked Tail
	275	0.6790	57	
	276	0.4277	56	
	277	0.3948	56	
	Median:	**0.716**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.709**	**57.850**	
	SEM:	**0.043**	**0.302**	
	%CV:	**23.069**	**17.729**	
	Median:	**0.716**	**59**	
	Mode:		**59**	
	Total Mean:	**0.708**	**58.125**	
	SEM:	**0.026**	**0.209**	
	%CV:	**19.185**	**17.367**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00054 **Test No: 1**

Test Chemical: Methimazole (MTZM) **Target Conc: 12.5 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	278	0.9441	57	
	279	0.8701	57	
	280	0.9158	57	
	281	1.1246	57	Kinked Tail
	282	1.3642	57	Kinked Tail
	283	0.6218	57	
	284	1.0720	57	Kinked Tail
	285	0.4744	56	
	286	0.7727	57	
	287	0.7518	59	Kinked Tail
	288	0.9463	57	Kinked Tail
	289	0.4422	56	
	290	0.8853	57	
	291	0.5124	56	Kinked Tail
	292	1.0617	57	Kinked Tail
	293	0.7979	57	
	294	0.7129	57	Kinked Tail
	295	0.6771	57	Kinked Tail
	296	0.6786	57	
	297	1.1091	57	
	Median:	**0.834**	**57**	
	Mode:		**57**	
	Mean:	**0.837**	**56.950**	
	SEM:	**0.054**	**0.135**	
	%CV:	**26.202**	**8.014**	
D	298	1.6091	57	
	299	0.8251	57	
	300	0.9484	57	Kinked Tail
	301	1.0076	57	Kinked Tail
	302	0.9982	57	Kinked Tail
	303	1.0186	57	
	304	1.0028	57	
	305	1.0194	57	
	306	1.0432	57	Kinked Tail
	307	0.5820	57	
	308	0.8356	57	
	309	0.5914	57	
	310	0.9557	56	Kinked Tail
	311	1.3645	57	Kinked Tail
	312	0.8024	57	Kinked Tail
	313	0.7804	56	Kinked Tail
	314	0.8903	57	Kinked Tail
	315	0.5340	56	Kinked Tail
	316	0.5204	56	
	317	0.5176	57	
	Median:	**0.919**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.892**	**56.800**	
	SEM:	**0.062**	**0.092**	
	%CV:	**29.323**	**5.445**	
	Median:	**0.878**	**57**	
	Mode:		**57**	
	Total Mean:	**0.865**	**56.875**	
	SEM:	**0.041**	**0.082**	
	%CV:	**27.663**	**6.839**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00054 **Test No: 1**

Test Chemical: Methimazole (MTZM) **Target Conc: 25.0 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	318	0.6045	57	
	319	0.7651	57	Kinked Tail
	320	0.7659	57	
	321	0.8159	57	
	322	0.8035	57	
	323	0.6915	57	
	324	1.0752	57	Kinked Tail
	325	0.6955	55	
	326	0.7741	57	Kinked Tail
	327	0.5164	56	
	328	0.8295	57	Kinked Tail
	329	0.7039	57	Kinked Tail
	330	0.4680	56	
	331	1.0572	56	Kinked Tail
	332	1.0023	56	
	333	0.6425	56	
	334	1.2657	57	Kinked Tail
	335	0.6993	57	
	336	0.6713	57	Kinked Tail
	337	0.8509	57	Kinked Tail
	Median:	**0.766**	**57**	
	Mode:		**57**	
	Mean:	**0.785**	**56.650**	
	SEM:	**0.043**	**0.131**	
	%CV:	**21.884**	**7.801**	
D	338	0.9876	56	Kinked Tail
	339	0.8172	56	Kinked Tail
	340	1.0791	57	Kinked Tail
	341	0.2996	55	
	342	1.0183	58	
	343	0.5395	56	Kinked Tail
	344	0.6022	55	Kinked Tail
	345	0.7680	57	Kinked Tail
	346	0.5122	57	
	347	0.4983	56	Kinked Tail
	348	0.7770	57	Kinked Tail
	349	0.6438	56	Kinked Tail
	350	0.6116	56	
	351	0.6070	57	Kinked Tail
	352	0.5162	57	
	353	0.9464	57	Kinked Tail
	354	0.7365	57	
	355	1.0805	56	Kinked Tail
	356	0.6988	56	
	357	0.7531	56	
	Median:	**0.718**	**56**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.725**	**56.400**	
	SEM:	**0.048**	**0.169**	
	%CV:	**25.203**	**10.039**	
	Median:	**0.745**	**57**	
	Mode:		**57**	
	Total Mean:	**0.755**	**56.525**	
	SEM:	**0.032**	**0.107**	
	%CV:	**23.496**	**9.030**	

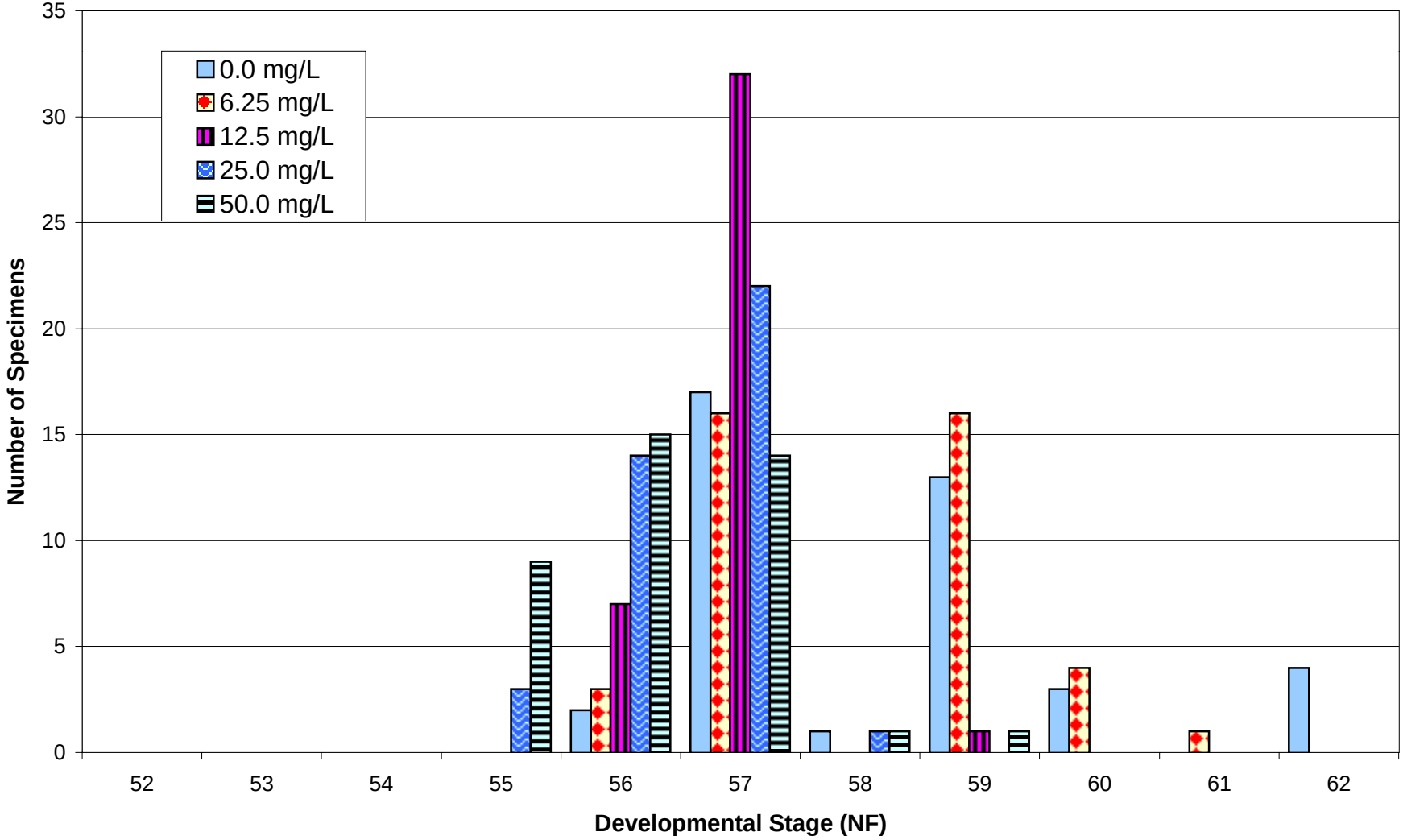
TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00054 **Test No: 1**

Test Chemical: Methimazole (MTZM) **Target Conc: 50.0 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	358	1.1700	55	
	359	0.9843	58	Kinked Tail
	360	0.8272	57	
	361	0.8189	57	Kinked Tail
	362	0.4465	56	
	363	0.3811	55	
	364	0.5850	56	Kinked Tail
	365	0.5721	55	
	366	0.6652	57	
	367	0.8029	56	Kinked Tail
	368	0.8409	57	
	369	0.9388	57	Kinked Tail
	370	0.5101	56	
	371	0.7364	57	Kinked Tail
	372	0.6892	57	
	373	0.9355	55	Kinked Tail
	374	0.7535	57	Kinked Tail
	375	0.5994	56	
	376	0.7161	57	Kinked Tail
	377	0.8548	57	Kinked Tail
	Median:	**0.745**	**57**	
	Mode:		**57**	
	Mean:	**0.741**	**56.400**	
	SEM:	**0.043**	**0.197**	
	%CV:	**22.485**	**11.752**	
D	378	1.2775	57	
	379	1.2899	56	Kinked Tail
	380	0.9230	59	
	381	1.1225	57	Kinked Tail
	382	0.6009	56	
	383	1.1265	56	
	384	0.9085	56	Kinked Tail
	385	0.7597	56	
	386	0.6010	56	
	387	0.7724	56	Kinked Tail
	388	0.3684	55	
	389	0.7908	56	
	390	0.7782	57	
	391	0.4620	57	
	392	0.4480	56	
	393	0.6222	56	
	394	0.4856	55	Kinked Tail
	395	0.8162	55	
	396	0.4923	55	
	397	0.6078	55	Kinked Tail
	Median:	**0.766**	**56**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.763**	**56.100**	
	SEM:	**0.062**	**0.216**	
	%CV:	**31.596**	**12.923**	
	Median:	**0.757**	**56**	
	Mode:		**56**	
	Total Mean:	**0.752**	**56.250**	
	SEM:	**0.037**	**0.147**	
	%CV:	**27.159**	**12.357**	

BATT02-00054
Methimazole
Stage Distribution for Initial Stage 54 Test



Methimazole
Metamorphosis Assay:
Statistical Data – Stage and Growth

One Way Analysis of Variance

Data source: BATT02-00054_MTMZ_Stage 51_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0mg/L	40	0	57	56	57
6.25mg/L	40	0	56	56	57
12.5mg/L	40	0	55	54	56
25.0mg/L	40	0	54	54	55
50.0mg/L	37	0	54	54	55

H = 110.149 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
50.0mg/L vs 0.0mg/L	100.015	7.691	Yes	
25.0mg/L vs 0.0mg/L	98.188	7.702	Yes	
12.5mg/L vs 0.0mg/L	58.763	4.609	Yes	**LOEC**
6.25mg/L vs 0.0mg/L	13.1	1.028	No	**NOEC**

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00054_MTMZ_Stage 51_Growth

Normality Test: Failed (P = 0.004)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0mg/L	40	0	0.563	0.422	0.62
6.25mg/L	40	0	0.57	0.455	0.734
12.5mg/L	40	0	0.481	0.375	0.608
25.0mg/L	40	0	0.513	0.36	0.654
50.0mg/L	37	0	0.561	0.443	0.695

H = 7.697 with 4 degrees of freedom. (P = 0.103)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.103)

One Way Analysis of Variance

Data source: BATT02-00054_MTMZ_Stage 54_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0mg/L	40	0	58.5	57	59
6.25mg/L	40	0	59	57	59
12.5mg/L	40	0	57	57	57
25.0mg/L	40	0	57	56	57
50.0mg/L	40	0	56	56	57

H = 76.562 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
50.0mg/L vs 0.0mg/L	81.762	6.318	Yes	
25.0mg/L vs 0.0mg/L	67.087	5.184	Yes	
12.5mg/L vs 0.0mg/L	46.925	3.626	Yes	**LOEC**
6.25mg/L vs 0.0mg/L	2.85	0.22	No	**NOEC**

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00054_MTMZ_Stage 54_Growth

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.083)

Group Name	N	Missing	Mean	Std Dev	SEM
0.0mg/L	40	0	0.636	0.165	0.026
6.25mg/L	40	0	0.708	0.161	0.0255
12.5mg/L	40	0	0.865	0.257	0.0407
25.0mg/L	40	0	0.755	0.204	0.0323
50.0mg/L	40	0	0.752	0.236	0.0372

Source of Variation	DF	SS	MS	F	P
Between Groups	4	1.103	0.276	6.373	<0.001
Residual	195	8.44	0.0433		
Total	199	9.543			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.978

Multiple Comparisons versus Control Group (Dunnett's Method) :

Comparisons for factor:

Comparison	Diff of Means	q'	P	P<0.050	
0.0mg/L vs. 12.5mg/L	0.228	4.903	--	Yes	**LOEC**
0.0mg/L vs. 25.0mg/L	0.118	2.543	--	Yes	
0.0mg/L vs. 50.0mg/L	0.116	2.484	--	Yes	
0.0mg/L vs. 6.25mg/L	0.0713	1.533	--	No	**NOEC**

Note: The P values for Dunnett's and Duncan's tests are currently unavailable except for reporting that the P's are greater or less than the critical values of .05 and .01.

Methimazole
Test Chemical Analytical Data from Battelle:
Purity / Stability Report

Battelle
Putting Technology To Work

Chemical Repository Services for the EDSP
EPA Contract No. 68-W-01-023

Chemistry Report for WA 3-11
Methimazole in Well Water

October 25, 2004

Prepared By:

Timothy Fortman — 10-25-04 (Date)
Chemical Repository Analyst

Approved By:

Richard M. Ecker — 10/25/04 (Date)
Director, Marine Sciences Laboratory

Micheal E Cobb — 10-25-04 (Date)
Chemical Repository Study Director

Battelle Marine Sciences Laboratory
1529 West Sequim Bay Road
Sequim, WA 98382

Submitted to:

Douglas J. Fort, Ph.D.
Fort Environmental Laboratories
Stillwater, Oklahoma 74074

Chemistry Report for WA 3-11
Methimazole in Well Water

Reviewed by: Mary E Lynn
Mary Lynn, EDSP Quality Assurance Representative
Windward Company, Gig Harbor

Date: 10/25/07

Personnel participating in this study:

Analyst: Tim Fortman

Analyst: Rebecca Wood

Statistician: Valerie Cullinan

Chemical Repository Study Director: Michael Cobb

Chemistry Report for WA 3-11
Methimazole in Well Water

Parameter	Chemical
Compound Name	Methimazole
CAS #	60-56-0
Central File No.	CF-2144-1
Initial Receipt Date	12/30/03
Expiration Date	12/30/2008
Manufacturer	Sigma, Inc.
Lot Number	073K3519
Battelle Study #	WA 3-11
Method	Battelle Method EDSP.H4-002

Executive Summary

The chemical purity of methimazole determined by the manufacturer was 99.9%. The purity result from Battelle-Sequim by HPLC-UV/Vis was determined to be 100%. Chemical stability was evaluated based on the average of the Day 0 concentration for both a stock and a diluted concentration with targets of 2500 mg/L and 25 mg/L, respectively. Based on the final regression model and the lower 95% confidence limit of the slope, the stock concentration of methimazole was expected to stay greater than or equal to 90% of the Day 0 concentration of 2630 mg/L for up to an estimated 7 weeks. Thus, the methimazole stock solution in water was considered stable at the 2630 mg/L concentration for the testing period of 21 days. Using the same statistical analysis but adding a day 60 measurement[1] for the diluted concentration sample, based on the final regression model and the lower 95% confidence limit of the slope, the diluted concentration of Methimazole was expected to stay greater than or equal to 90% of the Day 0 concentration of 26 mg/L for 15 days. To insure validity of Methimazole results for all in-life samples, analyses will be carried out within 15 days of each sampling event.

[1] The protocol specified a 21 day stability study – we continued monitoring the stability of the 26 mg/L sample out to 60 days - as a result, a deviation (EDSP.H4-002-01-02) was generated and is on file with the EDSP Chemical Repository Study Director.

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1.0 INTRODUCTION

The goal of the Battelle-Sequim, Marine Sciences Laboratory (MSL) Chemical Repository for the Endocrine Disruptor Screening Program (EDSP) is to provide the participating laboratory with requested chemicals of documented quality at required concentrations and in a matrix appropriate for different toxicological tests. The EDSP Chemical Repository supplies the manufacturer's information regarding purity and stability, the material safety data sheet (MSDS) chemical information, and independent analysis of purity and stability in a matrix specified by the Study Protocol: *Purity and Stability of Test Substances for Work Assignment 3-11* [EDSP Study Number: EDSP.311-01], made in collaboration with the requesting Principal Investigator. Under Work Assignment (WA) 3-11, Fort Environmental Laboratories requested purity and stability testing of methimazole (Figure 1).

2.0 GENERAL METHODS

Methods of standard operation of the Chemical Repository are currently addressed in MSL SOPs numbered R-001 through R-017. These procedures address chemical procurement including procurement of controlled substances, when applicable, which have unique permitting, ordering, handling, inventory, and storage requirements; chemical receipt and chain of custody, chemical log-in and labeling, inventory, chemical storage; stock solution preparation, documentation and archiving; test solution preparation, documentation and shipping; chemical disposal, and repository maintenance over time. The quality assurance (QA) requirements for procurement of chemicals for use in the Chemical Repository are addressed in Version 2 of the *Quality Assurance Project Plan for EDSP Chemical Repository.*

2.1 Chemical Procurement

As requested by Douglas J. Fort, Ph.D., the Principal Investigator for the Study, methimazole, (CAS No. 60-56-0) was purchased for purity and stability analysis and used as the test chemical in a one-generational study on frogs (Figure 1). Methimazole was purchased from Sigma, Inc. and lot number 073K3519 was received on 12/30/03 with an expiration date of 12/30/2008 (Table 1). The chemical was logged in to the Chemical Management System (CMS) and given a CMS barcode and unique log-in (central file) number (CF-2144-1) as per the QA Project Plan (QAPP) for the EDSP Chemical Repository. The chemical was stored in a clean, dry location at room temperature, away from direct sunlight.

EDSP Chemical Request Form

For EPA WA: 3-11

Study Director:
Name: Douglas J. Fort, Ph.D.
Affiliation: Fort Environmental Laboratories
Location: Stillwater, Oklahoma 74074
Telephone number: 405-624-6771

Bioassay Information:

Proposed Bioassay: Frog Metamorphosis Assay Demonstration

Test Chemical: Methimazole

Carrier(s): NA

Concentrations/Dilution Series: 6.25, 12.5, 25.0, 50.0 mg/L

*Consider if analysis method detection limit which may be determined in Purity analysis is above or below desired test concentrations?

In vitro or *in vivo* tests? *In vivo*

Organism to be tested: *Xenopus laevis* (South African Clawed Frog)

Method of test solution administration: Flow-through exposure/diluter system

Planned/proposed test duration: 14 and 21 days

Chemical Information:

Chemical Name: Methimazole

CAS: 60-56-0

Any known purity information: may refer to attached documentation

Any known stability information: Contact Dr. Joe Tietge, EPA, Duluth, MN

Desired purity (%) for test? ≥98%

Is there a known calibration standard available?
Name:
CAS:
Manufacturer:

Figure 1. EDSP Requisition Form for Methimazole

Table 1. Chemical Procurement Information

Parameter	Chemical
Compound Name	Methimazole
CAS #	60-56-0
Central File No.	CF-2144-1
Initial Receipt Date	12/30/03
Expiration Date	12/30/2008
Manufacturer	Sigma, Inc.
Lot Number	073K3519
Manufacturer's Purity	99.9%
Storage Conditions	Clean, dry place/room temp.
Battelle Study #	WA 3-11
Method	Battelle Method EDSP.H4-002

2.2 Chemical Purity

Chemical purity was verified by chromatographic analysis to determine areas under peaks other than the principal peak and compared to the manufacturer's certificate of analysis/purity (Appendix A). No statistical analyses were performed for the verification of chemical purity.

Purity verification was conducted by making a solution in Methanol/Water of about 100 µg/mL. This matrix was then run on an HPLC with a UV/Vis detector. A methanol/water blank was also analyzed on the system. The purity was determined by first identifying the peaks in the methimazole chromatogram that were the same as the peaks in the analysis of the blank. The areas associated with these common peaks were then eliminated by inhibiting integration and the remaining peaks were reported as a percentage of the total peak area. The percentage associated with the largest peak represented the purity of methimazole. The HPLC was set up with an autosampler and a 250X4.6 mm reversed-phase HPLC column. The HPLC system was run isocratically, with a 25% methanol/75% water eluent, the detector was set to a wavelength of 258 nm. One replicate was analyzed.

2.3 Preparation of Stock Matrices for Stability Analysis

A general study plan for stability testing based on the WA 3-11 request from Douglas J. Fort, Ph.D. was developed as the stability test protocol and is presented in Appendix B. A stock solution and one diluter solution were prepared to arrive at the chemical concentrations requested for stability analysis (Table 2). All samples were analyzed in triplicate for calculation of a mean concentration and relative standard deviation (RSD). Methimazole stock matrices were prepared on 2/20/04 for testing as described in Table 2. Briefly, for the 2500 mg/L methimazole, 2.4991 g was weighed into a 1 liter amber bottle and 1.0 L of well water was added using a 1 liter class A graduated cylinder. The solution was agitated by hand shaking for approximately five minutes until all of the methimazole was dissolved. For the 25 mg/L methimazole, 10 mL of the above 2500 mg/L stock was measured into another 1 L amber bottle containing 990 mL well water. All measurements were done with Class A graduated cylinders. The solution was agitated to insure uniformity. Solutions were stored on the counter at room temperature for the duration of the test.

Table 2. Stock Matrix Composition for Stability Testing

Study and Duration	Test Chemical	Target Concentration	Sample ID	Stock Matrix
WA 3-11 21 days[2]	Methimazole	2500 mg/L	stock	2.4991 g in 1L water
		25 mg/L	dil	10 mL of 2500 mg/L solution to 1 L water

2.4 Analytical Chemistry for Stability Testing

Chemical stability was evaluated under storage conditions and matrix specifications as requested by the participating laboratory. At initiation and at each time period throughout the duration of the test, the chemical concentration was determined by chromatographic analysis. Triplicate aliquots were tested. The frequency of determinations and the duration of testing were determined by the requesting principal investigator and the chemists based on *a priori* knowledge about chemical stability. Methimazole stock solution was sampled by taking 0.025 mL of the stock using a positive displacement pipette and diluting it to 1 mL with 0.975 mL of mobile phase, using a 1 mL syringe into a 1.8 mL vial. A transfer pipette was used to mix this solution and transfer it to an autosampler vial compatible with the HPLC autosampler. Methimazole diluter solution was sampled by taking 0.75 mL of the diluter sample using a syringe and diluting it to 1 mL with 0.25 mL of Methanol, using a 1 mL syringe into a 1.8 mL vial. A transfer pipette was used to mix this solution and transfer it to an autosampler vial compatible with the HPLC autosampler. Water blanks were prepared the same way. Spike solution, prepared similarly to the stability solutions, but stored at 4 deg. C. was used as a QA/QC sample, this sample was prepared for analysis in the same way as the stability solutions. This solution was then analyzed on the HPLC/UV/Vis for quantification. Initial calibration verification and continuing calibration verification (ICV/CCV) samples were analyzed to demonstrate on-going calibration accuracy.

Table 3. HPLC Conditions

Detector	UV/Vis, Wavelength, 258 nm
Flow Rate	1 ml/minute
Injection Volume	25 ul
Eluent; flow pattern	25% Methanol:75% water, Isocratic
Column	Reverse Phase, Phenominex Synergi RP

2.5 Statistical Analysis of Stability

Log linear degradation curves were fit to the data to describe the chemical concentration over time and their dependence on storage conditions and solvent matrix. Lack of fit and residual plots were evaluated to determine the form of the regression. Power calculations based on the observed variability were used to determine the sensitivity of the test to detect degraded concentrations. General methods are documented in SOP EDSP.D-012-01.

[2] See footnote #1 on page 3 for clarification of study duration.

3.0 RESULTS

3.1 Chemical Purity

Battelle-Sequim conducted an HPLC-UV/Vis purity scan on the methimazole. The chromatogram, blank correction not needed, no peaks were detected in the blank, showed only one large peak that had the appropriate retention time for methimazole with all other peaks consistent with the blank. The area of the methimazole peak was 100% of the total area of all peaks in the chromatogram. Chemical purity of methimazole determined by the manufacturer was 99.9% (Appendix A).

3.2 Analytical Chemistry for Stability Testing

Chemical stability testing was initiated on 2/20/04. Chemical concentrations were determined 8 times over a period of 25 days. The Study Protocol states that this stability study is a 21 day study, the study was done for 25 days due to sampling schedule. A 60 day sample was also done to see if Methimazole was actually stable for longer than statistical evaluations suggested (see footnote #1 page three for clarification). Concentrations measured in the stock Stability test ranged from 105% to 117% of the target concentration. Concentrations measured in the diluter Stability test ranged from 88% to 104% of the target concentration. The analytical and quality control (QC) results all met acceptance criteria and are presented in Appendix C. Blanks and matrix spikes were analyzed with every batch for QC purposes. There was less than 3 times the detection limit of methimazole in the blanks, spike recoveries were all within 10% of the nominal value. ICV/CCV results ranged from 94.9% to 101.7%. All QC data were within acceptance criteria. The MDL was determined to be 0.034 mg/L for diluter samples and 3.25 for stock samples.

Table 4. Calibration Acceptance

Calibration Date	R2 Value (= .995)	Curve Fit
2/19/04	0.999914	Linear
2/20/04	0.999983	Linear
2/24/04	0.999997	Linear
2/27/04	0.999994	Linear
3/2/04	0.999994	Linear
3/5/04	0.999997	Linear
3/9/04	0.999981	Linear
3/12/04	0.999979	Linear
3/16/04	0.999970	Linear
4/20/04	0.999982	Linear

3.3 Statistical Results of Stability Trial

A plot of methimazole with a target concentration of 2500 mg/L against time suggested very little chemical decay (Figure 2). There were no data points less than 90% of the target concentration or 90% of the average Day 0 concentration (2630 mg/L). The average Day 0 concentration was used to evaluate stability through time. Based on the final regression model and the lower 95% confidence limit of the slope, the concentration of methimazole was expected to stay greater than or equal to 90% of the Day 0 concentration for up to an estimated 7 weeks (Table 5). Thus, this stock solution was

considered stable for the 3-week testing period. The complete statistical analysis is presented in Appendix D.
Eight observations of methimazole with a target concentration of 25 mg/L were less than 90% of the average Day 0 concentration of 26 mg/L (Figure 2). Again, the average Day 0 concentration was used to evaluate stability through time. Based on the final regression model and the lower 95% confidence limit of the slope, the concentration of methimazole was expected to stay greater than or equal to 90% of the average Day 0 concentration for 15 days (Figure 2). The complete statistical analysis is presented in Appendix D. It should be noted that a 60 day stability point was added to the data to further characterize the stability of the low concentration test sample (increasing the stability projection from 11 days to 15 days). Figure 2 shows plots of concentration vs. time out to both 25 and 60 days.

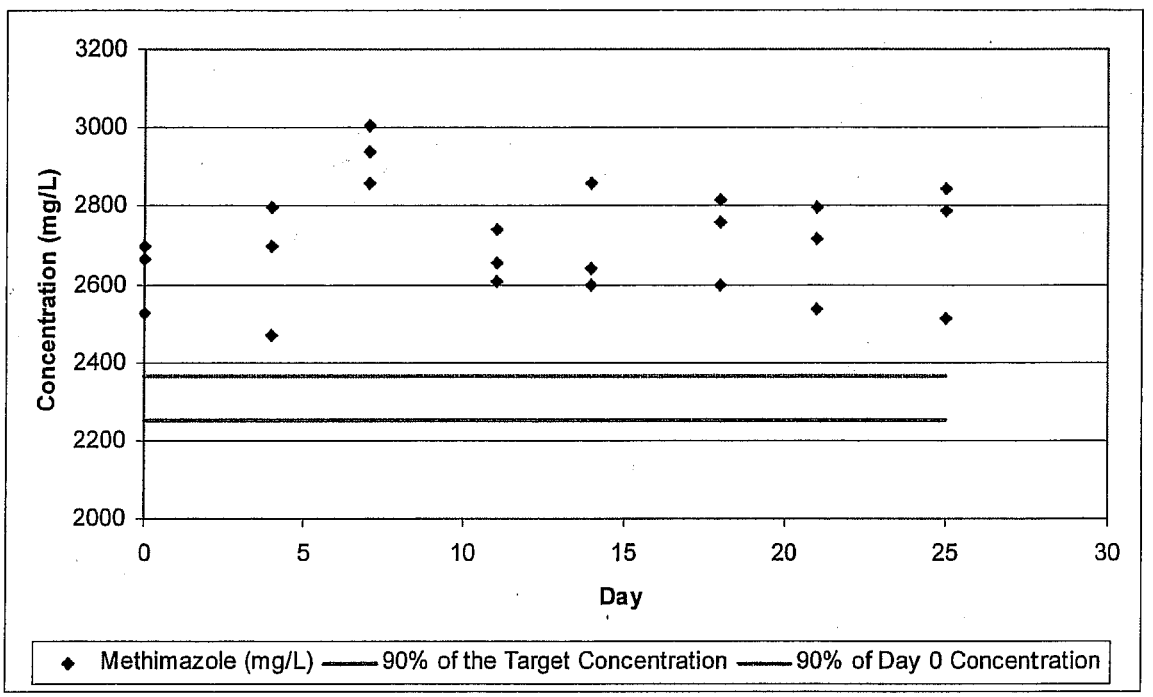


Figure 2a. Observed concentration of methimazole with a target concentration of 2500 mg/L against time

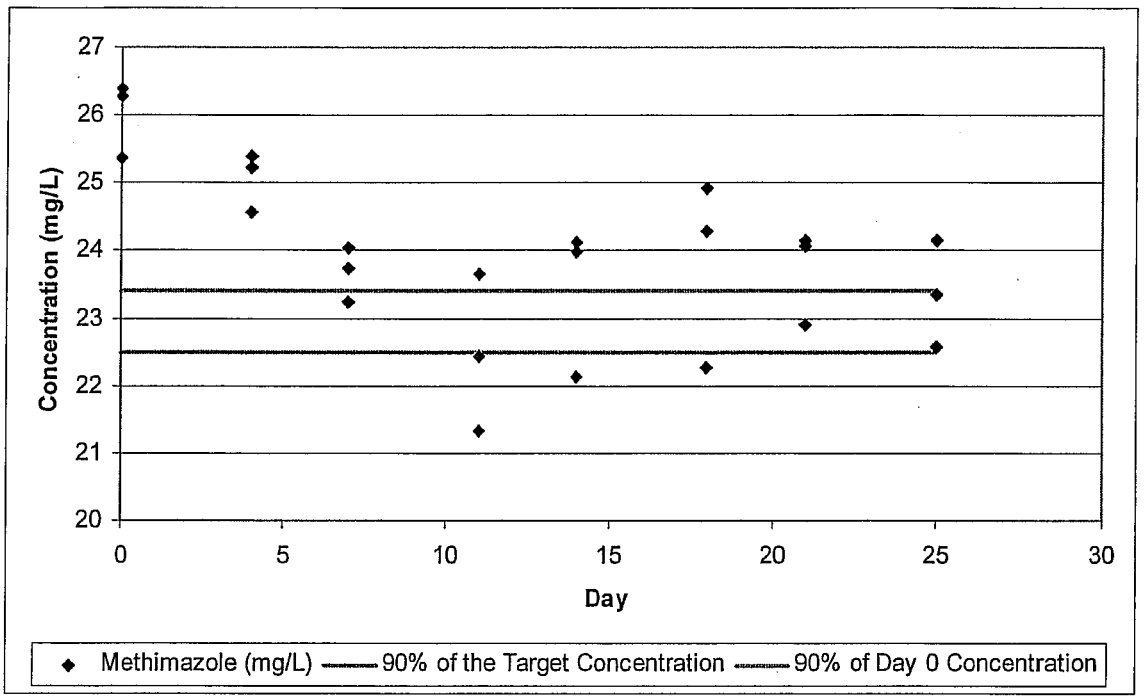


Figure 2b. Observed concentration of methimazole with a target concentration of 25 mg/L against time (25 days)

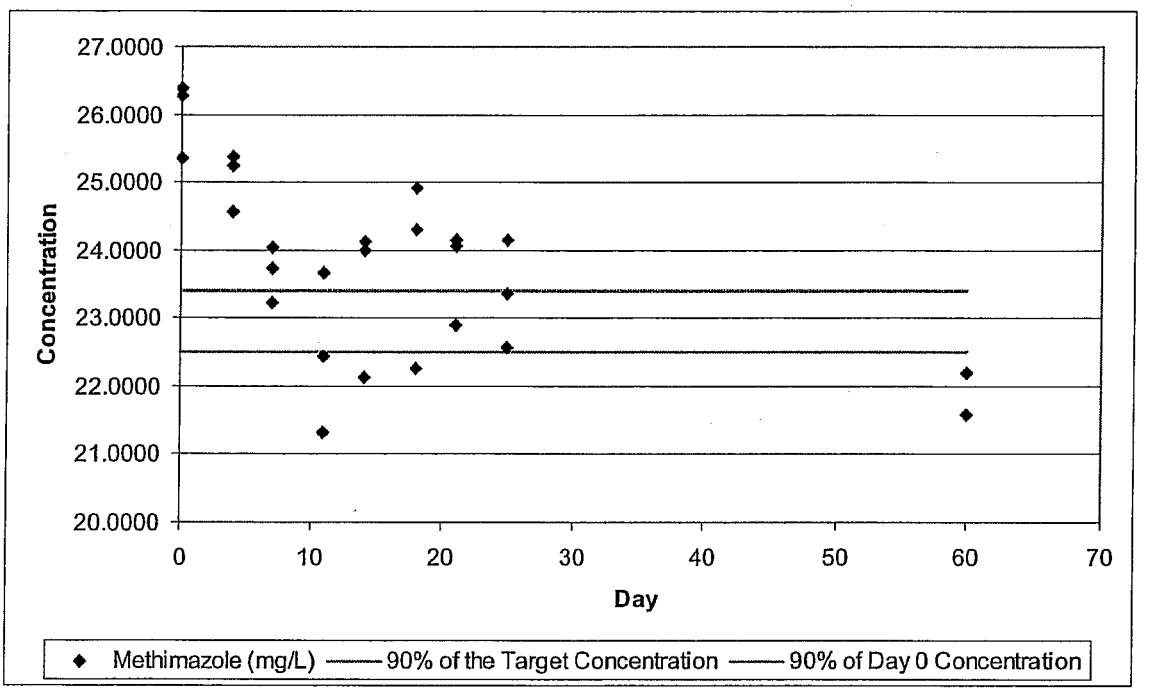


Figure 2c. Observed concentration of methimazole with a target concentration of 25 mg/L against time (60 days)

Table 5. Summary of Statistical Results for Methimazole

WA 3-11 Statistical Analysis conducted by Valerie Cullinan Using Minitab Version 13.32, Minitab Inc., 1999.	Methimazole 2500 mg/L	Methimazole 25 mg/L
Average Day 0 Concentration (mg/L)	2630	26
Number of determinations	1	1
Number of days tested	25	60
Number of replicates per day	3	3
Number of outliers removed	0	0
Number of observations removed	0	0
Overall Mean Concentration	2713	23.7
95% Upper CL	2762	24.2
error degrees of freedom	23	26
1-sample t-test of Ho: μ >= Day 0 concentration	NS [a]	S [b]
estimated intercept of ln(concentration) against time	7.9025	3.2019
estimated slope of ln(concentration) against time	0.0002	-0.0021
standard error of slope	0.0013	0.0005
error degrees of freedom	22	25
Significance test of lack-of-fit for final model	NS	S
Significance test of Ho: $\beta = 0$ vs. H1: $\beta = 0$	NS	S
Lower 95% CL of β	-0.003	-0.003
Upper 95% CL of β	0.003	-0.001
Maximum Percent Loss over 8 days (using LCL)	2.0%	2.5%
Mean Percent Loss (using bhat)	-0.1%	1.7%
LN(90% of Target)	7.7693	3.1531
Number of days until at 90% of Target (using LCL)	52	15
Conclusion using Day 0 Concentration:	**Stable for 3 wks**	**Stable for 15 days**

[a] Not significant at $\alpha = 0.05$
[b] Significant at $\alpha = 0.05$

4.0 CONCLUSIONS

The chemical purity determined by Battelle-Sequim was 100% and by the manufacturer was 99.9%. Stability testing of methimazole in water concluded that the 2500 mg/L stock solution was stable for 3 weeks and the 25 mg/L concentration was stable for a minimum holding period of 15 days (per the 60 day data analysis). Though the noise in the measurement for the diluted samples creates some uncertainty in the statistical conclusions, it appears that there may be some initial low-level plating of the Methimazole onto the walls of the dilutor sample container, reaching equilibrium within the first several days of operation. Beyond that period, the samples appear to remain stable. To insure that the in-life samples data is a reflection of actual dilutor performance, and not affected by stability concerns, all in-life samples will be tested within a 15 day holding period.

APPENDIX A

MANUFACTURER'S CERTIFICATE OF ANALYSIS/PURITY

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Methimazole
Product Number	M8506
Product Brand	SIGMA
CAS Number	60-56-0
Molecular Formula	$C_4H_6N_2S$
Molecular Weight	114.2

TEST	SPECIFICATION	LOT 073K3519 RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR TO SLIGHTLY HAZY COLORLESS TO YELLOW SOLUTION AT 50 MG/ML IN WATER	CLEAR COLORLESS SOLUTION
IR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS
PURITY BY HPLC	MINIMUM 98%	99.9%
SHELF LIFE SOP QC-12-006		JULY 2008
QC ACCEPTANCE DATE		JULY 2003

Lori Schulz, Manager
Analytical Services

APPENDIX B

PURITY AND STABILITY TESTING PROTOCOL

Study Protocol:
Purity and Stability of Test Substances for Work Assignment 3-11
EDSP Study Number: EDSP.311-01

Study Objective:

To verify the purity of 6 test substances (listed in Table 1), the solubility, (at the concentrations specified in Table 2), and to determine the stability of same dissolved in well water over a 21 day period. This study is in support of EPA contract number 68-W-01-023, MSL Work Assignment Number 3-11, Task 3, Amphibian Metamorphosis Assay Demonstration Study.

Address of Testing Facility and Sponsor's Representative:

Battelle – Marine Science Laboratory
1529 West Sequim Bay Road
Sequim, Washington

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propyl-2-thiouracil	PTU
L-Thyroxine	T4
Phenobarbital	F Barb
Pregnenolone-16-Alpha-carbonitrile	P16ac
Dexamethosone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	F Barb	P16ac	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	= 97%	= 97%	= 97%	= 97%	= 97%	= 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	= 770 mg/L	= 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	SOP MSL-O-	TBA	TBA	TBA	TBA	TBA

TABLE 3
Reference Substance Specifications:

Chemical Name	PTU	T4	F Barb	P16ac	DxM	Mtmz
Manufacturer	TCI America	Fluka	TBA	TBA	TBA	Aldrich
CAS #	51-52-5	51-48-9	TBA	TBA	TBA	60-56-0
Lot #	0630144	411874/1	TBA	TBA	TBA	06109DP
Purity req.	= 98%	= 98%	= 98%	= 98%	= 98%	= 98%
Mfg. Purity claim	> 99%	99.6%	TBA	TBA	TBA	99.7%

Proposed experimental start and termination dates:

Start Date – November 1, 2003
Termination Date – June 22, 2004

Experimental Design:

- Solubility of the substances will be determined as per SOP EDSP.C-0XX-01 (EDSP Chemical Characterization-TBA) in the diluent (well water) at a concentration approximately 10% above the stock solution concentration (see Table 2).
- If the solubility of the test substance is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP EDSP.C-0XX-01. Note: exposure concentrations won't change, so stability of an exposure solution will be carried out at the target concentration.
- Substance purity will be verified using HPLC, GC/MS or IC/ICP/MS as appropriate, and should be 98% or greater and be within ±2% of the value provided on the Certificate of Analysis by the manufacturer
- Stock solution – made up to concentrations delineated in Table 2 (approximate levels) in well water (obtained from MSL).
- Stability test solutions – Stability testing will be carried out on stock solutions and at a second level near or within the in-life exposure dose range. Nominal concentrations to be tested are delineated in Table 2 in well water (obtained from MSL).
- Storage and Labeling Requirements of Test Solution – stored in the dark (i.e., same storage conditions of solutions employed in the in-life studies of WA 3-11) at room temperature (20-25°C). Minimally, containers will be labeled with the name of the test substance, the date of preparation, the solution concentration, and the study number.
- Test Schedule – Samples will be analyzed the day of collection from the test solution
- Replicates – 3 per sample tested at each analysis time point
- Sampling Timing – Sampling will be carried out on the first day and then as appropriate for statistical analysis for duration of the 21 day study
- For details of the analytical methods see the appropriate SOP associated with the analytical method employed for the specific substance: MSL-O-018-00 (HPLC), MSL-X-XXX-XX (GC/MS)-TBA, MSL-X-XXX-XX (ICP/MS)-TBA.

Statistical Analysis:

Log linear degradation curves will be fit to the stability data to describe the trend of the chemical concentration over time. Lack of fit and residual plots will be evaluated to determine the form of

the regression. Power calculations based on the observed variability will be used to determine the sensitivity of the test to detect degraded concentrations.

Regulatory requirements:

This study will be conducted in compliance with EPA FIFRA Good Laboratory Practices (40CFR, Part 160).

Records to be maintained:

All records and the final report generated as a result of this study will be transported to and maintained in the EDSP Data Coordination Center for archival purposes at the following address:

EDSP Data Coordination Center
Battelle Columbus Operations (PNL)
505 King Ave
Columbus, OH 43201

Sponsor Representative_____ _____
Michael Blanton **Date**

Study Director_____ _____
Michael Cobb **Date**

PROTOCOL AMENDMENT
STUDY NUMBER: EDSP.311-01
AMENDMENT NUMBER: A-1
DATE: FEBRUARY 4, 2004
Page 1 of 2

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01			DATE: February 4, 2004		
AMENDMENT NUMBER: A-1			WAL/STUDY DIRECTOR:		
NOTEBOOK NUMBER: N/A			Michael Blanton/Michael Cobb		
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

1. Solubility of the substances will be determined as per SOP EDSP.C-0XX-01 (EDSP Chemical Characterization-TBA)...
   If the solubility of the test substance is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP EDSP.C-0XX-01.
2. For details of the analytical methods see the appropriate SOP associated with the analytical method employed for the specific substance: MSL-O-018-00 (HPLC), MSL-X-XXX-XX (GC/MS)-TBA, MSL-X-XXX-XX (ICP/MS)-TBA.
3. All records and the final report generated as a result of this study will be transported to and maintained in the EDSP Data Coordination Center for archival purposes at the following address:
   EDSP Data Coordination Center
   Battelle Columbus Operations (PNL)
   505 King Ave
   Columbus, OH 43201
4. Sponsor Representative_____ _____
   Michael Blanton Date

AMENDMENT:

1. Sentences amended to read: "Solubility of the substances will be determined as per SOP MSL-R-009 (Chemical Characterization)"...
   "If the solubility of the test substances is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP MSL-R-009."
   Last several SOPs identified in #2 below.
   Sentence amended to read: "For details of the analytical methods, see the method identified in Table 2 and provided in each specific chemical characterization notebook. These methods are supported by specific operation and maintenance SOPs appropriate to the analytical method employed for the particular substance under study: MSL-R-012 (Operation and Maintenance of HPLC Systems), MSL-O-008-02 (Operation and Maintenance of Gas Chromatographs (GC) and Gas Chromatograph/Mass
2. Spectrometer (GC/MS) Systems), MSL-R-014 (Operation & Maintenance of IC ICP/MS Systems – currently being drafted).

PROTOCOL AMENDMENT
STUDY NUMBER: EDSP.311-01
AMENDMENT NUMBER: A-1
DATE: FEBRUARY 4, 2004
Page 2 of 2

3. Sentence amended to read: "All records and the final report generated as a result of this study will be maintained and archived per SOP MSL-D-003 (Archiving of Records, Data and Retired SOPs). In addition, the documentation will be transported in electronic form to the EDSP Data Coordination Center for archival purposes at the following address:

   EDSP Data Coordination Center
   Battelle Columbus Operations
   505 King Ave
   Columbus, OH 43201

4. Sponsor Representative_____ _____
   David Houchens Date

REASON FOR CHANGE:

1. The SOP numbers were not available (in the "Experimental Design" section) when the protocol was written. These SOP numbers are now available and are provided in this amendment.
2. Our new documentation approach involves generation of a specific "Analytical Method" document developed for the chemical characterization and provided in the final report. Each specific "Method" will correspond to a generic SOP.
3. Original document section in "Records to be maintained" section requires clarification. Battelle Columbus Operations incorrectly labeled as PNL.
4. Correct Sponsor Representative is EDSP Program Manager.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01				DATE: February 24, 2004	
AMENDMENT NUMBER: A-2				WAL/STUDY DIRECTOR:	
NOTEBOOK NUMBER: N/A				Michael Blanton/Michael Cobb	
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

Table entries that are earmarked for change in this amendment are highlighted in Yellow

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	SOP MSL-O-018-00 (HPLC)	TBA	TBA	TBA	TBA	TBA

AMENDMENT:
New table entries are highlighted in Orange

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	PTU	T4	F Barb	P16ac	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	= 97%	= 97%	= 97%	= 97%	= 97%	= 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	= 770 mg/L	= 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

REASON FOR CHANGE:

Information was not available when protocol was originally generated.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01				DATE: March 30, 2004	
AMENDMENT NUMBER: A-3				WAL/STUDY DIRECTOR:	
NOTEBOOK NUMBER: N/A				Michael Blanton/Michael Cobb	
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

Table entries that are earmarked for change in this amendment are presented underlined and italicized in ***Georgia Font***

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propyl-*2*-thiouracil	PTU
L-Thyroxine	T4
Phenobarbital	F Barb
Pregnenolone-16-Alpha-carbonitrile	P16ac
Dexamethosone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	F Barb	P16ac	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	= 97%	= 97%	= 97%	= 97%	= 97%	= 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	= 770 mg/L	= 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	*0*, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, *100* mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

TBA = To Be Amended

AMENDMENT:

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propylthiouracil	PTU
L-Thyroxine	T4
Phenobarbital	F Barb
Pregnenolone-16-Alpha-carbonitrile	P16ac
Dexamethasone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	PTU	T4	F Barb	P16ac	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	= 97%	= 97%	= 97%	= 97%	= 97%	= 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	= 770 mg/L	= 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

REASON FOR CHANGE:

Information was not available when protocol was originally generated.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

NOTE 1: Signed copies of this protocol and all relevant Amendments are available in the files of the Chemical Repository Study Director in MSL 5.

NOTE : A signed Deviation is on file with the EDSP Chemical Repository Study Director (#EDSP.H4-002-01-01) indicating the use of a linear fit for the calibration curves instead of the specified non-linear quadratic fit described in Method EDSP.H4-002-01.

APPENDIX C

Table C1. Methimazole concentration in Well Water (mg/L)

Target Conc. (mg/L)	Sample ID	Date	Methimazole (mg/L)	Average (mg/L)	Recovery	RSD
2499	stock 1R-1	2/20/2004	2528.17			
2499	stock 1 R-2	2/20/2004	2699.40	2629.85	105.24%	3.42%
2499	stock 1 R-3	2/20/2004	2661.98			
2499	stock 2 R-1	2/24/2004	2470.03			
2499	stock 2 R-2	2/24/2004	2695.11	2654.02	106.20%	6.30%
2499	stock 2 R-3	2/24/2004	2796.93			
2499	stock 3 R-1	2/27/2004	3004.75			
2499	stock 3 R-2	2/27/2004	2936.92	2933.49	117.39%	2.49%
2499	stock 3 R-3	2/27/2004	2858.79			
2499	stock 4 R-1	3/2/2004	2607.72			
2499	stock 4 R-2	3/2/2004	2654.26	2667.53	106.74%	2.53%
2499	stock 4 R-3	3/2/2004	2740.62			
2499	stock 5 R-1	3/5/2004	2639.71			
2499	stock 5 R-2	3/5/2004	2597.70	2698.84	108.00%	5.20%
2499	stock 5 R-3	3/5/2004	2859.12			
2499	stock 6 R-1	3/9/2004	2595.56			
2499	stock 6 R-2	3/9/2004	2759.03	2722.96	108.96%	4.18%
2499	stock 6 R-3	3/9/2004	2814.29			
2499	stock 7 R-1	3/12/2004	2537.53			
2499	stock 7 R-2	3/12/2004	2797.01	2683.56	107.39%	4.95%
2499	stock 7 R-3	3/12/2004	2716.14			
2499	stock 8 R-1	3/16/2004	2511.67			
2499	stock 8 R-2	3/16/2004	2843.23	2714.49	108.62%	6.55%
2499	stock 8 R-3	3/16/2004	2788.59			
2499	stock 9 R-1	4/20/2004	2675.46			
2499	stock 9 R-2	4/20/2004	2633.85	2668.01	106.76%	1.17%
2499	stock 9 R-3	4/20/2004	2694.71			

Table C1 continued. Methimazole concentration in Well Water (mg/L)

Target Conc. (mg/L)	Sample ID	Date	Methimazole (mg/L)	Average (mg/L)	Recovery	RSD
25	dil 1 R-1	2/20/2004	25.35			
25	dil 1 R-2	2/20/2004	26.40	26.01	104.04%	2.21%
25	dil 1 R-3	2/20/2004	26.28			
25	dil 2 R-1	2/24/2004	24.57			
25	dil 2 R-2	2/24/2004	25.24	25.07	100.27%	1.74%
25	dil 2 R-3	2/24/2004	25.39			
25	dil 3 R-1	2/27/2004	23.24			
25	dil 3 R-2	2/27/2004	23.73	23.67	94.68%	1.70%
25	dil 3 R-3	2/27/2004	24.04			
25	dil 4 R-1	3/2/2004	21.32			
25	dil 4 R-2	3/2/2004	23.66	22.47	89.94%	5.21%
25	dil 4 R-3	3/2/2004	22.44			
25	dil 5 R-1	3/5/2004	22.13			
25	dil 5 R-2	3/5/2004	23.98	23.41	93.65%	4.76%
25	dil 5 R-3	3/5/2004	24.13			
25	dil 6 R-1	3/9/2004	22.26			
25	dil 6 R-2	3/9/2004	24.91	23.82	95.29%	5.83%
25	dil 6 R-3	3/9/2004	24.30			
25	dil 7 R-1	3/12/2004	22.90			
25	dil 7 R-2	3/12/2004	24.16	23.71	94.84%	2.96%
25	dil 7 R-3	3/12/2004	24.07			
25	dil 8 R-1	3/16/2004	22.56			
25	dil 8 R-2	3/16/2004	23.36	23.36	93.44%	3.42%
25	dil 8 R-3	3/16/2004	24.16			
25	dil 9 R-1	4/20/2004	21.57			
25	dil 9 R-2	4/20/2004	22.19	21.99	87.99%	1.64%
25	dil 9 R-3	4/20/2004	22.20			

Table C2. Spike Recoveries (mg/L)

Target Conc. (mg/L)	Sample ID	Date	Methimazole (mg/L)	Average (mg/L)	Recovery	RSD
214	Spike 1 R-1	2-20-04	212.26			
214	Spike 1 R-2	2-20-04	215.87	215.90	100.89%	1.69%
214	Spike 1 R-3	2-20-04	219.57			
214	Spike 2 R-1	2-24-04	213.94			
214	Spike 2 R-2	2-24-04	220.81	217.95	101.84%	1.64%
214	Spike 2 R-3	2-24-04	219.09			
214	Spike 3 R-1	2-27-04	211.98			
214	Spike 3 R-2	2-27-04	223.29	219.70	102.66%	3.04%
214	Spike 3 R-3	2-27-04	223.82			
214	Spike 4 R-1	3-02-04	212.19			
214	Spike 4 R-2	3-02-04	217.23	216.91	101.36%	2.11%
214	Spike 4 R-3	3-02-04	221.32			
214	Spike 5 R-1	3-05-04	216.70			
214	Spike 5 R-2	3-05-04	220.16	218.67	102.18%	0.81%
214	Spike 5 R-3	3-05-04	219.15			
214	Spike 6 R-1	3-09-04	202.60			
214	Spike 6 R-2	3-09-04	216.90	213.27	99.66%	4.41%
214	Spike 6 R-3	3-09-04	220.30			
214	Spike 7 R-1	3-12-04	195.85			
214	Spike 7 R-2	3-12-04	197.67	200.96	93.91%	3.65%
214	Spike 7 R-3	3-12-04	209.36			
214	Spike 8 R-1	3-16-04	204.81			
214	Spike 8 R-2	3-16-04	210.98	209.71	98.00%	2.10%
214	Spike 8 R-3	3-16-04	213.35			
214	Spike 9 R-1	4-20-04	214.74			
214	Spike 9 R-2	4-20-04	214.42	215.09	100.51%	0.41%
214	Spike 9 R-3	4-20-04	216.10			

Table C3. ICV/CCV Data for Methimazole Concentration in Well Water

Date	Sample Name	Methimazole concentration, measured (µg/mL)	Methimazole concentration, expected (µg/mL)	Recovery
2-20-04	Meth4 ICV	19.76	19.88	99.41%
2-20-04	meth2C CCV	10.16	10.20	99.61%
2-20-04	meth2C CCV	10.37	10.20	101.67%
2-20-04	meth2C CCV	10.24	10.20	100.40%
2-24-04	Meth4 ICV	19.92	19.88	100.22%
2-24-04	meth2C CCV	10.25	10.20	100.49%
2-24-04	meth2C CCV	10.31	10.20	101.05%
2-24-04	meth2C CCV	10.19	10.20	99.87%
2-27-04	Meth4 ICV	19.20	19.88	96.59%
2-27-04	meth2C CCV	10.09	10.20	98.90%
2-27-04	meth2C CCV	10.16	10.20	99.63%
2-27-04	meth2C CCV	10.01	10.20	98.12%
2-27-04	meth2C CCV	10.17	10.20	99.69%
3-02-04	Meth4 ICV	19.55	19.88	98.36%
3-02-04	meth2C CCV	9.99	10.20	97.99%
3-02-04	meth2C CCV	10.04	10.20	98.46%
3-02-04	meth2C CCV	9.93	10.20	97.30%
3-02-04	meth2C CCV	9.97	10.20	97.70%
3-05-04	Meth4 ICV	19.73	19.88	99.26%
3-05-04	meth2C CCV	10.13	10.20	99.35%
3-05-04	meth2C CCV	10.22	10.20	100.21%
3-05-04	meth2C CCV	10.13	10.20	99.30%
3-05-04	meth2C CCV	10.15	10.20	99.49%
3-09-04	Meth4 ICV	19.22	19.88	96.69%
3-09-04	meth2C CCV	9.96	10.20	97.64%
3-09-04	meth2C CCV	9.93	10.20	97.36%
3-09-04	meth2C CCV	9.93	10.20	97.34%
3-12-04	Meth4 ICV	19.21	19.88	96.63%
3-12-04	meth2C CCV	9.68	10.20	94.95%
3-12-04	meth2C CCV	9.75	10.20	95.59%
3-12-04	meth2C CCV	9.76	10.20	95.65%
3-16-04	Meth4 ICV	19.08	19.88	95.97%
3-16-04	meth2C CCV	9.93	10.20	97.33%
3-16-04	meth2C CCV	9.89	10.20	96.98%
3-16-04	meth2C CCV	9.83	10.20	96.39%
4-20-04	Meth4 ICV	19.17	19.88	96.43%
4-20-04	meth2C CCV	9.73	10.20	95.36%
4-20-04	meth2C CCV	9.91	10.20	97.11%
4-20-04	meth2C CCV	9.81	10.20	96.16%
4-20-04	meth2C CCV	9.82	10.20	96.28%

APPENDIX D

STATISTICAL REPORT

WA-3-11

1.1 Statistical Analysis conducted by Valerie Cullinan

Using Minitab Version 13.32, Minitab Inc., 1999.

4/22/2004 10:42:12 AM

Analysis of Methimazole – 26 mg/L Stability Study

- Test to determine if the data are from a population with mean of 26 mg/L.

Descriptive Statistics: Methimazole (mg/L)

Variable	N	Mean	Median	TrMean	StDev	SE Mean
Methimaz	27	23.723	23.984	23.712	1.365	0.263

Variable	Minimum	Maximum	Q1	Q3
Methimaz	21.322	26.395	22.445	24.566

```
Macro performs a one-sample t-test for mu less than TARGET &
What is the target value for  Methimazole
DATA> 26
```

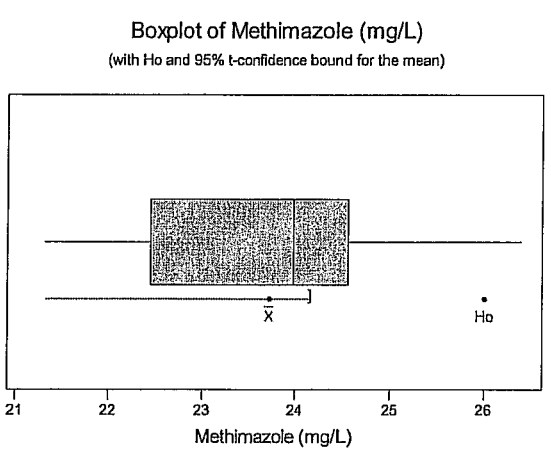
One-Sample T: Methimazole (mg/L)

```
Test of mu = 26 vs mu < 26
```

Variable	N	Mean	StDev	SE Mean
Methimazole	27	23.723	1.365	0.263

Variable	95.0% Upper Bound	T	P
Methimazole	24.171	-8.67	0.000

t Boxplot of Methimazole (mg/L)



Transforms data to natural log

Day	Ln Methimazole
0	3.2329
0	3.2732
0	3.2688
4	3.2014
4	3.2283
4	3.2342
7	3.1457
7	3.1667
7	3.1798
11	3.0598
11	3.1637
11	3.1111
14	3.0970
14	3.1774
14	3.1834
18	3.1029
18	3.2155
18	3.1903
21	3.1311
21	3.1845
21	3.1809
25	3.1163
25	3.1509
25	3.1846
60	3.0713
60	3.0996
60	3.1003

Conducts Simple Linear Regression

Regression Analysis: LnConc versus Day

The regression equation is
LnConc = 3.20 - 0.00208 Day

Predictor	Coef	SE Coef	T	P
Constant	3.20191	0.01292	247.77	0.000
Day	-0.0020835	0.0005290	-3.94	0.001

S = 0.04606 R-Sq = 38.3% R-Sq(adj) = 35.8%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.032915	0.032915	15.51	0.001
Residual Error	25	0.053040	0.002122		
Lack of Fit	7	0.029147	0.004164	3.14	0.024
Pure Error	18	0.023892	0.001327		
Total	26	0.085955			

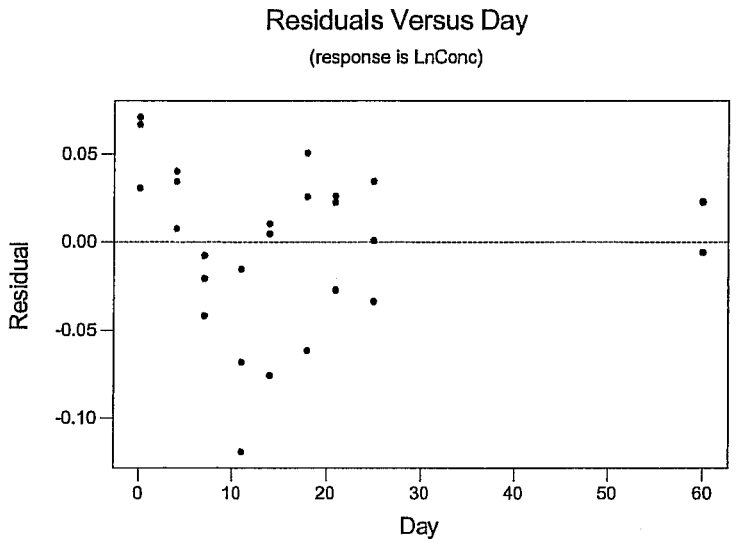
Unusual Observations

Obs	Day	LnConc	Fit	SE Fit	Residual	St Resid
10	11.0	3.05976	3.17899	0.00956	-0.11923	-2.65R
25	60.0	3.07133	3.07690	0.02403	-0.00557	-0.14 X
26	60.0	3.09958	3.07690	0.02403	0.02269	0.58 X
27	60.0	3.10029	3.07690	0.02403	0.02339	0.60 X

R denotes an observation with a large standardized residual

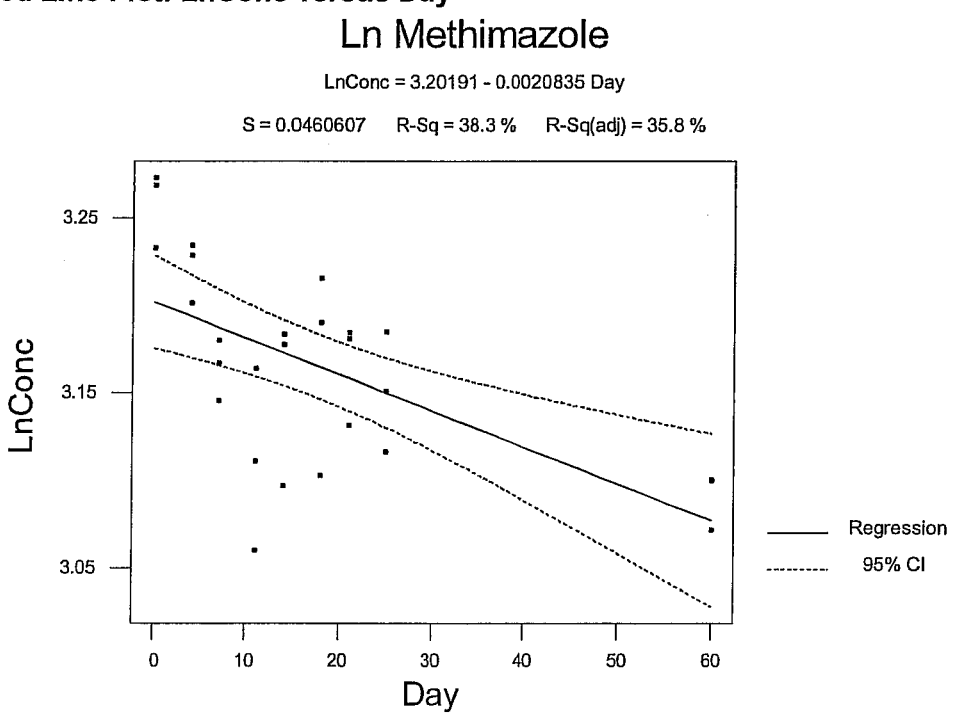
X denotes an observation whose X value gives it large influence.

Residuals from LnConc vs Day



Fit 95% confidence bands about the fitted simple linear model

Fitted Line Plot: LnConc versus Day



- **Conclude 15 days**

Analysis of Methimazole – 2630 mg/L Stability Study

- Test to determine if the data are from a population with mean of 2630 mg/L.

Results for: Stock

```
MTB > %stability c1-c3;
SUBC> start 3;
SUBC> week c1.
Executing from file: C:\Program Files\MTBWIN\MACROS\stability.MAC
Executing from file: C:\Program Files\MTBWIN\MACROS\Fitline.MAC

Macro performs a one-sample t-test for mu less than TARGET &
What is the target value for X    3
DATA> 2630
```

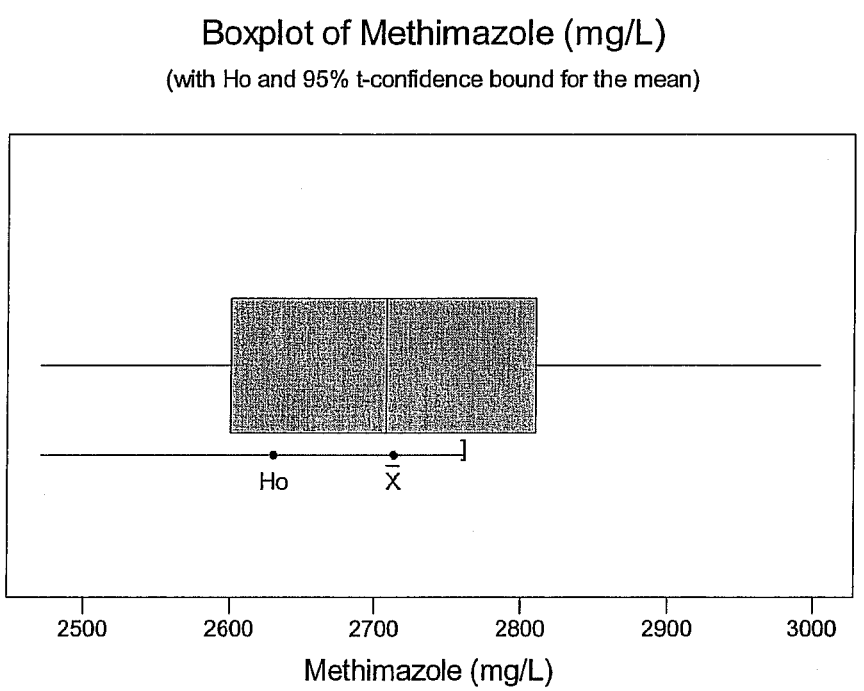
One-Sample T: Methimazole (mg/L)

```
Test of mu = 2630 vs mu < 2630

Variable          N      Mean     StDev   SE Mean
Methimazole      24    2713.1     138.7      28.3

Variable      95.0% Upper Bound         T      P
Methimazole              2761.6      2.94  0.996
```

t Boxplot of Methimazole (mg/L)



Outliers are < Median-3*IQD OR > Median+3*IQD

Boundary for outliers are values < 2078.48 and > 3337.07

No outliers

- Transform data to natural logarithm and conduct regression analysis.

Day	Rep	Ln(Concentration)
0	1	7.8352
0	2	7.9008
0	3	7.8868
4	1	7.8120
4	2	7.8992
4	3	7.9363
7	1	8.0079
7	2	7.9851
7	3	7.9582
11	1	7.8662
11	2	7.8839
11	3	7.9159
14	1	7.8784
14	2	7.8624
14	3	7.9583
18	1	7.8616
18	2	7.9226
18	3	7.9425
21	1	7.8389
21	2	7.9363
21	3	7.9070
25	1	7.8287
25	2	7.9527
25	3	7.9333

Conducts Simple Linear Regression

Regression Analysis: Methimazole (mg/L) versus Day

The regression equation is
Methimazole (mg/L) = 7.90 + 0.00016 Day

Predictor	Coef	SE Coef	T	P
Constant	7.90254	0.01963	402.54	0.000
Day	0.000165	0.001319	0.12	0.902

S = 0.05220 R-Sq = 0.1% R-Sq(adj) = 0.0%

Analysis of Variance

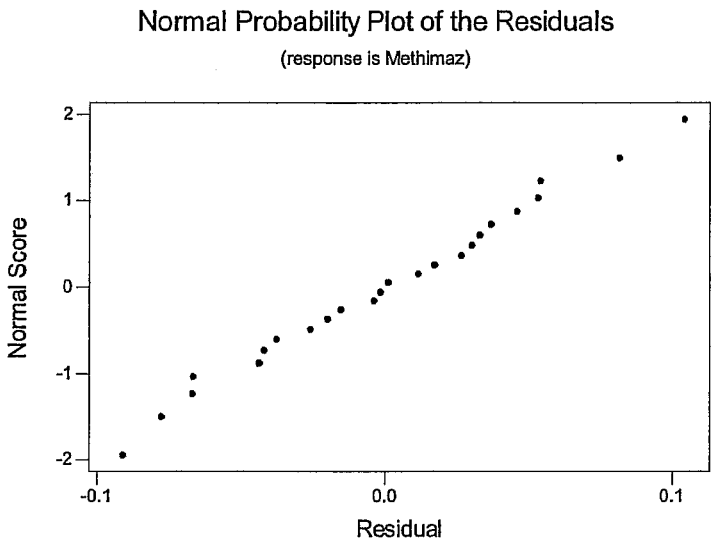
Source	DF	SS	MS	F	P
Regression	1	0.000042	0.000042	0.02	0.902
Residual Error	22	0.059946	0.002725		
Lack of Fit	6	0.024189	0.004032	1.80	0.162
Pure Error	16	0.035756	0.002235		
Total	23	0.059988			

Unusual Observations

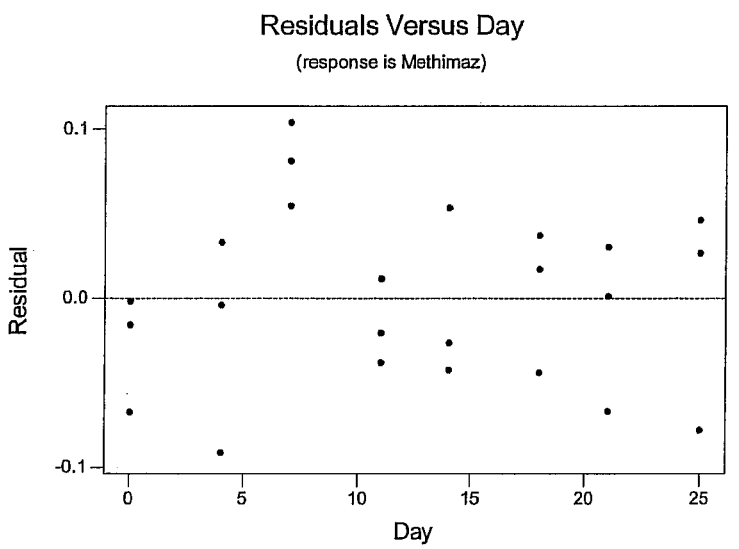
Obs	Day	Methimaz	Fit	SE Fit	Residual	St Resid
7	7.0	8.0079	7.9037	0.0129	0.1043	2.06R

```
R denotes an observation with a large standardized residual
```

Normplot of Residuals for Methimaz



Residuals from Methimaz vs Day




```
Break to allow evaluation of lack-of-fit and residuals
* Entering PAUSE mode. Type RESUME or R to leave PAUSE.
%MTB > r
* Leaving PAUSE mode.

Do you want to remove any data points? (yes OR no)
n

Should a quadratic be fit? (yes OR no)
```

```
n

Power analysis for t-test of slope less than zero
```

Power and Sample Size

```
1-Sample t Test

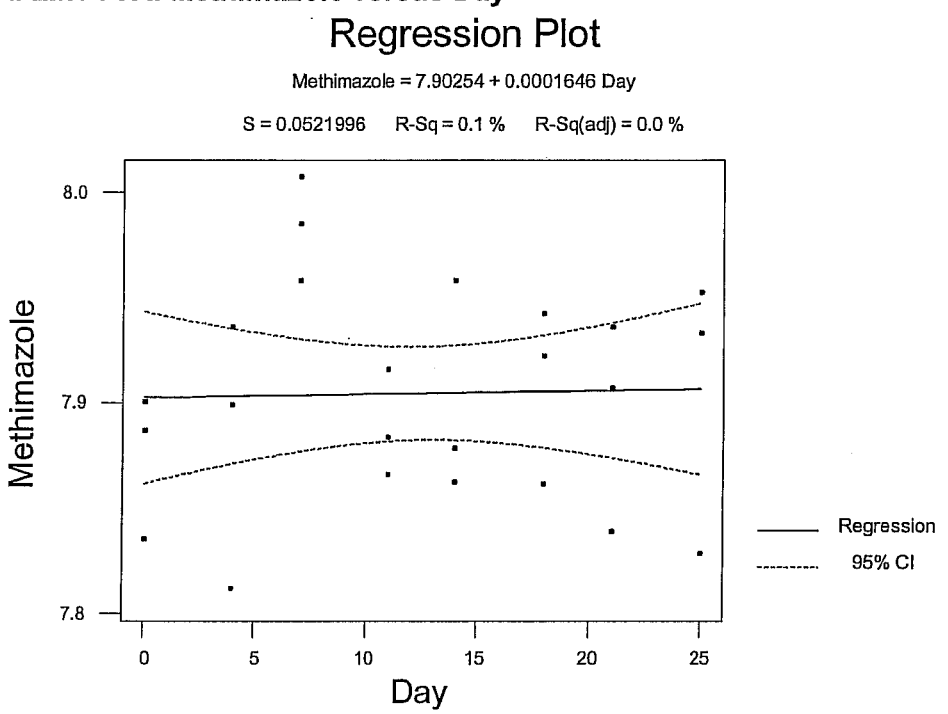
Testing mean = null (versus < null)
Calculating power for mean = null + difference
Alpha = 0.05  Sigma = 0.0521996

Sample
  Size   Power  Difference
    22  0.9900     -0.0457
```

- That means we would detect a mean of 7.829 as significantly less than ln(2630) = 7.875 or a change of 2513 from 2630 = 4.4% loss.

```
Fit 95% confidence bands about the fitted simple linear model
```

Fitted Line Plot: Methimazole versus Day



- **Conclusion – stable for 25 days.**

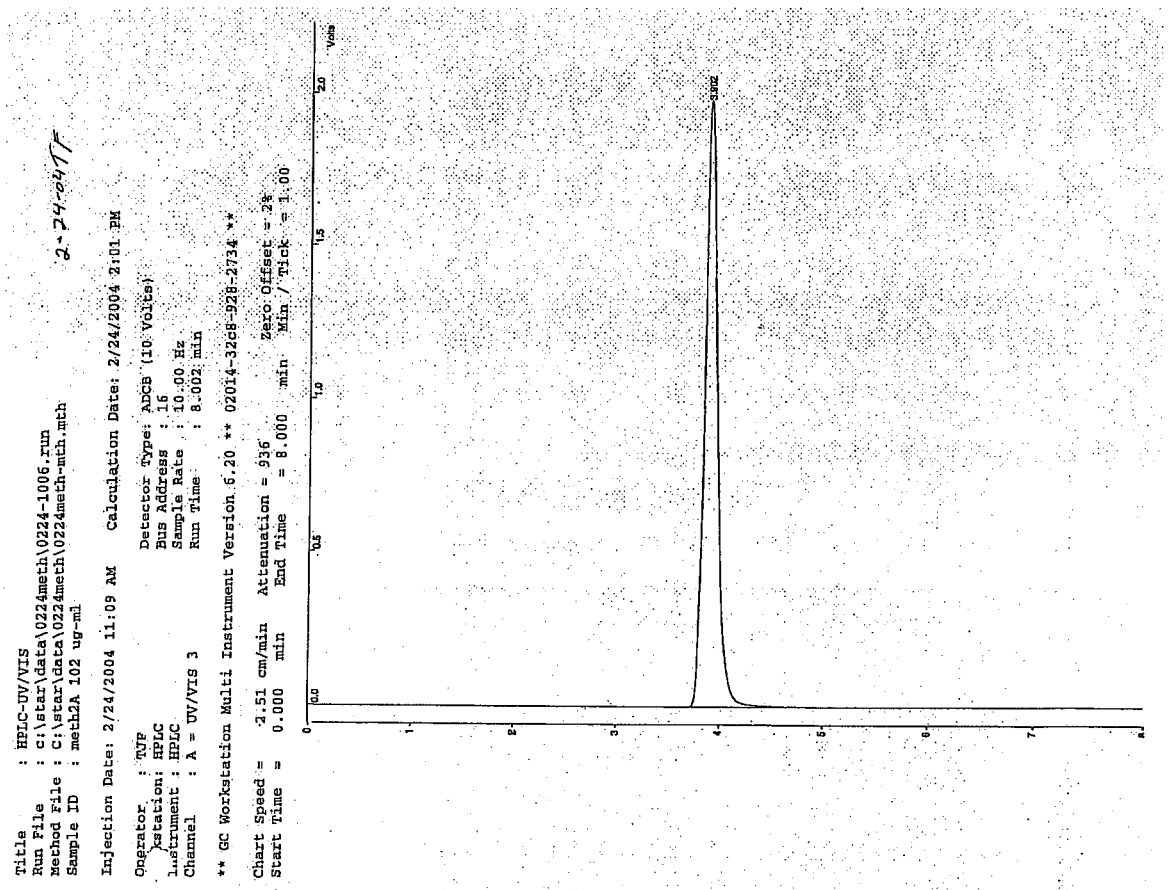


Figure 3. Typical Chromatogram, 102 ug/ml Methimazole

Methimazole
Test Chemical Analytical Data from Battelle:
In-Life Test Chemical Analysis Report

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Nadia Noll
P.O. #:	

BATT02-00054

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
002	DeCl$_2$ Tap H_2O	4/8/04 1600	1	G	Blue Ice	Methimazole
003	6.25 mg/L Methimazole		1			
004	12.5 mg/L Methimazole		1			
005	25.0 mg/L Methimazole		1			
006	50.0 mg/L Methimazole		2			
007	2500 mg/L Methimazole		1			

SPECIAL INSTRUCTIONS: Please send back ice chest

DATE:
INITIALS:

Nadia Noll	4/8/04 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE. USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road

Stillwater, OK 74074

405-624-6771 Fax 405-377-3388

mail@fortlabs.com

Client	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Nold
P.O. #	

For Lab Use Only

Lab Project # / WO #: BATT02-00054

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	For Lab Use Only: Temp	pH
008	DeCl$_2$ Tap H_2O	4/16/04 1600	1	G	Blue Ice	Methimazole		
009	6.25 mg/L Methimazole		1					
010	12.5 mg/L Methimazole		1					
011	25.0 mg/L Methimazole		2					
012	50.0 mg/L Methimazole		1					
013	2500 mg/L Methimazole		1					

SPECIAL INSTRUCTIONS: Please return ice chest

DATE:

INITIALS:

Sample Relinquished By:	Date/Time:	Sample Received By:	Date/Time:
John Haley	4/19/04 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Noll
P.O. #:	

Lab Use Only
Batch/Project # (Lab #)
BATT02-00054

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only: Init.	Temp.	pH
014	$DrCl_2$ Tap H_2O	4/22/04 1600	1	G	Blue Ice	Methimazole			
015	6.25 mg/L Methimazole		1						
016	12.5 mg/L Methimazole		1						
017	25.0 mg/L Methimazole		1						
018	50.0 mg/L Methimazole		2						
019	2500 mg/L Methimazole		1						

DATE:
INITIALS:

SPECIAL INSTRUCTIONS:
Please send back ice chest

Sampled By (Signature)	Date/Time	Received By (Signature)	Date/Time
Andrea Noll	4/22/04 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road

Stillwater, OK 74074

405-624-8771 Fax 405-377-3389

mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andria Noll
P.O. #:	

BATT02-00054

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
020	$DeCl_2$ Tap H_2O	4/29/04 1600	1	G	Blue Ice	Methimazole
021	6.25 mg/L Methimazole	↓	1	↓	↓	↓
022	12.5 mg/L Methimazole		1			
023	25.0 mg/L Methimazole		1			
024	50.0 mg/L Methimazole		1			
025 ~~029~~	2500 mg/L Methimazole		2			

SPECIAL INSTRUCTIONS:

Please send back ice chest

DATE:

INITIALS:

Relinquished by:	Date/Time	Received by:	Date/Time
Andria Noll	4/29/04 - 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

① Wrong entry - AN - 4/29/04

Methimazole
Thyroid Histology Data from EPL:
Sample Identification Key
Thyroid Pathology Report

EPL Sample Key for Methimazole

FEL Sample Number	Individual Specimen Number[1]	Conc. Replicate Number	Initial Specimen Stage	Chemical Conc. (mg/L)
026	002, 006, 007, 014, 017 021, 027, 032, 035, 038	A B	51	0
027	044, 045, 050, 056, 058 062, 063, 066, 074, 075	A B	51	6.25
028	083, 084, 088, 095, 098 106, 107, 110, 113, 116	A B	51	12.5
029	127, 130, 131, 138, 139 142, 145, 148, 150, 160	A B	51	25.0
030	162, 171, 174, 175, 179 181, 183, 184, 191, 196	A B	51	50.0
031	203, 205, 206, 211, 215 219, 227, 231, 232, 235	C D	54	0
032	238, 245, 246, 247, 255 263, 268, 271, 274, 275	C D	54	6.25
033	279, 281, 283, 290, 292 299, 305, 309, 310, 316	C D	54	12.5
034	320, 322, 324, 331, 335 342, 345, 347, 349, 356	C D	54	25.0
035	358, 364, 368, 374, 377 381, 382, 385, 386, 392	C D	54	50.0

[1] Individual specimens for thyroid histology were randomly selected using *Principles and Procedures of Statistics, A Biometrical Approach*, 2nd Ed., by Robert G.D. Steel and James H. Torrie, McGraw-Hill Book Co., USA, 1980.

EPL®
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February 2, 2005

Douglas J. Fort, Ph.D.
Fort Environmental Laboratories, Inc.
1414 South Sangre Road
Stillwater, OK 74074

Dear Dr. Fort:

Enclosed is a copy of the draft pathology report for Battelle, Study Number BATT02-00054, "Amphibian Metamorphosis Assay Demonstration Study," (EPL Project Number 237-009).

Upon approval, a final pathology report will be issued.

Sincerely,

SARAH L. HALE, DVM, Diplomate, ACVP
Veterinary Pathologist

SLH/cb
Enclosure

EPL®

EXPERIMENTAL PATHOLOGY LABORATORIES, INC.

BATTELLE
STUDY NUMBER BATT02-00054
EPL PROJECT NUMBER 237-009

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

DRAFT PATHOLOGY REPORT

Submitted by:

Experimental Pathology Laboratories, Inc.
P.O. Box 474
Herndon, VA 20172-0474
(703) 471-7060

Submitted to:

Battelle
Columbus, OH 43201

February 2, 2005

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DRAFT PATHOLOGY SUMMARY

BATTELLE
STUDY NUMBER BATT02-00054
EPL PROJECT NUMBER 237-009

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

DRAFT PATHOLOGY SUMMARY

Histomorphological evaluation was performed on thyroid glands of *Xenopus laevis* (South African clawed frogs) that were exposed to various concentrations of methimazole to determine the differential sensitivity of Stage 51 and Stage 54 larvae and to establish the necessary duration of exposure. Stage 51 larvae were exposed to methimazole for 21 days and Stage 54 larvae were exposed to methimazole for 14 days. The experimental designs were as follows:

Stage 51 Frog Larvae Exposed for 21 Days

Group/Replicate	Methimazole (mg/L)	No. of Frogs/Group	Individual Frog Numbers
026A	0.0	5	002, 006, 007, 014, 017
026B	0.0	5	021, 027, 032, 035, 038
027A	6.25	5	044, 045, 050, 056, 058
027B	6.25	5	062, 063, 066, 074, 075
028A	12.5	5	083, 084, 088, 095, 098
028B	12.5	5	106, 107, 110, 113, 116
029A	25.0	5	127, 130, 131, 138, 139
029B	25.0	5	142, 145, 148, 150, 160
030A	50.0	5	162, 171, 174, 175, 179
030B	50.0	5	181, 183, 184, 191, 196

Stage 54 Frog Larvae Exposed for 14 Days

Group/Replicate	Methimazole (mg/L)	No. of Frogs/Group	Individual Frog Numbers
031C	0.0	5	203, 205, 206, 211, 215
031D	0.0	5	219, 227, 231, 232, 235
032C	6.25	5	238, 245, 246, 247, 255
032D	6.25	5	263, 268, 271, 274, 275
033C	12.5	5	279, 281, 283, 290, 292
033D	12.5	5	299, 305, 309, 310, 316
034C	25.0	5	320, 322, 324, 331, 335
034D	25.0	5	342, 345, 347, 349, 356
035C	50.0	5	358, 364, 368, 374, 377
035D	50.0	5	381, 382, 385, 386, 392

MATERIALS AND METHODS

Slides were prepared by Experimental Pathology Laboratories, Inc. (EPL®). Head samples were trimmed from the frog larvae and placed in cassettes with the cut side down so that the heads were sectioned in a transverse plane from caudal to rostral. Three step sections (32 microns apart) were taken from each block at the level of the thyroid glands and were mounted on one slide. The slides were examined by a technician to assure that a sufficient amount of thyroid tissues was present bilaterally in the sections. If necessary to meet these criteria, additional step sections were cut from the block. The slides were stained with hematoxylin and eosin.

Microscopic findings for thyroid glands from each frog are listed in the attached Histopathology Incidence Tables. The changes were graded from 1 to 5 depending upon severity (1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high). Non-gradable findings were designated as present (P). All lesions are summarized by group in the Summary Incidence Tables. The sexes of the frogs were not reported.

RESULTS

Stage 51 *Xenopus laevis* exposed to methimazole for 21 days

There were exposure-related changes in the thyroid glands; these included follicular cell hyperplasia, decreased colloid and diffuse enlargement of the thyroid gland. At the three highest exposure concentrations (12.5 mg/L, 25 mg/L and 50 mg/L), the colloid in several animals varied in appearance between follicles. In some follicles, the colloid was homogeneous and in others it was pale and foamy. Two frogs exposed at 50 mg/L had only pale, foamy colloid. The incidence of decreased colloid in follicles occurred in a dose dependent fashion; one animal exposed at 6.25 mg/L, three animals exposed at 12.5 mg/L, seven animals exposed at 25 mg/L and all ten animals exposed at 50 mg/L had decreased colloid.

Hyperplasia of follicular cells occurred in all animals exposed at 25 mg/L and 50 mg/L; the severity of this change ranged from slight/mild to moderately severe at exposures of 50 mg/L and from minimal to moderately severe at 25 mg/L. Eight animals exposed to methimazole at 12.5 mg/L had follicular cell hyperplasia that ranged from minimal to moderate, and two animals exposed at 6.25 mg/L had minimal follicular cell hyperplasia. Diffuse thyroid gland enlargement was present in nine animals exposed at 50 mg/L and ranged in severity from slight/mild to moderately severe. Six animals exposed at 25 mg/L had diffuse thyroid enlargement that ranged widely in severity from minimal to severe. Minimal to slight/mild diffuse enlargement was noted in six animals exposed at 12.5 mg/L and minimal diffuse enlargement was present in two animals in the 6.25 mg/L exposure groups.

Three animals exposed to the highest concentration had one to several brightly eosinophilic droplets within scattered follicular epithelial cells; this change was not evident in animals exposed to lower concentrations. Distension of follicles was most prevalent in the animals exposed to lower doses of methimazole. Four animals exposed at 6.25 mg/L had follicular distension graded as minimal to slight/mild. Three animals exposed at 12.5 mg/L had distension of follicles graded as minimal to moderate, and one animal exposed at 25 mg/L had minimal follicular distension. See Table 1 for a tabulation of the prevalence of selected changes in the thyroid glands described above in Stage 51 frogs.

Table 1: Prevalence, With Replicates Combined, of Selected Changes in Thyroid Glands of Stage 51 Frogs After Exposure to Various Concentrations of Methimazole for 21 Days

Group	26 A & B	27 A & B	28 A & B	29 A & B	30 A & B
Exposure Conc.	0 mg/L	6.25 mg/L	12.5 mg/L	25.0 mg/L	50.0 mg/L
Thyroid (No. Examined)	9	10	10	10	10
Colloid, Decreased	0	1	3	7	10
Colloid, Foamy & Pale	0	0	6	3	7
Distension, Follicles	0	4	3	1	0
Enlargement, Diffuse	0	2	6	6	9
Eosinophilic Droplets, Follicular Cell	0	0	0	0	3
Hyperplasia, Follicular Cell	0	2	8	10	10

Interfollicular pigment was present in many of the thyroid glands from all exposure concentrations and varied in severity from minimal to moderate. There was no relationship between either the incidence or severity of this finding with exposure concentration of methimazole.

See Appendix A for photographs of thyroid glands from the Stage 51 frogs. The photographs are annotated to illustrate various features of thyroid glands and changes that resulted from exposure to methimazole.

Stage 54 *Xenopus laevis* larvae exposed to methimazole for 14 days

There were exposure-related changes in the thyroid glands, which included follicular cell hyperplasia, distension of thyroid follicles, decreased colloid, and diffuse glandular enlargement. At the three highest exposure levels (12.5 mg/L, 25 mg/L, and 50 mg/L), the colloid in many animals varied between follicles from the homogeneous appearance present in the control animals to pale and foamy. Five animals exposed at 50 mg/L and four animals exposed at 25 mg/L had only pale, foamy colloid in the examined follicles. All of the thyroids from animals exposed to 50 mg/L and 25 mg/L had decreased colloid, and nine of ten thyroids from animals exposed at 12.5 mg/L had decreased colloid. One animal exposed at 50 mg/L had no colloid evident. All of the animals in the three highest exposure groups had follicular cell hyperplasia. This change ranged from minimal to moderately severe in the animals exposed at 25 and 50 mg/L and from minimal to moderate in the group exposed at 12.5 mg/L.

Diffuse enlargement of the thyroid gland was most severe in the groups exposed at 12.5 mg/L and 25 mg/L and ranged from minimal to moderately severe. In the group exposed at 50 mg/L, diffuse enlargement was present in seven animals and ranged in severity from minimal to moderate. Distension of thyroid follicles was present only in animals from groups exposed to 12.5 mg/L and 25 mg/L; severity of this change ranged from minimal to moderate in the 12.5 mg/L exposure group and from minimal to slight/mild in the 25 mg/L exposure group. Two animals exposed at 50 mg/L and three animals exposed at

25 mg/L had variably sized eosinophilic droplets present in follicular epithelial cells. See Table 2 for a tabulation of the prevalence of selected changes in thyroid glands described above in Stage 54 frogs.

Table 2: Prevalence, With Replicates Combined, of Selected Changes in Thyroid Glands of Stage 54 Frogs After Exposure to Various Concentrations of Methimazole for 14 Days

Group	31 C & D	32 C & D	33 C & D	34 C & D	35 C & D
Exposure Conc.	0 mg/L	6.25 mg/L	12.5 mg/L	25.0 mg/L	50.0 mg/L
Thyroid (No. Examined)	10	9	10	10	9
Colloid, Decreased	0	0	9	10	9
Colloid, Foamy & Pale	0	0	9	7	6
Distension, Follicles	0	0	6	3	0
Enlargement, Diffuse	0	0	8	8	7
Eosinophilic Droplets, Follicular Cell	0	0	0	3	2
Hyperplasia, Follicular Cell	0	0	10	10	9

Interfollicular pigment was present in variable amounts in thyroid glands of several animals in each group. There was no relationship between the prevalence or severity of this change to the exposure concentration of methimazole.

See Appendix B for photographs of thyroid glands from Stage 54 frogs. The photographs are annotated to illustrate various features of thyroid glands and changes that resulted from exposure to various concentrations of methimazole.

DISCUSSION

Methimazole is a known thyrotoxin that blocks synthesis of thyroid hormone. Its mechanism of action is to inhibit thyroid peroxidase, which prevents

the oxidation of inorganic iodide necessary for binding of iodide to tyrosyl residues in thyroglobulin.[1] Administration of methimazole decreases circulating thyroid hormone concentration, which prompts release of thyrotropin-releasing hormone (TRH) by the hypothalamus. TRH in turn stimulates release of thyroid-stimulating hormone by the pituitary. The resulting continuous stimulation causes hypertrophy and hyperplasia of thyroid follicular cells.[2]

Dose dependent effects of methimazole on the thyroid follicular epithelial cells of larval African clawed frogs were apparent both in frogs that were exposed at Stage 51 for 21 days and at Stage 54 for 14 days. All Stage 51 frogs exposed at the two highest concentrations (25 mg/L and 50 mg/L) and all Stage 54 frogs exposed at the three highest concentrations (12.5 mg/L, 25 mg/L, and 50 mg/L) had thyroid follicular cell hyperplasia. There was a decreasing incidence of thyroid follicular cell hyperplasia in Stage 51 frogs with decreasing dose, while in Stage 54 frogs there was an apparent threshold effect of dose on incidence. None of the Stage 54 frogs exposed at 6.25 mg/L had follicular cell hyperplasia, but all Stage 54 larvae exposed to concentrations of methimazole of 12.5 mg/L and higher had follicular cell hyperplasia.

Changes in amount and appearance of colloid were present in both Stage 51 and Stage 54 larvae exposed to the higher doses of methimazole. All of the Stage 51 frogs exposed to the highest concentration of methimazole had decreased colloid. In Stage 54 frogs, all of the animals exposed at the two highest doses and nine of ten animals exposed to 12.5 mg/L had decreased colloid. Most of the Stage 51 and Stage 54 animals exposed to 12.5 mg/L of methimazole had pale and foamy colloid in some thyroid follicles; this type of colloid was not evident in any of the control animals or animals exposed at 6.25 mg/L. Brightly eosinophilic droplets within scattered follicular epithelial cells were present in low numbers of Stage 54 animals exposed at both 25.0 mg/L and

50 mg/L and in low numbers of Stage 51 animals exposed at 50.0 mg/L. Both the incidence and severity of diffuse thyroid gland enlargement generally increased with increasing exposure concentration. Some of the unexpected variation in diffuse gland enlargement at higher doses was likely because the glands were not all sectioned at the widest dimension, hindering direct comparison of sizes. Distension of follicles was most prevalent in the groups exposed to low and mid-range concentrations.

CONCLUSION

Exposure of *Xenopus laevis* larvae to methimazole resulted in changes in thyroid glands that included follicular cell hyperplasia, diffuse thyroid gland enlargement, decreased colloid and colloid with an abnormal pale, foamy appearance. These changes were most common in Stage 51 frogs exposed to the two highest concentrations and in Stage 54 frogs exposed to the three highest concentrations; however, several Stage 51 frogs exhibited some of these changes at the two lowest exposure concentrations. Both Stage 51 and Stage 54 frogs appeared to be similarly sensitive to development of follicular cell hyperplasia, diffuse enlargement and pale, foamy colloid, but Stage 54 frogs in this study appeared more likely to have decreased colloid at exposure concentrations of 12.5 mg/L and 25.0 mg/L than were Stage 51 frogs.

SARAH L. HALE, DVM, Diplomate, ACVP Date
Veterinary Pathologist

SLH/cb

REFERENCES

[1] Capen, CC, DeLellis RA, and Yarrington JT. (1991). Endocrine System. In "Handbook of Toxicologic Pathology", (WM Haschek and CG Rousseaux, eds.), pp. 718-719. Academic Press, San Diego.

[2] Capen CC. (1993). The Endocrine Glands. In "Pathology of Domestic Animals," (KVF Jubb, PC Kennedy, and N Palmer, eds.), pp. 307 and 315. Academic Press, San Diego.

2/2/05 DRAFT

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Amphibian Metamorphosis Assay Demonstration Study

Client Study: BATT02-00054 EPL Project Coordinator: Marilyn J. Wolfe

EPL Project Number: 237-009 EPL Pathologist: Marilyn J. Wolfe

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 Project Coordinator and Management are indicated below.

	Dates	
Area Inspected	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management _____

Date of last quarterly facility inspection 1/05

_____ _____

EPL Quality Assurance Unit Date

SUMMARY INCIDENCE TABLES

SUMMARY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African clawed frog)

	GROUP 026A	GROUP 026B	GROUP 027A	GROUP 027B	GROUP 028A	GROUP 028B
THYROID (NO. EXAMINED)	(5)	(4)	(5)	(5)	(5)	(5)
Colloid, Decreased			1		2	1
Colloid, Foamy and Pale					4	2
Colloid, Homogeneous	5	4	5	5	5	5
Distension, Follicles			1	3	2	1
Enlargement, Diffuse			1	1	3	3
Eosinophilic Droplets, Follicular Cell						
Hyperplasia, Follicular Cell			2		5	3
Interfollicular Pigment	1	2	3		3	4

SUMMARY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African clawed frog)

	GROUP 029A	GROUP 029B	GROUP 030A	GROUP 030B	GROUP 031C	GROUP 031D
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Colloid, Decreased	2	5	5	5		
Colloid, Foamy and Pale		3	4	3		
Colloid, Homogeneous	5	5	4	4	5	5
Distension, Follicles		1				
Enlargement, Diffuse	3	3	5	4		
Eosinophilic Droplets,						
Follicular Cell			3			
Hyperplasia, Follicular Cell	5	5	5	5		
Interfollicular Pigment		1	2	3	1	1

SUMMARY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African clawed frog)

	GROUP 032C	GROUP 032D	GROUP 033C	GROUP 033D	GROUP 034C	GROUP 034D
THYROID (NO. EXAMINED)	(5)	(4)	(5)	(5)	(5)	(5)
Colloid, Decreased			5	4	5	5
Colloid, Foamy and Pale			4	5	4	3
Colloid, Homogeneous	5	4	3	1	4	2
Distension, Follicles			3	3	1	2
Enlargement, Diffuse			4	4	5	3
Eosinophilic Droplets,						
Follicular Cell					2	1
Hyperplasia, Follicular Cell			5	5	5	5
Interfollicular Pigment	2	3	4	2	2	1

SUMMARY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African clawed frog)

	GROUP 035C	GROUP 035D				
THYROID (NO. EXAMINED)	(4)	(5)				
Colloid, Decreased	4	5				
Colloid, Foamy and Pale	4	2				
Colloid, Homogeneous	1	2				
Distension, Follicles						
Enlargement, Diffuse	4	3				
Eosinophilic Droplets, Follicular Cell	1	1				
Hyperplasia, Follicular Cell	4	5				
Interfollicular Pigment	3	3				

HISTOPATHOLOGY INCIDENCE TABLES

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African Clawed)

	GROUP 026A						GROUP 026B						GROUP 027A							
ANIMAL	51-002	51-006	51-007	51-014	51-017		51-021	51-027	51-032	51-035	51-038		51-044	51-045	51-050	51-056	51-058			
THYROID							N													
Colloid, Decreased																	1			
Colloid, Foamy and Pale																				
Colloid, Homogeneous	P	P	P	P	P			P	P	P	P		P	P	P	P	P			
Distension, Follicles													1							
Enlargement, Diffuse														1						
Eosinophilic Droplets, Follicular Cell																				
Hyperplasia, Follicular Cell													1				1			
Interfollicular Pigment	3							3		3				2	2	3				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African clawed frog)

	GROUP 027B						GROUP 028A						GROUP 028B						
ANIMAL	51-062	51-063	51-066	51-074	51-075		51-083	51-084	51-088	51-095	51-098		51-106	51-107	51-101	51-113	51-116		
THYROID																			
Colloid, Decreased							3		2								2		
Colloid, Foamy and Pale							P	P	P	P				P			P		
Colloid, Homogeneous	P	P	P	P	P		P	P	P	P	P		P	P	P	P	P		
Distension, Follicles		2	1	1					1	2							3		
Enlargement, Diffuse		1					1		2	2			2			1	2		
Eosinophilic Droplets, Follicular Cell																			
Hyperplasia, Follicular Cell							3	1	3	3	1		2			2	3		
Interfollicular Pigment							2	3		2			3	3	2	3			

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1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African clawed frog)

	GROUP 029A						GROUP 029B						GROUP 030A							
ANIMAL	51-1127	51-1130	51-1133	51-1138	51-1139		51-1142	51-1144	51-1148	51-1150	51-1160		51-1162	51-1171	51-1174	51-1175	51-1179			
THYROID																				
Colloid, Decreased			2	1			4	2	1	3	2		2	3	3	3	2			
Colloid, Foamy and Pale							P	P			P			P	P	P	P			
Colloid, Homogeneous	P	P	P	P	P		P	P	P	P	P		P		P	P	P			
Distension, Follicles								1												
Enlargement, Diffuse		1	5	4			2	3			5		3	3	3	2	4			
Eosinophilic Droplets, Follicular Cell													1	2	2					
Hyperplasia, Follicular Cell	1	2	4	4	2		3	3	1	2	4		4	3	3	4	4			
Interfollicular Pigment							3							2		2				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
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HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African Clawed)

	GROUP 030B						GROUP 031C						GROUP 031D							
ANIMAL	51-118 1	51-118 3	51-118 4	51-118 9 1	51-119 6		54-202 3	54-202 5	54-202 6	54-201 1	54-201 5		54-201 9	54-202 7	54-203 1	54-203 2	54-203 5			
THYROID																				
Colloid, Decreased	2	2	3	3	2															
Colloid, Foamy and Pale		P	P	P																
Colloid, Homogeneous	P	P	P		P		P	P	P	P	P		P	P	P	P	P			
Distension, Follicles																				
Enlargement, Diffuse	2	2	3	2																
Eosinophilic Droplets, Follicular Cell																				
Hyperplasia, Follicular Cell	3	3	4	3	2															
Interfollicular Pigment		2	1		3					3						2				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African clawed frog)

	GROUP 032C						GROUP 032D						GROUP 033C							
ANIMAL	54-2238	54-2245	54-2246	54-2247	54-2255		54-2263	54-2268	54-2271	54-2274	54-2275		54-2279	54-2281	54-2283	54-2290	54-2292			
THYROID										N										
Colloid, Decreased													2	2	4	2	2			
Colloid, Foamy and Pale													P	P	P	P				
Colloid, Homogeneous	P	P	P	P	P		P	P	P		P		P	P			P			
Distension, Follicles													2			3	1			
Enlargement, Diffuse													3		1	4	4			
Eosinophilic Droplets, Follicular Cell																				
Hyperplasia, Follicular Cell													3	2	2	2	2			
Interfollicular Pigment		2		3			2	2			2		1		2	1	2			

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
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HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African Clawed Frog)

	GROUP 033D						GROUP 034C						GROUP 034D								
ANIMAL	54-29 9	54-30 5	54-30 9	54-31 0	54-31 6		54-32 0	54-32 2	54-32 4	54-33 1	54-33 5		54-34 2	54-34 5	54-34 7	54-34 9	54-34 6				
THYROID																					
Colloid, Decreased	2	2		3	2		3	3	2	3	3		4	3	1	3	4				
Colloid, Foamy and Pale	P	P	P	P	P		P	P	P		P		P	P			P				
Colloid, Homogeneous			P				P		P	P	P				P	P					
Distension, Follicles	3	3			2			2					1	2							
Enlargement, Diffuse	1	4		2	1		2	1	4	3	2		4	2			3				
Eosinophilic Droplets, Follicular Cell									1		1		3								
Hyperplasia, Follicular Cell	2	2	1	2	2		3	3	3	3	3		4	3	2	1	3				
Interfollicular Pigment		3		1						2	3		2								

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

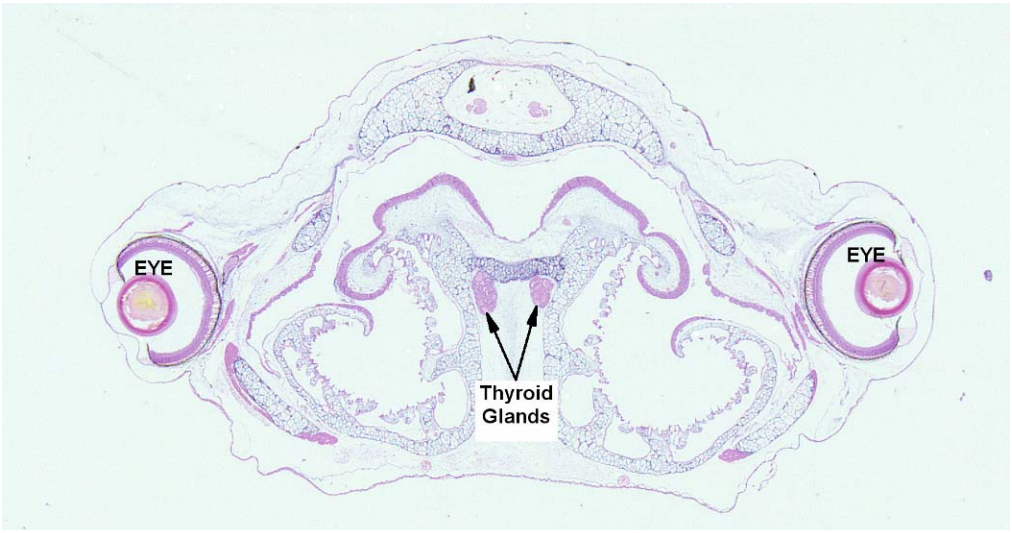
HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00054
Terminal Sacrifice
Xenopus laevis (South African Clawed Frog)

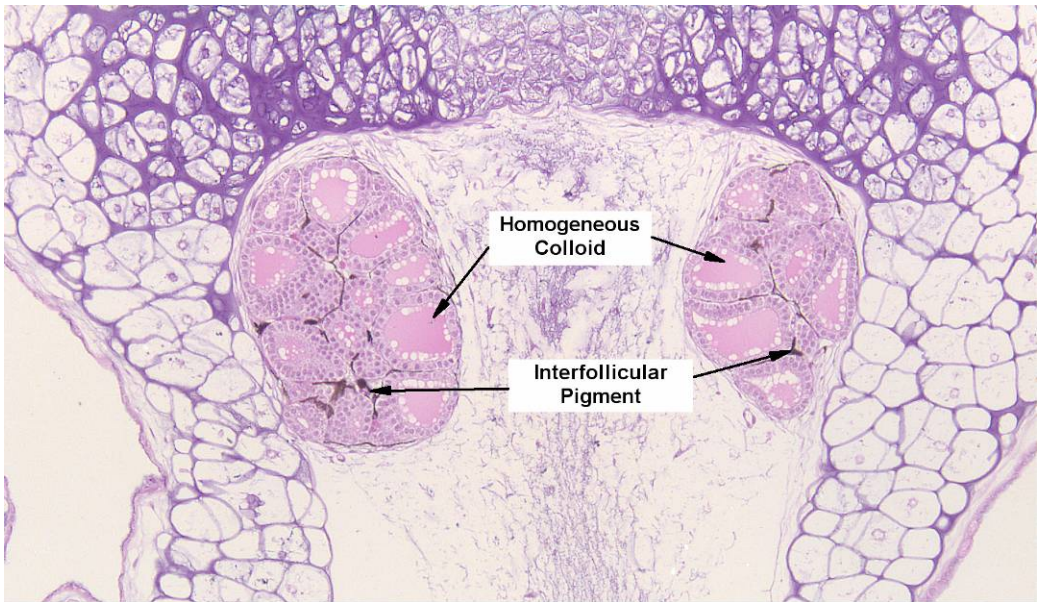
	GROUP 035C						GROUP 035D															
ANIMAL	54-33581	54-33644	54-33668	54-33674	54-33737		54-33881	54-33882	54-33885	54-33886	54-33892											
THYROID	N																					
Colloid, Decreased		4	3	4	3		4	4	5	3	1											
Colloid, Foamy and Pale		P	P	P	P		P	P														
Colloid, Homogeneous					P					P	P											
Distension, Follicles																						
Enlargement, Diffuse		1	1	1	1		3	1	1													
Eosinophilic Droplets, Follicular Cell			1					2														
Hyperplasia, Follicular Cell		3	3	2	3		4	2	3	3	1											
Interfollicular Pigment		2		3	2			2		2	3											

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

APPENDIX A: PHOTOGRAPHS – STAGE 51 FROGS

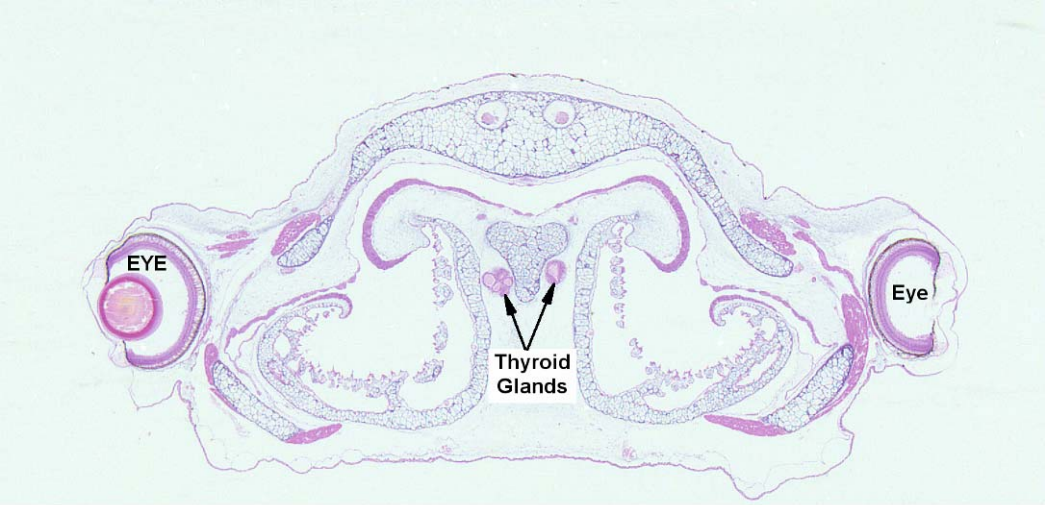


Z12117 African Clawed Frog 51-038 Group 26B
BATT02-00054 0.0 mg/L Methimazole H&E 1.25x

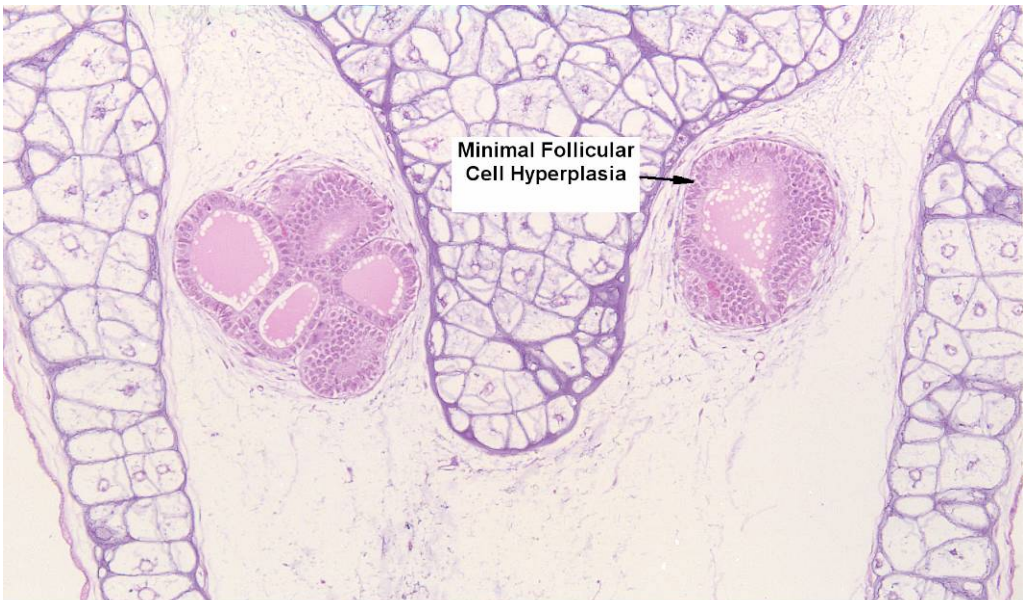


Z12132 African Clawed Frog 51-002 Group 26A
BATT02-00054 0.0 mg/L Methimazole H&E 10x

Z12117 and Z12132. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from control Stage 51 *Xenopus laevis* (African clawed frog). The colloid is homogeneous and light eosinophilic and the follicles are lined by simple cuboidal epithelium.

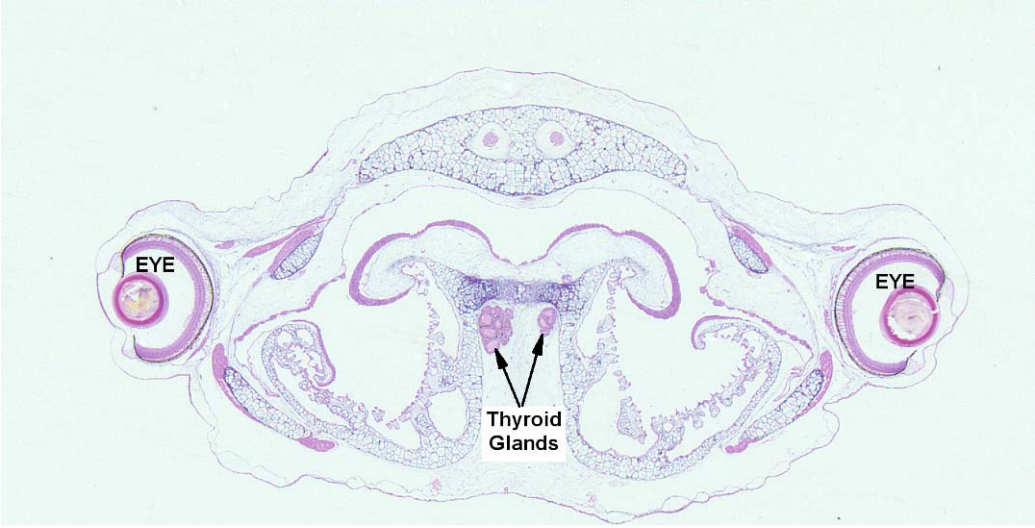


Z12123 African Clawed Frog 51-044 Group 27A
BATT02-00054 6.25 mg/L Methimazole H&E 1.25x

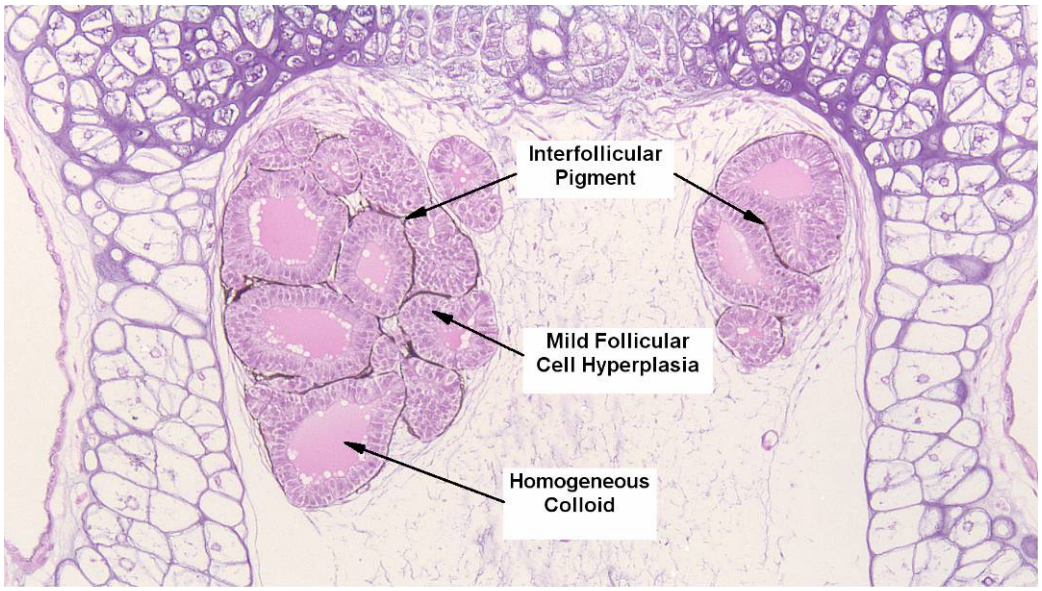


Z12139 African Clawed Frog 51-044 Group 27A
BATT02-00054 6.25 mg/L Methimazole H&E 10x

Z12123 and Z12139. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* (African clawed frog) exposed to 6.25 mg/L methimazole for 21 days. There is minimal follicular cell hyperplasia.

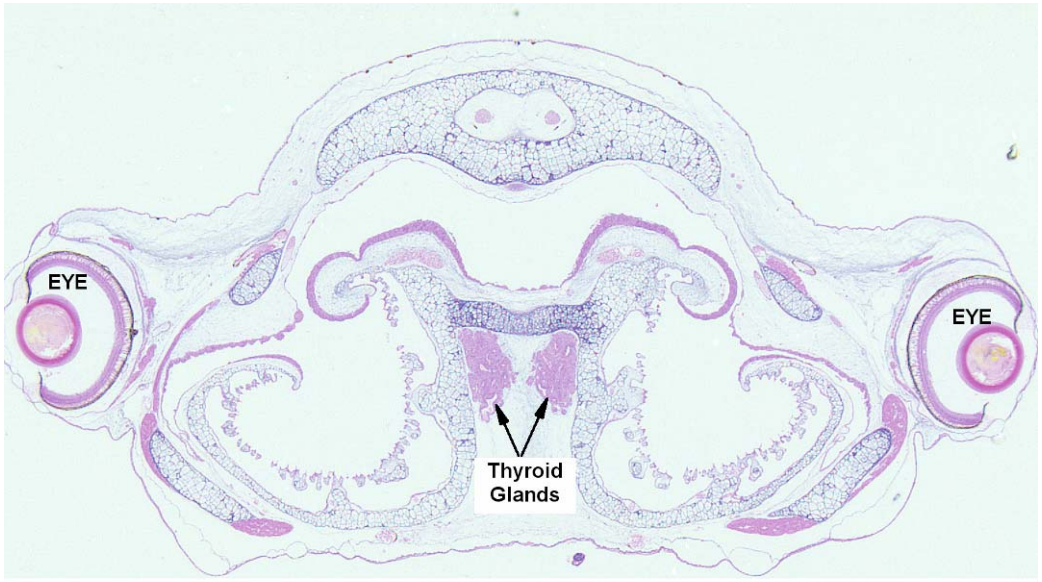


Z12122 African Clawed Frog 51-106 Group 28B
BATT02-00054 12.5 mg/L Methimazole H&E 1.25x

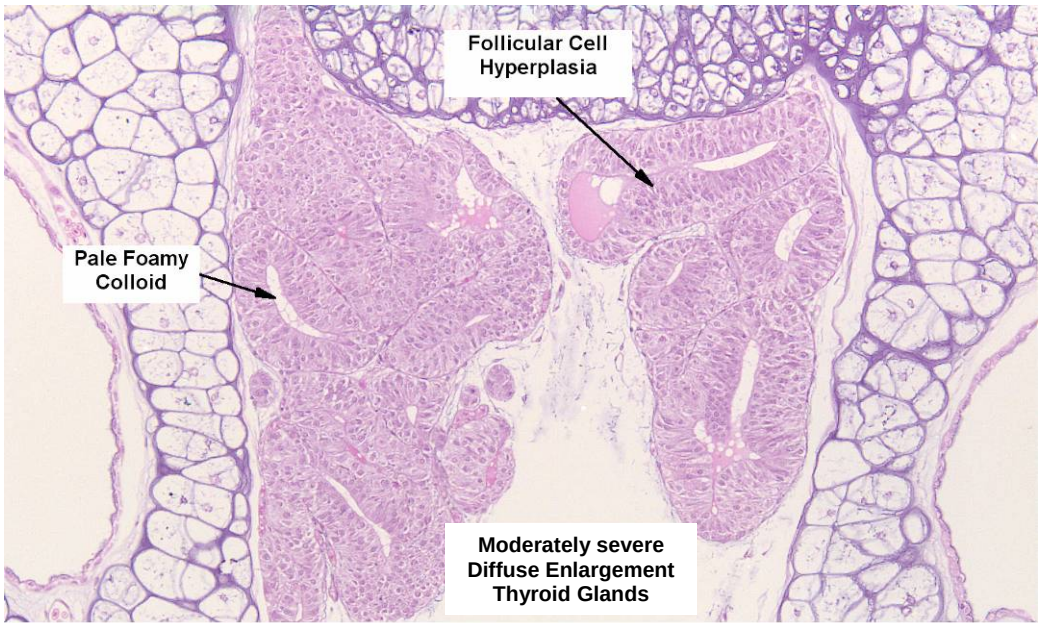


Z12138 African Clawed Frog 51-106 Group 28B
BATT02-00054 12.5 mg/L Methimazole H&E 10x

Z12122 and Z12138. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* (African clawed frog) exposed to 12.5 mg/L methimazole for 21 days. There is mild follicular cell hyperplasia.

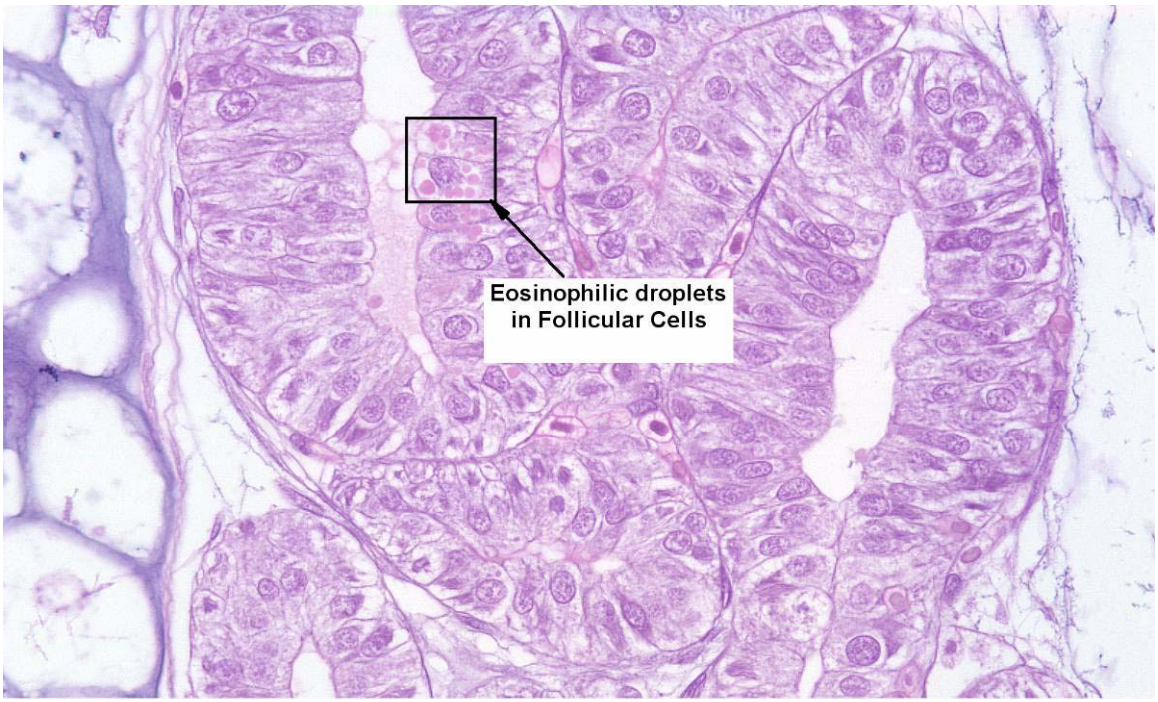


Z12120 African Clawed Frog 51-184 Group 30B
BATT02-00054 50 mg/L Methimazole H&E 1.25x



Z12134 African Clawed Frog 51-179 Group 30A
BATT02-00054 50 mg/L Methimazole H&E 10x

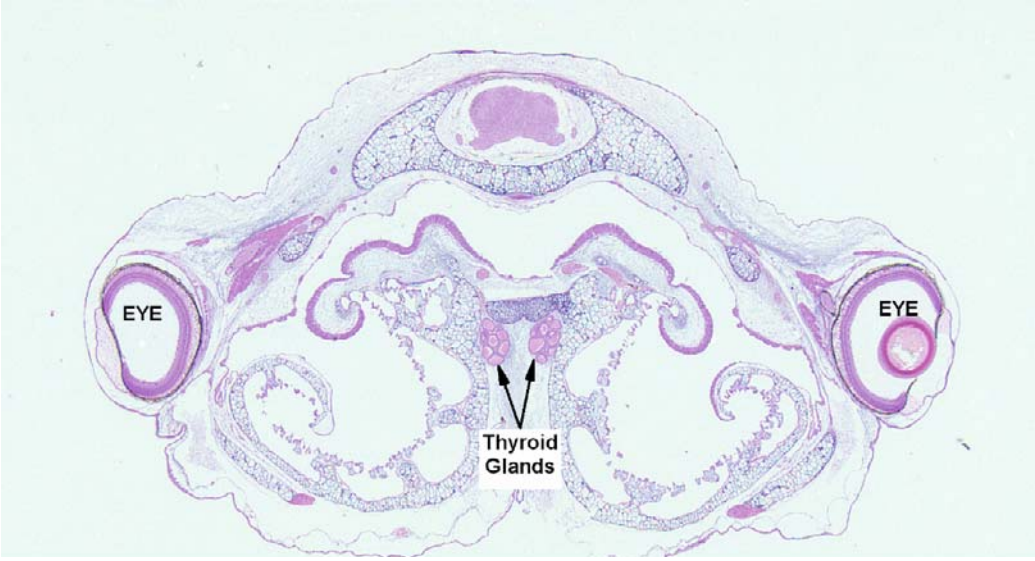
Z12120 and Z12134. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* (African clawed frog) exposed to 50 mg/L methimazole for 21 days. There is moderately severe, diffuse enlargement of the thyroid glands, follicular cell hyperplasia, and pale, foamy colloid.



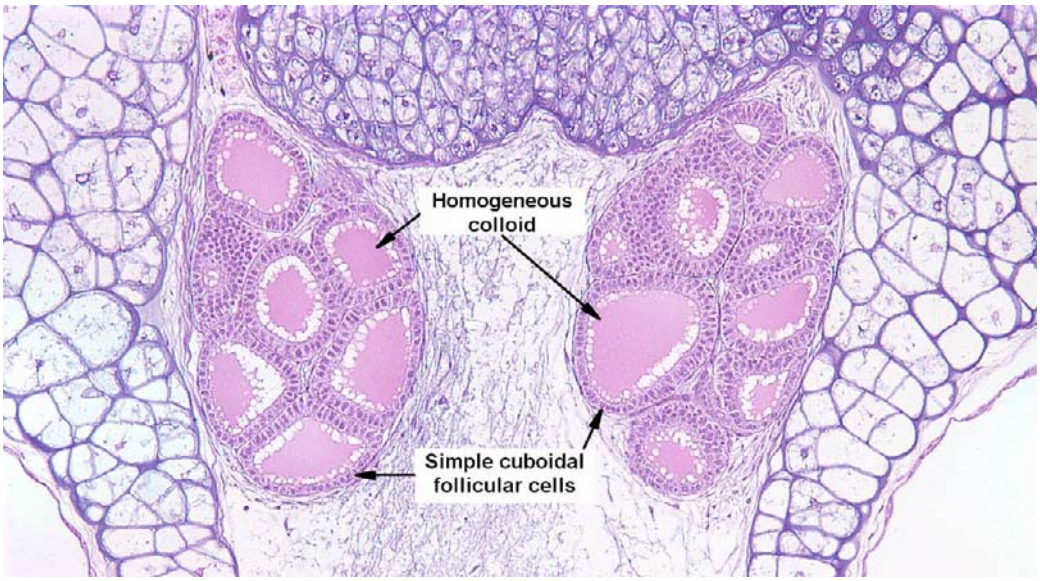
Z12141 African Clawed Frog 51-174 Group 30A
BATT02-00054 50 mg/L Methimazole H&E 40x

Z12141. High (40x) magnification photomicrograph of thyroid gland from a Stage 51 *Xenopus laevis* (African clawed frog) exposed to 50 mg/L methimazole for 21 days. Note the eosinophilic droplets in follicular cells.

APPENDIX B: PHOTOGRAPHS – STAGE 54 FROGS

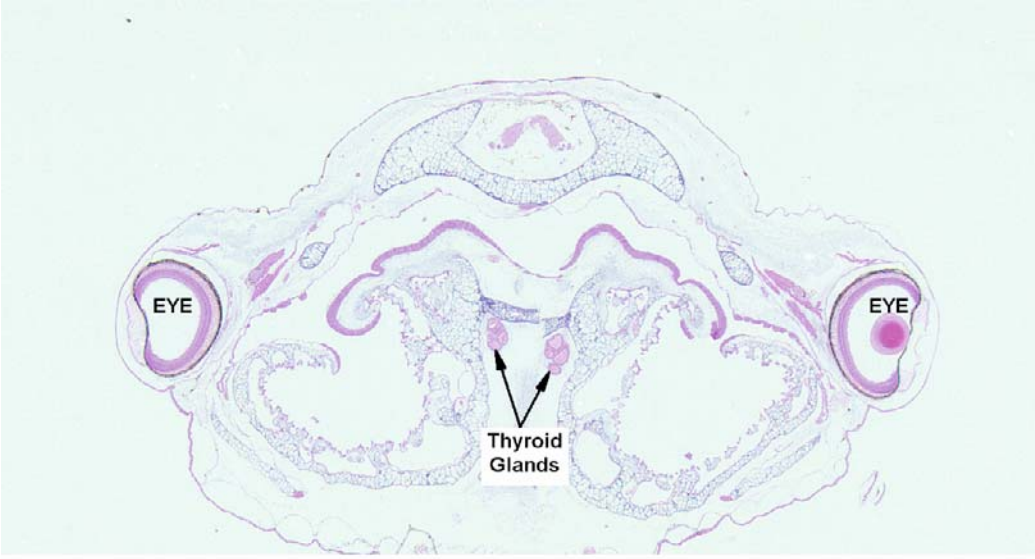


Z12108 African Clawed Frog 54-203 Group 31C
BATT02-00054 0.0 mg/L Methimazole H&E 1.25x

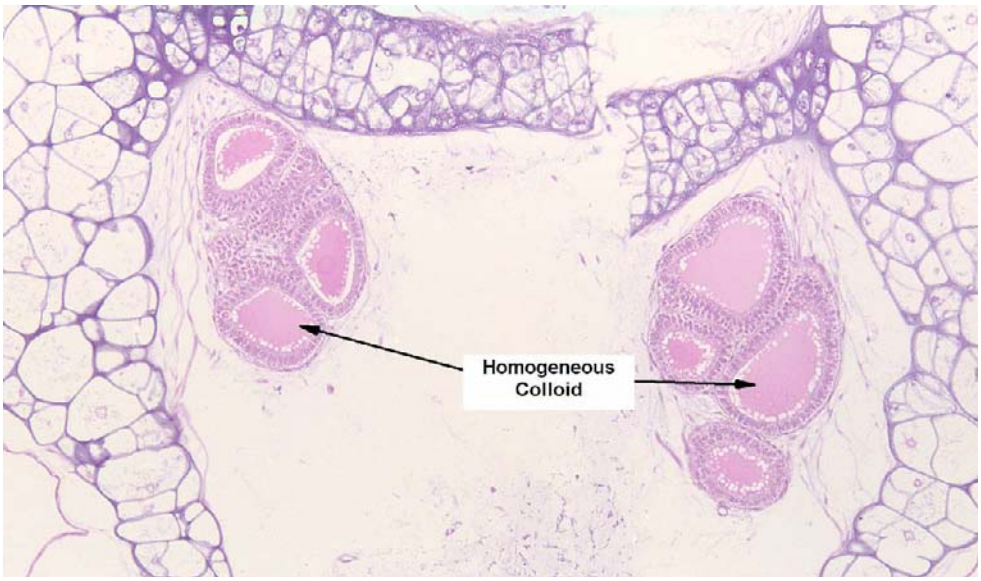


Z12124 African Clawed Frog 54-203 Group 31C
BATT02-00054 0.0 mg/L Methimazole H&E 10x

Z12108 and Z12124. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from control Stage 54 *Xenopus laevis* (African clawed frog). The colloid is eosinophilic and homogeneous, and the follicles are lined by simple cuboidal epithelium.

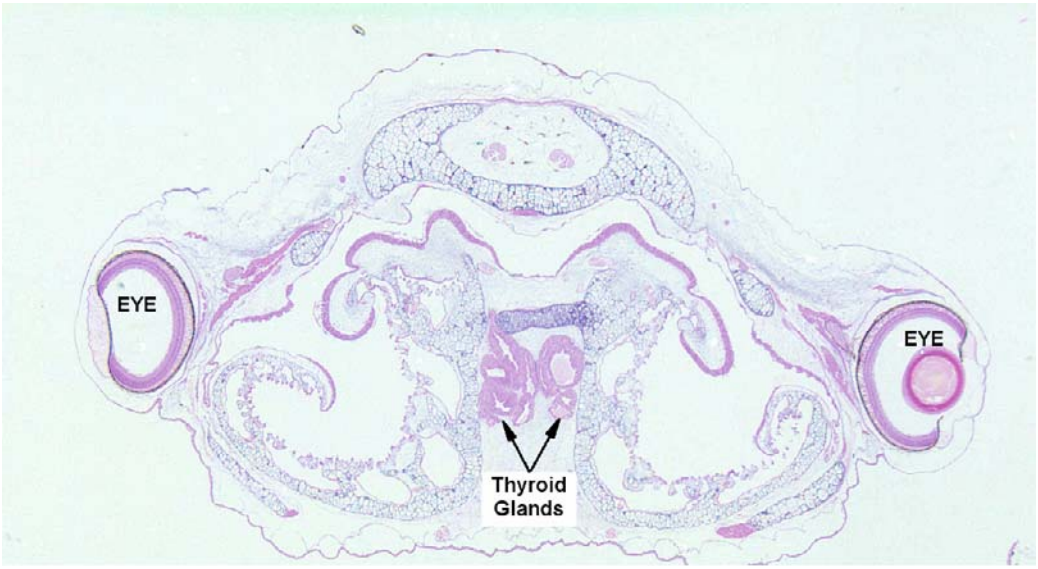


Z12115 African Clawed Frog 54-246 Group 32C
BATT02-00054 6.25 mg/L Methimazole H&E 1.25x

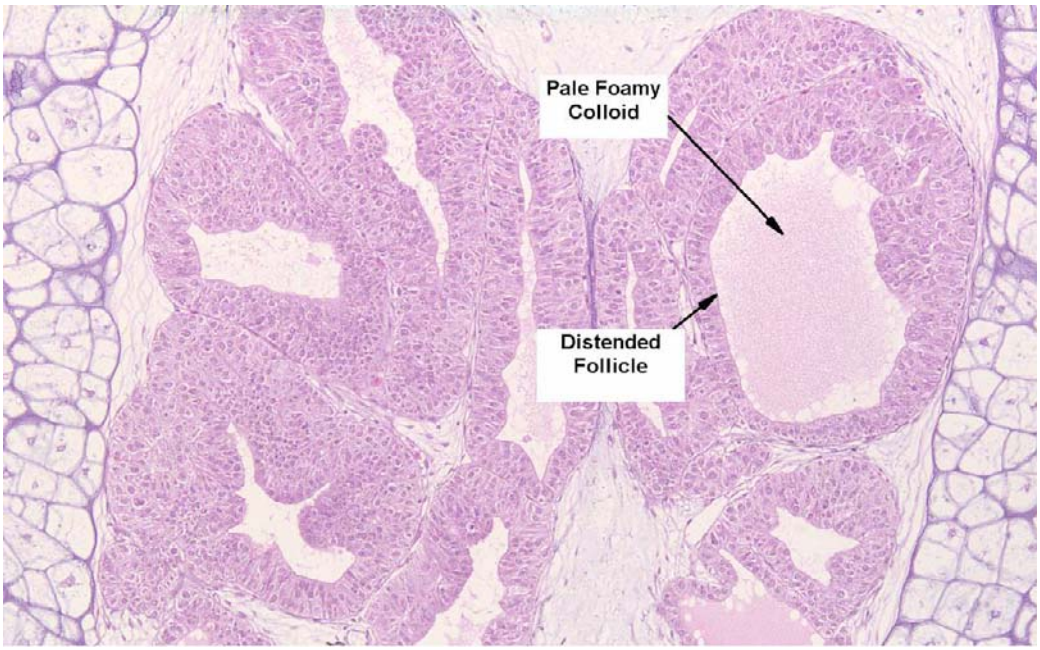


Z12131 African Clawed Frog 54-246 Group 32C
BATT02-00054 6.25 mg/L Methimazole H&E 10x

Z12115 and Z12131. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* (African clawed frog) exposed to 6.25 mg/L methimazole for 14 days. The colloid is homogeneous and eosinophilic, and follicles are lined by simple cuboidal epithelium.

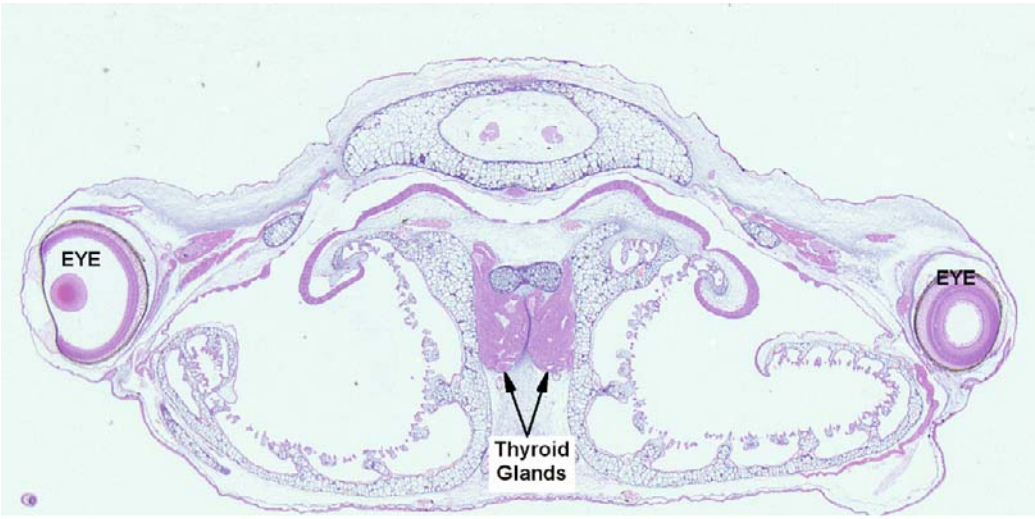


Z12114 African Clawed Frog 54-290 Group 33C
BATT02-00054 12.5 mg/L Methimazole H&E 1.25x

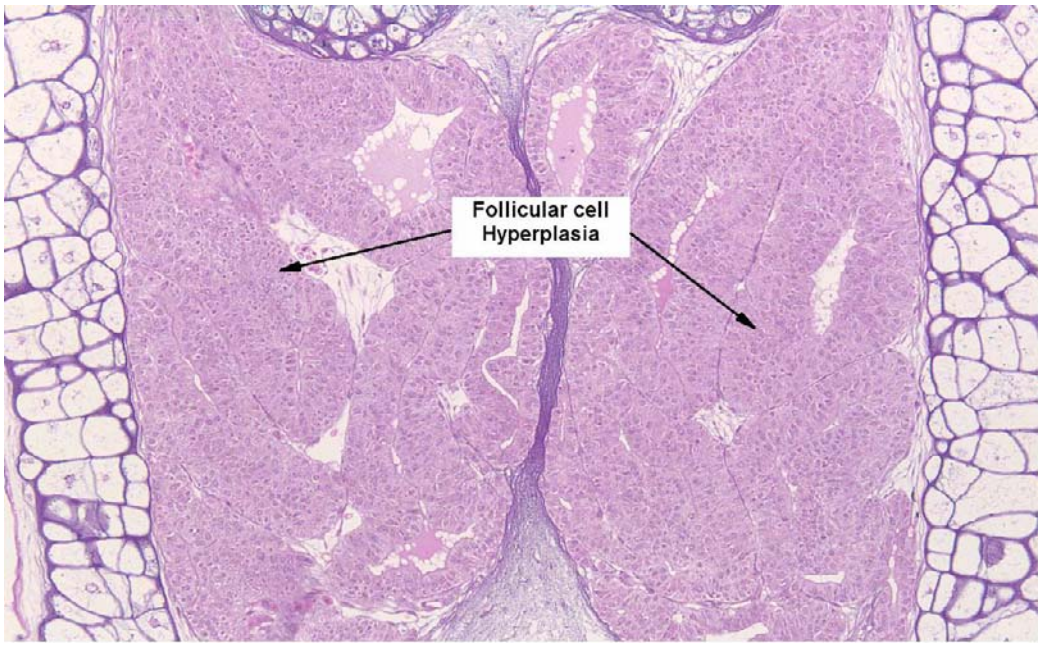


Z12130 African Clawed Frog 54-290 Group 33C
BATT02-00054 12.5 mg/L Methimazole H&E 10x

Z12114 and Z12130. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* (African clawed frog) exposed to 12.5 mg/L methimazole for 14 days. There is pale, foamy colloid and several follicles are distended.

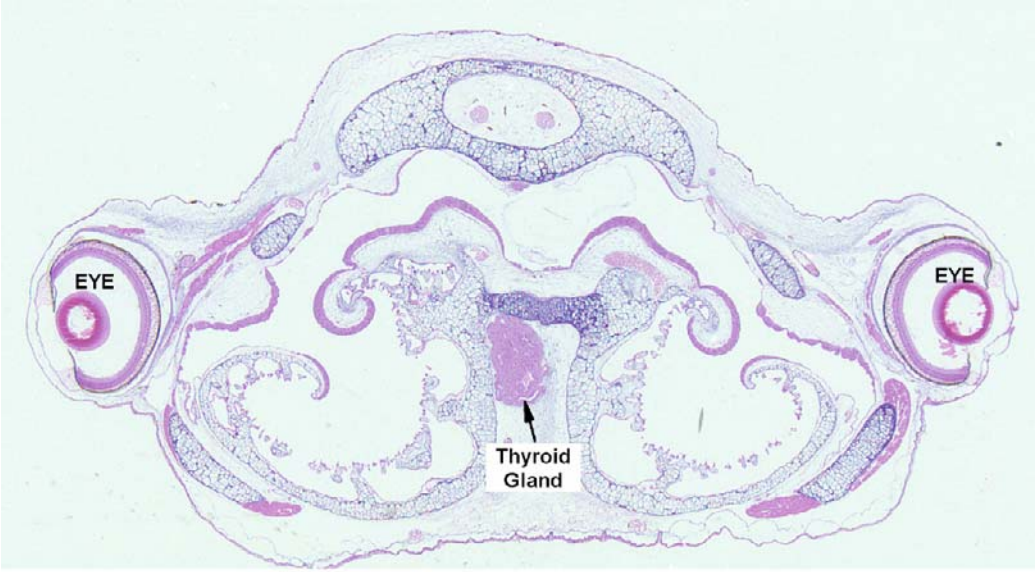


Z12112 African Clawed Frog 54-324 Group 34C
BATT02-00054 25 mg/L Methimazole H&E 1.25x

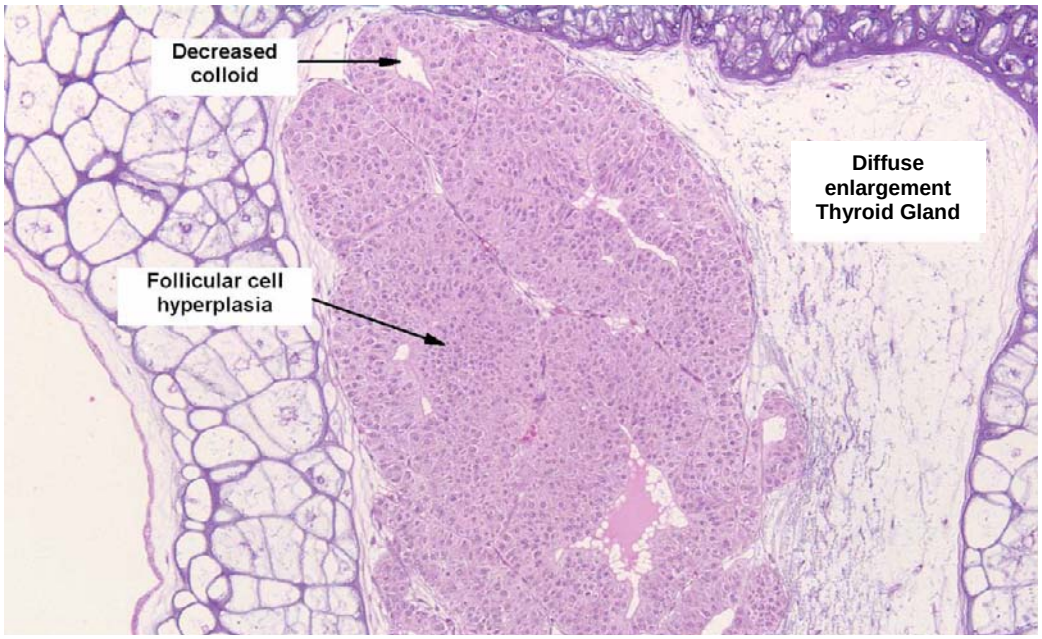


Z12128 African Clawed Frog 54-324 Group 34C
BATT02-00054 25 mg/L Methimazole H&E 10x

Z12112 and Z12128. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* (African clawed frog) exposed to 25 mg/L methimazole for 14 days. There is diffuse gland enlargement and follicular cell hyperplasia.

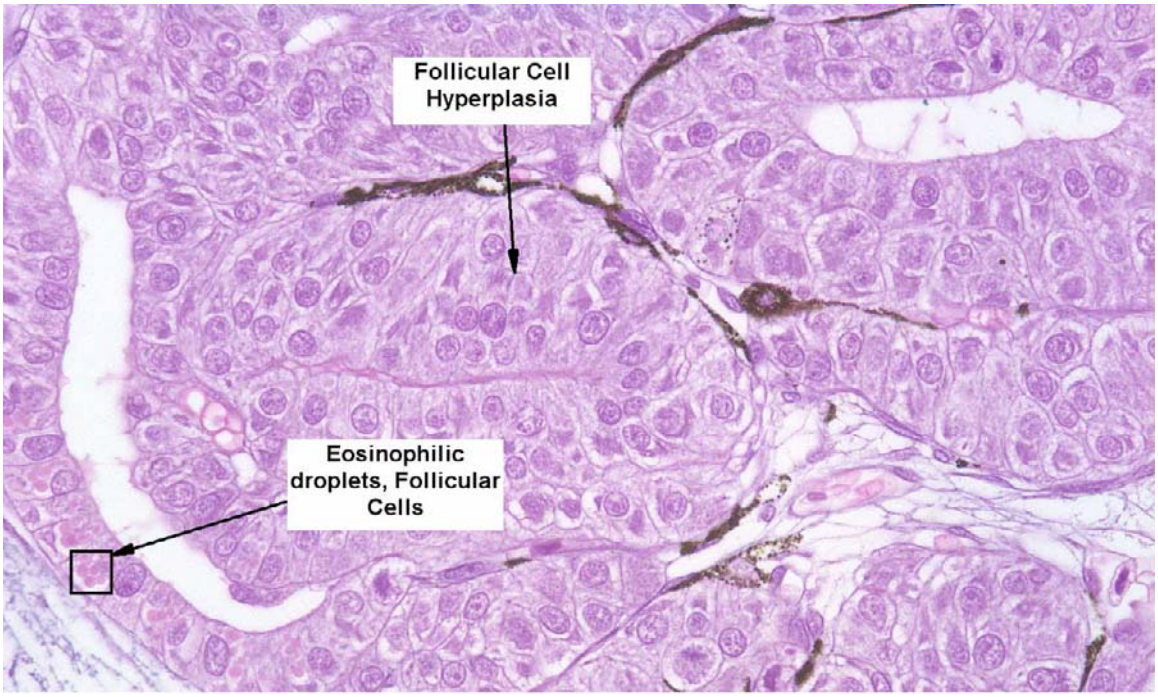


Z12111 African Clawed Frog 54-381 Group 35D
BATT02-00054 50 mg/L Methimazole H&E 1.25x



Z12127 African Clawed Frog 54-381 Group 35D
BATT02-00054 50 mg/L Methimazole H&E 10x

Z12111 and Z12127. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* (African clawed frog) exposed to 50 mg/L methimazole for 14 days. There is diffuse thyroid gland enlargement, decreased colloid and follicular cell hyperplasia.



Z12140 African Clawed Frog 54-382 Group 35D
BATT02-00054 50 mg/L Methimazole H&E 40x

Z12140. High (40x) magnification photomicrograph of thyroid gland from a Stage 54 *Xenopus laevis* (African clawed frog) exposed to 50 mg/L methimazole for 14 days. There are eosinophilic droplets in follicular cells and follicular cell hyperplasia.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

1 of 2

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	John Oldey
P.O. #:	

Lab Use Only
Client/Project # - WO #
BATT02- 00054

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only ID#	Lab Use Only Temp	Lab Use Only pH
026	Specimen #s: 002, 006, 007,	4/29/04 1600	10	G	10% Formalin	Histological examination of Thyroid gland as			
	014, 017, 021, 027, 032, 035	↓	↓	↓	↓	per protocol # BATT02-1			
	038	↓	↓	↓					
027	Specimen #s: 044, 045, 050,		10	G	10% Formalin				
	056, 058, 062, 063,		↓	↓	↓				
	066, 074, 075		↓	↓	↓				
028	Specimen #s: 083, 084, 088,		10	G	10% Formalin				
	095, 098, 106, 107, 110,		↓	↓	↓				
	113, 116		↓	↓	↓				
029	Specimen #s: 127, 130, 131,		10	G	10% Formalin				
	138, 139, 142, 145, 148,		↓	↓	↓				
	150, 160		↓	↓	↓				
030	Specimen #s: 162, 171, 174,		10	G	10% Formalin				
	175, 179, 181, 183, 184,		↓	↓	↓				
	191, 196	↓	↓	↓	↓				

DATE:
INITIALS:

SPECIAL INSTRUCTIONS: Organisms exposed to methimazole

Sample Relinquished By	Date-Time	Sample Received By	Date-Time
John Oldey	5/5/04 1700	Michael D. Hall	5/7/04 0830

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

2 of 2

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	
P.O. #:	

Lab Use Only	
Client/Project # - WO #	
BATT02-00054	

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only ID#	Temp	pH
031	Specimen #s: 203, 205, 206, 211, 215, 219, 227, 231 232, 235	4/30/04 1400	10	G	10% Formalin	Histological examination of Thyroid gland as per protocol # BATT02-1			
032	Specimen #s: 238, 245, 246, 247, 255, 263, 268, 271, 274, 275		10	G	10% Formalin				
033	Specimen #s: 279, 290, 292, 283, 281, 309, 299, 310, 305, 316		10	G	10% Formalin				
034	Specimen #s: 320, 322, 324, 331, 335, 342, 345, 347 349, 356		10	G	10% Formalin				
035	Specimen #s: 358, 364, 368, 374, 377, 381, 382, 385, 386, 392		10	G	10% Formalin				

SPECIAL INSTRUCTIONS: Organisms exposed to Methimazole

DATE:
INITIALS:

Sample Relinquished By	Date-Time	Sample Received By	Date-Time
	5/5/04 1700		5/7/04 09:30

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

Appendix E
L-Thyroxine (T4)

Metamorphosis Assay – Raw Data:

Daily Operational Data
Test Termination Data – Stage and Growth
Statistical Data – Stage and Growth

Test Chemical Analytical Data from Battelle:

Purity / Stability Report
In-Life Test Chemical Analysis Report

Thyroid Histology Data from EPL:

Sample Identification Key
Thyroid Pathology Report

L-Thyroxine (T4)
Metamorphosis Assay:
Daily Operational Data

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00062 **Test No: 1**
Test Chemical: L-Thyroxine (T4) **Target Conc: 0.0 µg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc[1] (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	06/10/04			100.0	22.0	7.4	8.8	1555	25			100.0	22.0	7.5	8.9	1274	25	100.0	22.0	7.5	8.9	1415	25	0.05
1	06/11/04		20	100.0	22.0	7.2	8.1	1442	25		20	100.0	22.0	7.3	8.4	1711	25	100.0	22.0	7.3	8.3	1577	25	
2	06/12/04		20	100.0	22.0	7.2	8.2	1538	25		20	100.0	22.0	7.2	8.2	1629	25	100.0	22.0	7.2	8.2	1584	25	
3	06/13/04		20	100.0	22.0	7.1	8.0	1740	25		20	100.0	22.0	7.1	8.0	1660	25	100.0	22.0	7.1	8.0	1700	25	
4	06/14/04		20	100.0	22.0	7.0	7.9	1690	25		20	100.0	22.1	7.1	8.0	1720	25	100.0	22.1	7.1	8.0	1705	25	
5	06/15/04		20	100.0	22.0	7.1	6.6	1509	25		20	100.0	22.0	7.2	6.7	1393	25	100.0	22.0	7.2	6.7	1451	25	
6	06/16/04		20	100.0	22.2	7.0	6.6	1380	25		20	100.0	22.2	7.1	6.7	1520	25	100.0	22.2	7.1	6.7	1450	25	
7	06/17/04		20	100.0	22.0	7.1	6.8	1365	25		20	100.0	22.0	7.1	6.6	1580	25	100.0	22.0	7.1	6.7	1473	25	0.05
8	06/18/04		20	100.0	20.8	7.0	5.9	1556	25		20	100.0	20.8	7.1	6.0	1413	25	100.0	20.8	7.1	6.0	1485	25	
9	06/19/04		20	100.0	21.0	6.9	6.2	1540	25		20	100.0	21.0	7.1	6.1	1625	25	100.0	21.0	7.0	6.2	1583	25	
10	06/20/04		20	100.0	21.0	7.4	5.8	1480	25		20	100.0	21.0	7.1	6.1	1620	25	100.0	21.0	7.3	6.0	1550	25	
11	06/21/04		20	100.0	21.7	7.2	5.9	1565	25		20	100.0	21.7	7.1	5.8	1635	25	100.0	21.7	7.2	5.9	1600	25	
12	06/22/04		20	100.0	21.4	7.1	6.5	1620	25		20	100.0	21.4	7.2	6.5	1621	25	100.0	21.4	7.2	6.5	1621	25	
13	06/23/04		20	100.0	21.4	7.2	6.6	1640	25		20	100.0	21.6	7.3	6.5	1620	25	100.0	21.5	7.3	6.6	1630	25	
14	06/24/04		20	100.0	21.5	7.1	5.8	1565	25		20	100.0	21.6	7.2	5.8	1535	25	100.0	21.6	7.2	5.8	1550	25	0.05
15	06/25/04		20	100.0	21.9	7.3	6.3	1658	25		20	100.0	21.8	7.3	6.7	1515	25	100.0	21.9	7.3	6.5	1587	25	
16	06/26/04		20	100.0	21.8	7.3	6.1	1650	25		20	100.0	21.8	7.3	6.0	1555	25	100.0	21.8	7.3	6.1	1603	25	
17	06/27/04		20	100.0	21.9	7.4	5.9	1465	25		20	100.0	21.8	7.4	5.9	1505	25	100.0	21.9	7.4	5.9	1485	25	
18	06/28/04		20	100.0	21.7	7.3	6.4	1625	25		20	100.0	21.7	7.3	6.1	1505	25	100.0	21.7	7.3	6.3	1565	25	
19	06/29/04		20	100.0	21.6	7.1	6.1	1842	25		20	100.0	21.8	7.2	5.7	1646	25	100.0	21.7	7.2	5.9	1744	25	
20	06/30/04		20	100.0	21.5	7.2	6.1	1120	25		20	100.0	21.6	7.4	6.2	1145	25	100.0	21.6	7.3	6.2	1133	25	
21	07/01/04		20	100.0							20	100.0						100.0						1.30
	Median:				**21.8**	**7.2**	**6.4**	**1556**	**25**				**21.8**	**7.2**	**6.5**	**1580**	**25**		**21.8**	**7.2**	**6.5**	**1577**	**25**	
	Mode:				**22.0**	**7.1**	**6.6**	**1565**	**25**				**22.0**	**7.1**	**6.7**	**1620**	**25**		**22.0**	**7.2**	**6.7**	**1550**	**25**	
	Mean:			**100.0**	**21.7**	**7.2**	**6.7**	**1550**	**25.0**			**100.0**	**21.7**	**7.2**	**6.7**	**1544**	**25.0**	**100.0**	**21.7**	**7.2**	**6.7**	**1547**	**25.0**	**0.363**
	SEM:			**0.00**	**0.08**	**0.03**	**0.20**	**32.93**	**0.00**			**0.00**	**0.08**	**0.03**	**0.21**	**30.87**	**0.00**	**0.00**	**0.08**	**0.03**	**0.21**	**28.12**	**0.00**	**0.31**
	% CV:			**0.00**	**8.35**	**5.30**	**35.58**	**383.35**	**0.00**			**0.00**	**8.17**	**4.50**	**37.65**	**360.02**	**0.00**	**0.00**	**8.22**	**4.49**	**36.44**	**327.59**	**0.00**	**103.81**

[1]All measured test concentrations reported by Battelle as less than method detection limit (MDL) [0.09 µg/L] or none detected (ND) are expressed as 0.05 µg/L (1/2 of MDL).

DAILY DATA

Workorder No: 00062
Test Chemical: L-Thyroxine (T4)

Test No: 1
Target Conc: 0.5 µg/L

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Conc[1] (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	06/10/04			100.0	22.0	7.5	8.8	1202	27			100.0	22.0	7.5	8.9	1468	27	100.0	22.0	7.5	8.9	1335	27	
1	06/11/04		20	100.0	22.0	7.3	7.9	1308	25		20	100.0	22.0	7.3	8.1	1366	25	100.0	22.0	7.3	8.0	1337	25	
2	06/12/04		20	100.0	22.0	7.3	8.0	1426	25		20	100.0	22.0	7.3	8.0	1548	25	100.0	22.0	7.3	8.0	1487	25	
3	06/13/04		20	100.0	22.0	7.2	7.9	1505	25		20	100.0	22.0	7.2	7.9	1701	25	100.0	22.0	7.2	7.9	1603	25	
4	06/14/04		20	100.0	22.0	7.2	8.0	1545	25		20	100.0	22.0	7.1	7.9	1895	25	100.0	22.0	7.2	8.0	1720	25	
5	06/15/04		20	100.0	22.0	7.2	6.7	1357	25		20	100.0	22.0	7.2	6.6	1609	25	100.0	22.0	7.2	6.7	1483	25	
6	06/16/04		20	100.0	22.4	7.1	6.5	1240	25		20	100.0	22.2	7.2	6.6	1450	25	100.0	22.3	7.2	6.6	1345	25	
7	06/17/04		20	100.0	22.0	7.2	6.5	1295	25		20	100.0	22.0	7.3	6.5	1450	25	100.0	22.0	7.3	6.5	1373	25	
8	06/18/04		20	100.0	20.7	7.1	6.0	1119	25		20	100.0	20.8	7.1	5.9	1484	25	100.0	20.8	7.1	6.0	1302	25	
9	06/19/04		20	100.0	21.0	7.2	6.4	1475	25		20	100.0	21.0	7.2	6.1	1335	25	100.0	21.0	7.2	6.3	1405	25	
10	06/20/04		20	100.0	21.0	7.1	5.9	1515	25		20	100.0	21.0	7.1	6.1	1195	25	100.0	21.0	7.1	6.0	1355	25	
11	06/21/04		20	100.0	21.8	7.2	5.9	1205	25		20	100.0	21.7	7.2	5.9	1495	25	100.0	21.8	7.2	5.9	1350	25	
12	06/22/04		20	100.0	21.4	7.3	6.8	1302	25		20	100.0	21.6	7.3	6.7	1263	25	100.0	21.5	7.3	6.8	1283	25	
13	06/23/04		20	100.0	21.6	7.3	6.4	1380	25		20	100.0	21.7	7.3	6.2	1270	25	100.0	21.7	7.3	6.3	1325	25	
14	06/24/04		20	100.0	21.7	7.2	6.0	1396	25		20	100.0	21.8	7.2	5.9	1705	25	100.0	21.8	7.2	6.0	1551	25	
15	06/25/04		20	100.0	21.9	7.3	6.1	1444	25		20	100.0	21.9	7.2	5.7	1480	25	100.0	21.9	7.3	5.9	1462	25	
16	06/26/04		20	100.0	21.8	7.3	6.1	1485	25		20	100.0	21.9	7.3	6.1	1460	25	100.0	21.9	7.3	6.1	1473	25	
17	06/27/04		20	100.0	21.8	7.4	5.8	1625	25		20	100.0	21.7	7.3	5.9	1555	25	100.0	21.8	7.4	5.9	1590	25	
18	06/28/04		20	100.0	21.7	7.2	6.1	1455	25		20	100.0	21.7	7.1	5.9	1420	25	100.0	21.7	7.2	6.0	1438	25	
19	06/29/04		20	100.0	22.0	7.2	5.6	1711	24		20	100.0	22.0	7.1	5.6	1676	24	100.0	22.0	7.2	5.6	1694	24	
20	06/30/04		20	100.0	21.7	7.3	6.4	1190	24		20	100.0	21.7	7.1	5.2	1245	24	100.0	21.7	7.2	5.8	1218	24	
21	07/01/04		20	100.0							20	100.0						100.0						
	Median:				**21.8**	**7.2**	**6.4**	**1396**	**25**				**21.9**	**7.2**	**6.1**	**1468**	**25**		**21.9**	**7.2**	**6.3**	**1405**	**25**	
	Mode:				**22.0**	**7.2**	**6.4**	**#N/A**	**25**				**22.0**	**7.3**	**5.9**	**1450**	**25**		**22.0**	**7.3**	**8.0**	**#N/A**	**25**	
	Mean:			**100.0**	**21.7**	**7.2**	**6.7**	**1390**	**25.0**			**100.0**	**21.7**	**7.2**	**6.6**	**1480**	**25.0**	**100.0**	**21.7**	**7.2**	**6.6**	**1435**	**25.0**	**#DIV/0!**
	SEM:			**0.00**	**0.09**	**0.02**	**0.20**	**33.31**	**0.12**			**0.00**	**0.08**	**0.02**	**0.22**	**37.95**	**0.12**	**0.00**	**0.09**	**0.02**	**0.21**	**29.58**	**0.12**	**#DIV/0!**
	% CV:			**0.00**	**8.75**	**3.64**	**35.04**	**409.51**	**10.95**			**0.00**	**8.04**	**3.84**	**39.04**	**452.11**	**10.95**	**0.00**	**8.36**	**3.50**	**36.64**	**357.85**	**10.95**	**#DIV/0!**

[1]Samples collected for this nominal test concentration were not analyzed per instructions from EPA.

DAILY DATA

Workorder No: 00062
Test Chemical: L-Thyroxine (T4)

Test No: 1
Target Conc: 1.0 µg/L

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Conc[1] (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	06/10/04			100.0	22.0	7.5	8.8	1303	25			100.0	22.0	7.5	8.8	1239	25	100.0	22.0	7.5	8.8	1271	25	
1	06/11/04		20	100.0	22.0	7.3	8.0	1214	25		20	100.0	22.0	7.3	8.1	1181	25	100.0	22.0	7.3	8.1	1198	25	
2	06/12/04		20	100.0	22.0	7.3	8.0	1208	25		20	100.0	22.0	7.3	8.0	1755	25	100.0	22.0	7.3	8.0	1482	25	
3	06/13/04		20	100.0	22.0	7.3	7.9	1508	25		20	100.0	22.0	7.3	7.9	1519	25	100.0	22.0	7.3	7.9	1514	25	
4	06/14/04		20	100.0	22.0	7.3	8.1	1760	25		20	100.0	22.0	7.1	8.2	1705	25	100.0	22.0	7.2	8.2	1733	25	
5	06/15/04		20	100.0	22.0	7.2	6.6	1102	25		20	100.0	21.9	7.2	6.7	1364	25	100.0	22.0	7.2	6.7	1233	25	
6	06/16/04		20	100.0	22.1	7.2	6.4	1295	25		20	100.0	22.2	7.2	6.4	1230	25	100.0	22.2	7.2	6.4	1263	25	
7	06/17/04		20	100.0	22.0	7.1	6.5	1305	25		20	100.0	22.0	7.2	6.6	1270	25	100.0	22.0	7.2	6.6	1288	25	
8	06/18/04		20	100.0	20.8	7.2	5.9	1226	25		20	100.0	20.8	7.2	5.9	1172	25	100.0	20.8	7.2	5.9	1199	25	
9	06/19/04		20	100.0	21.0	7.1	6.2	1420	25		20	100.0	21.0	7.0	5.9	1515	25	100.0	21.0	7.1	6.1	1468	25	
10	06/20/04		20	100.0	21.0	7.0	6.0	1235	25		20	100.0	21.0	7.1	5.9	1180	25	100.0	21.0	7.1	6.0	1208	25	
11	06/21/04		20	100.0	21.7	7.2	6.0	1245	25		20	100.0	21.7	7.3	5.8	1195	25	100.0	21.7	7.3	5.9	1220	25	
12	06/22/04	1	19	95.0	21.6	7.2	6.6	1697	25		20	100.0	21.5	7.3	6.6	1569	25	97.5	21.6	7.3	6.6	1633	25	
13	06/23/04		19	95.0	21.6	7.1	6.2	1685	25		20	100.0	21.6	7.3	6.3	1550	25	97.5	21.6	7.2	6.3	1618	25	
14	06/24/04		19	95.0	21.7	7.3	6.0	1432	25		20	100.0	21.6	7.3	5.9	1421	25	97.5	21.7	7.3	6.0	1427	25	
15	06/25/04		19	95.0	21.8	7.3	6.3	1416	25		20	100.0	21.8	7.3	6.2	1438	25	97.5	21.8	7.3	6.3	1427	25	
16	06/26/04		19	95.0	21.8	7.2	6.2	1425	25		20	100.0	21.8	7.2	6.1	1435	25	97.5	21.8	7.2	6.2	1430	25	
17	06/27/04		19	95.0	21.8	7.3	5.8	1485	25		20	100.0	21.9	7.3	5.8	1320	25	97.5	21.9	7.3	5.8	1403	25	
18	06/28/04		19	95.0	21.6	7.1	6.0	1465	25		20	100.0	21.7	7.0	6.2	1480	25	97.5	21.7	7.1	6.1	1473	25	
19	06/29/04		19	95.0	21.7	7.3	6.3	1142	23		20	100.0	21.8	7.3	6.0	1834	23	97.5	21.8	7.3	6.2	1488	23	
20	06/30/04		19	95.0	21.7	7.1	6.2	1335	24		20	100.0	21.8	7.2	6.5	1365	24	97.5	21.8	7.2	6.4	1350	24	
21	07/01/04		19	95.0							20	100.0						97.5						
	Median:				**21.8**	**7.2**	**6.3**	**1335**	**25**				**21.8**	**7.3**	**6.3**	**1421**	**25**		**21.8**	**7.2**	**6.3**	**1427**	**25**	
	Mode:				**22.0**	**7.3**	**6.2**	**#N/A**	**25**				**22.0**	**7.3**	**5.9**	**#N/A**	**25**		**22.0**	**7.3**	**5.9**	**#N/A**	**25**	
	Mean:			**97.7**	**21.7**	**7.2**	**6.7**	**1376**	**24.9**			**100.0**	**21.7**	**7.2**	**6.7**	**1416**	**24.9**	**98.9**	**21.7**	**7.2**	**6.7**	**1396**	**24.9**	**#DIV/0!**
	SEM:			**0.54**	**0.08**	**0.02**	**0.20**	**39.48**	**0.10**			**0.00**	**0.08**	**0.03**	**0.20**	**42.57**	**0.10**	**0.27**	**0.08**	**0.02**	**0.20**	**33.83**	**0.10**	**#DIV/0!**
	% CV:			**25.78**	**7.79**	**4.18**	**34.69**	**487.70**	**9.59**			**0.00**	**7.96**	**4.29**	**36.31**	**518.36**	**9.59**	**12.81**	**7.85**	**3.93**	**35.39**	**414.92**	**9.59**	**#DIV/0!**

[1]Samples collected for this nominal test concentration were not analyzed per instructions from EPA.

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00062 **Test No: 1**
Test Chemical: L-Thyroxine (T4) **Target Conc: 2.0 µg/L**

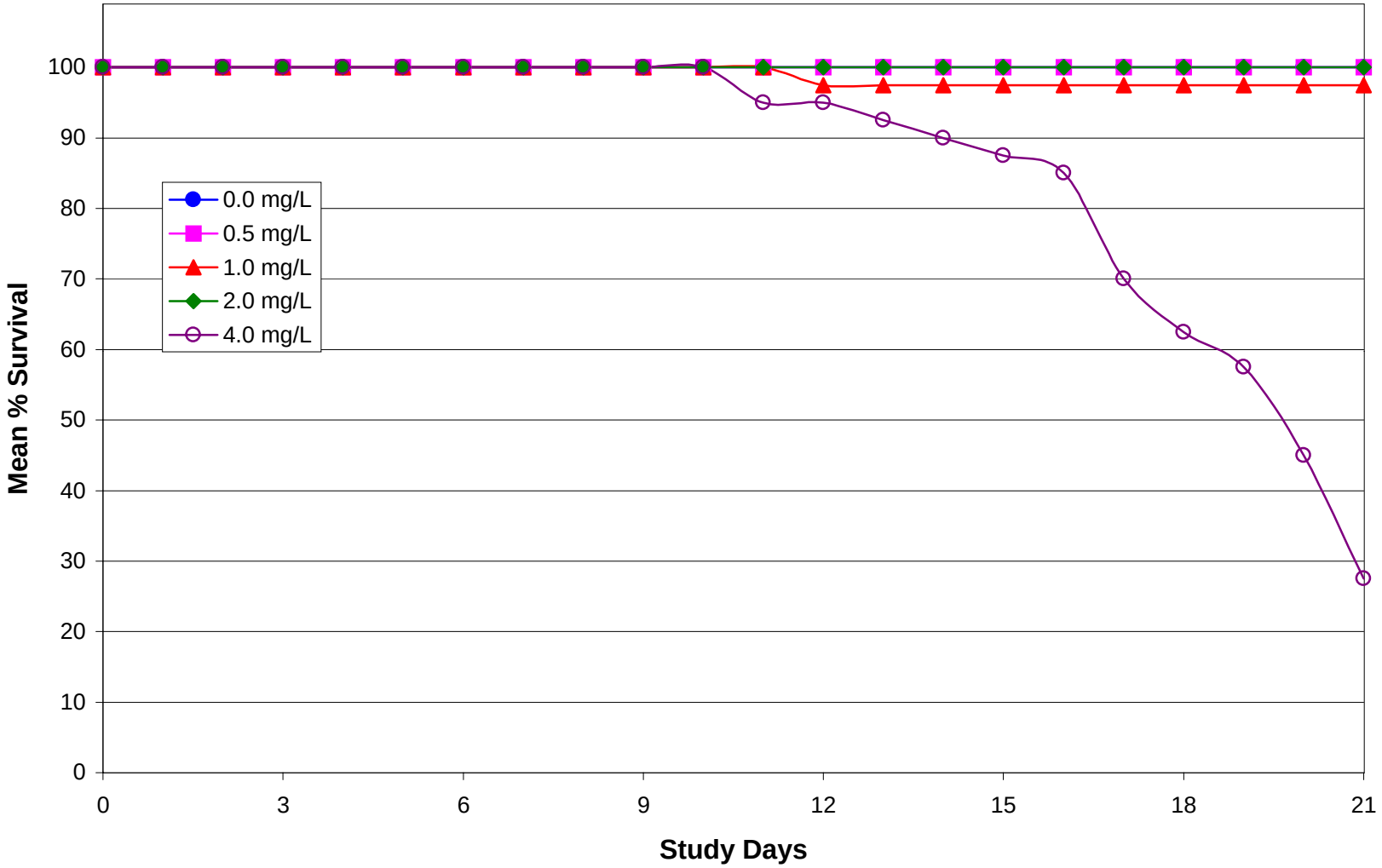
		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	06/10/04			100.0	22.0	7.5	8.9	1557	25			100.0	22.0	7.5	8.8	1257	25	100.0	22.0	7.5	8.9	1407	25	1.60
1	06/11/04		20	100.0	22.0	7.3	8.2	1641	25		20	100.0	22.0	7.3	8.2	1670	25	100.0	22.0	7.3	8.2	1656	25	
2	06/12/04		20	100.0	22.0	7.3	8.2	1619	25		20	100.0	22.0	7.3	8.1	1441	25	100.0	22.0	7.3	8.2	1530	25	
3	06/13/04		20	100.0	22.0	7.3	8.1	1822	25		20	100.0	22.0	7.2	7.9	1712	25	100.0	22.0	7.3	8.0	1767	25	
4	06/14/04		20	100.0	22.0	7.2	8.1	1665	25		20	100.0	22.0	7.2	8.1	1570	25	100.0	22.0	7.2	8.1	1618	25	
5	06/15/04		20	100.0	22.0	7.2	6.7	1700	25		20	100.0	22.0	7.2	6.7	1343	25	100.0	22.0	7.2	6.7	1522	25	
6	06/16/04		20	100.0	22.2	7.1	6.6	1615	25		20	100.0	22.3	7.2	6.2	1460	25	100.0	22.3	7.2	6.4	1538	25	
7	06/17/04		20	100.0	22.0	7.2	6.7	1425	25		20	100.0	22.0	7.1	6.4	1610	25	100.0	22.0	7.2	6.6	1518	25	1.75
8	06/18/04		20	100.0	20.7	7.2	6.0	1551	25		20	100.0	20.6	7.2	5.9	1466	25	100.0	20.7	7.2	6.0	1509	25	
9	06/19/04		20	100.0	21.0	7.2	6.2	1625	25		20	100.0	21.0	7.1	6.1	1480	25	100.0	21.0	7.2	6.2	1553	25	
10	06/20/04		20	100.0	21.0	7.2	5.8	1265	25		20	100.0	21.0	7.1	6.0	1305	25	100.0	21.0	7.2	5.9	1285	25	
11	06/21/04		20	100.0	21.7	7.2	5.6	1565	25		20	100.0	21.7	7.3	5.7	1505	25	100.0	21.7	7.3	5.7	1535	25	
12	06/22/04		20	100.0	21.6	7.3	6.6	1830	25		20	100.0	21.7	7.3	6.7	1637	25	100.0	21.7	7.3	6.7	1734	25	
13	06/23/04		20	100.0	21.5	7.3	6.2	1815	25		20	100.0	21.5	7.2	6.3	1605	25	100.0	21.5	7.3	6.3	1710	25	
14	06/24/04		20	100.0	21.6	7.3	6.1	1678	25		20	100.0	21.8	7.3	6.1	1534	25	100.0	21.7	7.3	6.1	1606	25	1.40
15	06/25/04		20	100.0	21.9	7.3	6.2	1601	25		20	100.0	22.0	7.4	6.4	1528	25	100.0	22.0	7.4	6.3	1565	25	
16	06/26/04		20	100.0	21.7	7.3	6.1	1635	25		20	100.0	21.9	7.4	6.1	1585	25	100.0	21.8	7.4	6.1	1610	25	
17	06/27/04		20	100.0	22.0	7.2	5.9	1055	25		20	100.0	21.9	7.2	6.0	1185	25	100.0	22.0	7.2	6.0	1120	25	
18	06/28/04		20	100.0	21.7	7.1	6.4	1620	25		20	100.0	21.7	7.1	6.5	1560	25	100.0	21.7	7.1	6.5	1590	25	
19	06/29/04		20	100.0	22.0	7.3	6.1	1460	25		20	100.0	22.0	7.3	6.3	1661	25	100.0	22.0	7.3	6.2	1561	25	
20	06/30/04		20	100.0	21.7	7.2	6.6	1440	25		20	100.0	21.7	7.3	6.2	1310	25	100.0	21.7	7.3	6.4	1375	25	
21	07/01/04		20	100.0							20	100.0						100.0						1.95
	Median:				**21.9**	**7.2**	**6.4**	**1619**	**25**				**21.9**	**7.2**	**6.3**	**1528**	**25**		**22.0**	**7.3**	**6.4**	**1553**	**25**	
	Mode:				**22.0**	**7.3**	**6.6**	**#N/A**	**25**				**22.0**	**7.3**	**6.1**	**#N/A**	**25**		**22.0**	**7.3**	**6.4**	**#N/A**	**25**	
	Mean:			**100.0**	**21.7**	**7.2**	**6.7**	**1580**	**25.0**			**100.0**	**21.8**	**7.2**	**6.7**	**1496**	**25.0**	**100.0**	**21.7**	**7.2**	**6.7**	**1538**	**25.0**	**1.675**
	SEM:			**0.00**	**0.09**	**0.02**	**0.21**	**39.51**	**0.00**			**0.00**	**0.09**	**0.02**	**0.20**	**31.73**	**0.00**	**0.00**	**0.09**	**0.02**	**0.20**	**32.31**	**0.00**	**0.12**
	% CV:			**0.00**	**8.48**	**3.24**	**36.92**	**455.44**	**0.00**			**0.00**	**8.88**	**4.00**	**35.37**	**375.85**	**0.00**	**0.00**	**8.64**	**3.40**	**35.99**	**377.49**	**0.00**	**17.98**

DAILY DATA

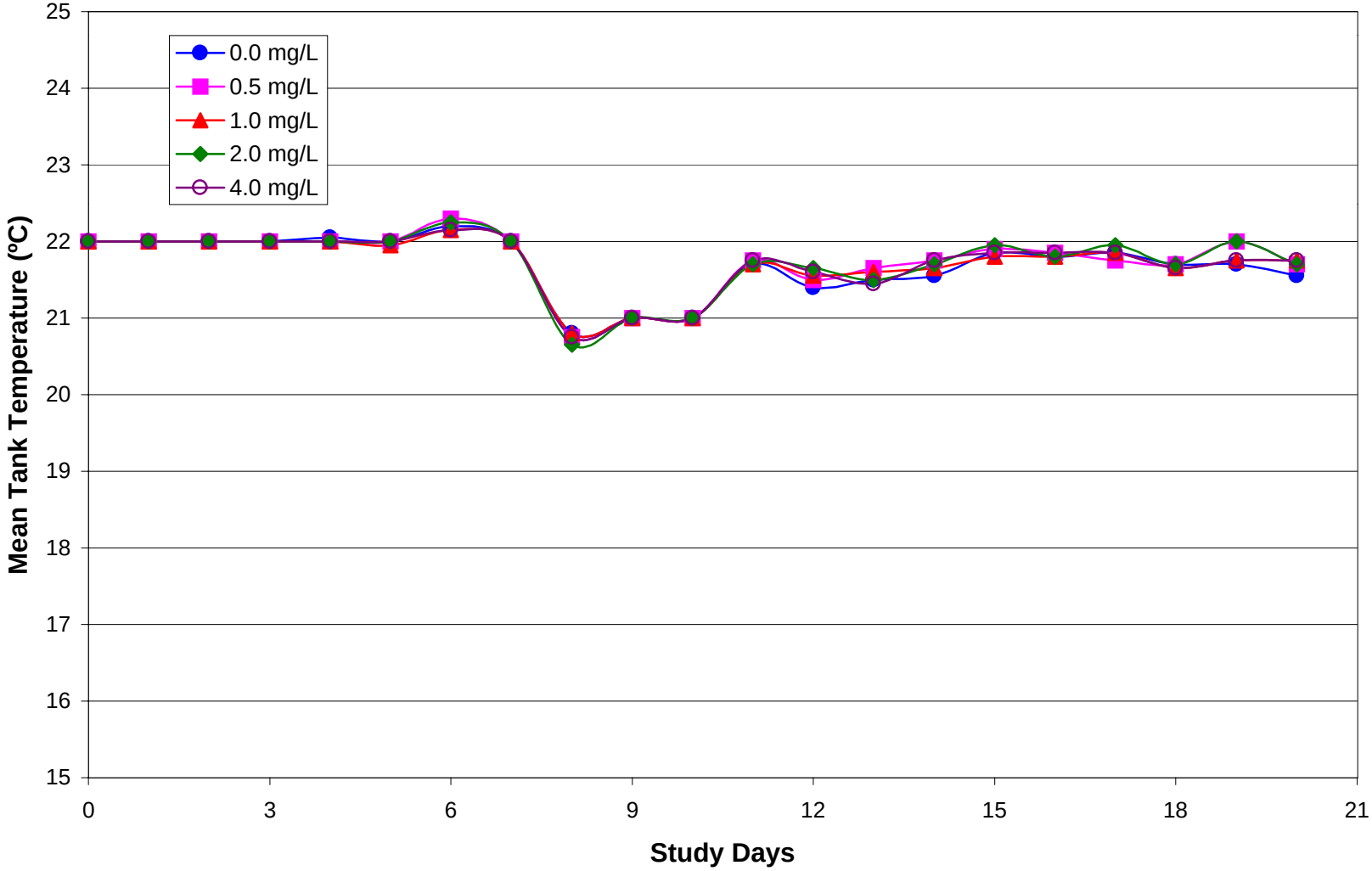
Workorder No: 00062 **Test No: 1**
Test Chemical: L-Thyroxine (T4) **Target Conc: 4.0 µg/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	06/10/04			100.0	22.0	7.5	8.9	1757	25			100.0	22.0	7.5	8.7	1850	25	100.0	22.0	7.5	8.8	1804	25	3.30
1	06/11/04		20	100.0	22.0	7.3	8.2	1673	25		20	100.0	22.0	7.3	8.0	1926	25	100.0	22.0	7.3	8.1	1800	25	
2	06/12/04		20	100.0	22.0	7.3	8.2	1636	25		20	100.0	22.0	7.4	8.0	1561	25	100.0	22.0	7.4	8.1	1599	25	
3	06/13/04		20	100.0	22.0	7.3	8.1	1996	25		20	100.0	22.0	7.3	7.7	1892	25	100.0	22.0	7.3	7.9	1944	25	
4	06/14/04		20	100.0	22.0	7.3	7.8	1915	25		20	100.0	22.0	7.2	8.1	1615	25	100.0	22.0	7.3	8.0	1765	25	
5	06/15/04		20	100.0	22.0	7.2	6.7	1803	25		20	100.0	22.0	7.2	6.7	1620	25	100.0	22.0	7.2	6.7	1712	25	
6	06/16/04		20	100.0	22.1	7.0	6.3	1605	25		20	100.0	22.2	7.1	6.7	1875	25	100.0	22.2	7.1	6.5	1740	25	
7	06/17/04		20	100.0	22.0	7.2	6.5	1645	25		20	100.0	22.0	7.2	6.4	1730	25	100.0	22.0	7.2	6.5	1688	25	3.20
8	06/18/04		20	100.0	20.7	7.2	6.1	1596	25		20	100.0	20.8	7.2	6.0	1551	25	100.0	20.8	7.2	6.1	1574	25	
9	06/19/04		20	100.0	21.0	7.1	6.3	1265	25		20	100.0	21.0	7.2	6.4	1330	25	100.0	21.0	7.2	6.4	1298	25	
10	06/20/04		20	100.0	21.0	7.0	6.0	1320	25		20	100.0	21.0	7.1	5.9	1365	25	100.0	21.0	7.1	6.0	1343	25	
11	06/21/04	2	18	90.0	21.8	7.4	5.8	1635	25		20	100.0	21.7	7.3	5.8	1585	25	95.0	21.8	7.4	5.8	1610	25	
12	06/22/04		18	90.0	21.7	7.3	6.8	1743	25		20	100.0	21.5	7.4	6.7	1906	25	95.0	21.6	7.4	6.8	1825	25	
13	06/23/04		18	90.0	21.5	7.2	6.4	1725	25	1	19	95.0	21.4	7.3	6.3	1920	25	92.5	21.5	7.3	6.4	1823	25	
14	06/24/04	1	17	85.0	21.8	7.3	6.3	1799	25		19	95.0	21.7	7.3	6.2	1956	25	90.0	21.8	7.3	6.3	1878	25	3.20
15	06/25/04		17	85.0	21.9	7.4	6.5	1961	25	1	18	90.0	21.8	7.3	6.3	1833	25	87.5	21.9	7.4	6.4	1897	25	
16	06/26/04		17	85.0	21.9	7.3	6.0	1900	25	1	17	85.0	21.8	7.4	5.8	1870	25	85.0	21.9	7.4	5.9	1885	25	
17	06/27/04	2	15	75.0	21.9	7.3	6.1	1885	25	4	13	65.0	21.8	7.2	6.0	1850	25	70.0	21.9	7.3	6.1	1868	25	
18	06/28/04	2	13	65.0	21.6	7.2	6.4	1905	25	1	12	60.0	21.7	7.2	6.2	1810	25	62.5	21.7	7.2	6.3	1858	25	
19	06/29/04	1	12	60.0	21.9	7.4	6.6	1551	23	1	11	55.0	21.6	7.4	6.5	1417	23	57.5	21.8	7.4	6.6	1484	23	
20	06/30/04	2	10	50.0	21.7	7.2	6.1	1295	24	3	8	40.0	21.8	7.3	6.4	1245	24	45.0	21.8	7.3	6.3	1270	24	
21	07/01/04	2	8	40.0						5	3	15.0						27.5						4.05
	Median:				**21.9**	**7.3**	**6.4**	**1725**	**25**				**21.8**	**7.3**	**6.4**	**1810**	**25**		**21.9**	**7.3**	**6.4**	**1765**	**25**	
	Mode:				**22.0**	**7.3**	**6.3**	**#N/A**	**25**				**22.0**	**7.3**	**6.7**	**1850**	**25**		**22.0**	**7.4**	**8.1**	**#N/A**	**25**	
	Mean:			**87.0**	**21.7**	**7.3**	**6.8**	**1696**	**24.9**			**86.4**	**21.7**	**7.3**	**6.7**	**1700**	**24.9**	**86.7**	**21.7**	**7.3**	**6.7**	**1698**	**24.9**	**3.438**
	SEM:			**3.85**	**0.08**	**0.03**	**0.20**	**46.18**	**0.10**			**5.09**	**0.08**	**0.02**	**0.19**	**48.41**	**0.10**	**4.44**	**0.08**	**0.02**	**0.19**	**44.45**	**0.10**	**0.21**
	% CV:			**193.34**	**8.26**	**4.63**	**34.43**	**513.92**	**9.59**			**256.79**	**8.10**	**3.87**	**33.12**	**537.95**	**9.59**	**223.46**	**8.11**	**4.04**	**33.58**	**494.33**	**9.59**	**22.17**

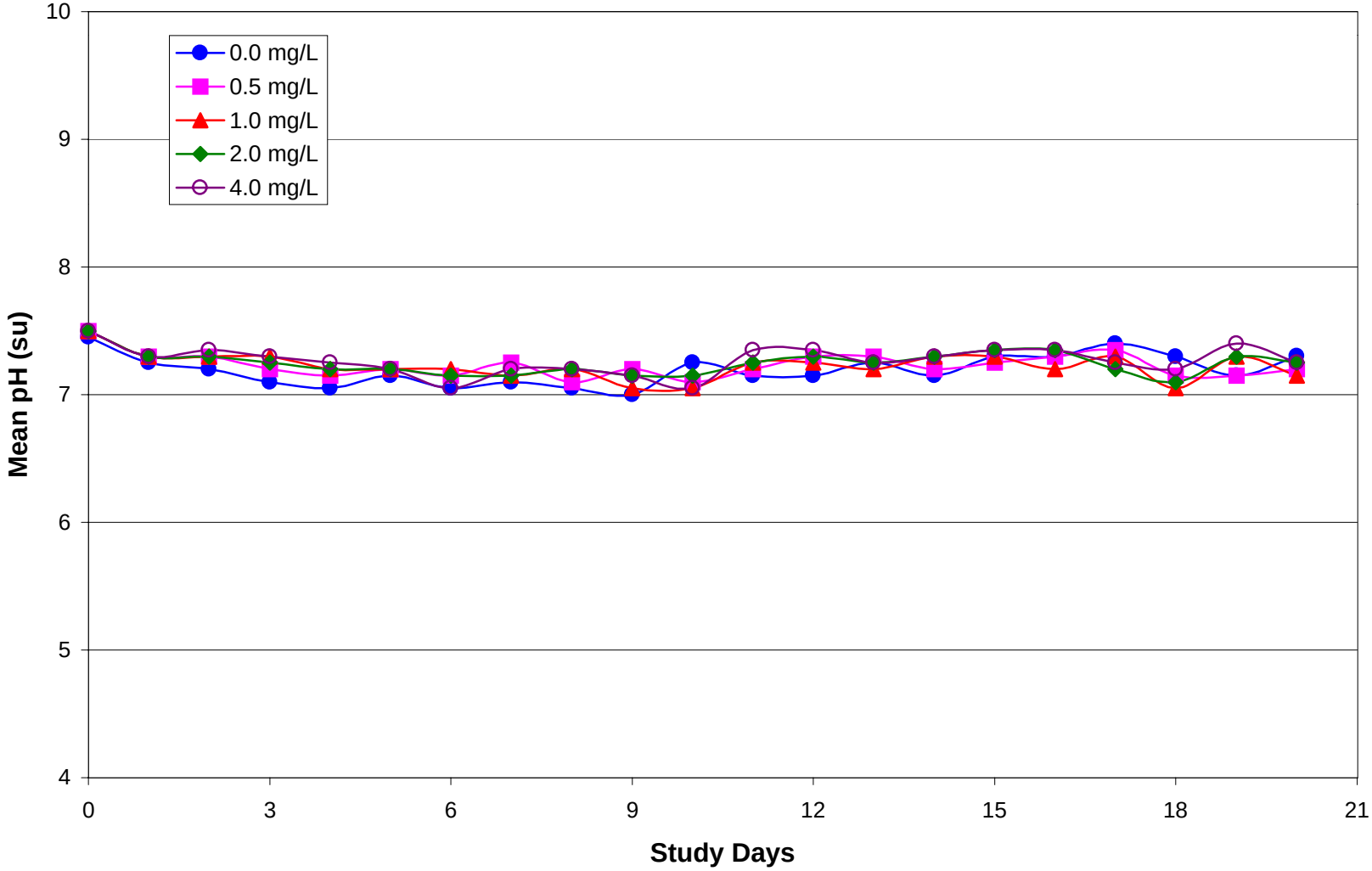
BATT02-00062
L-Thyroxine
Daily Average of Larval Survival for Initial Stage 51 Test



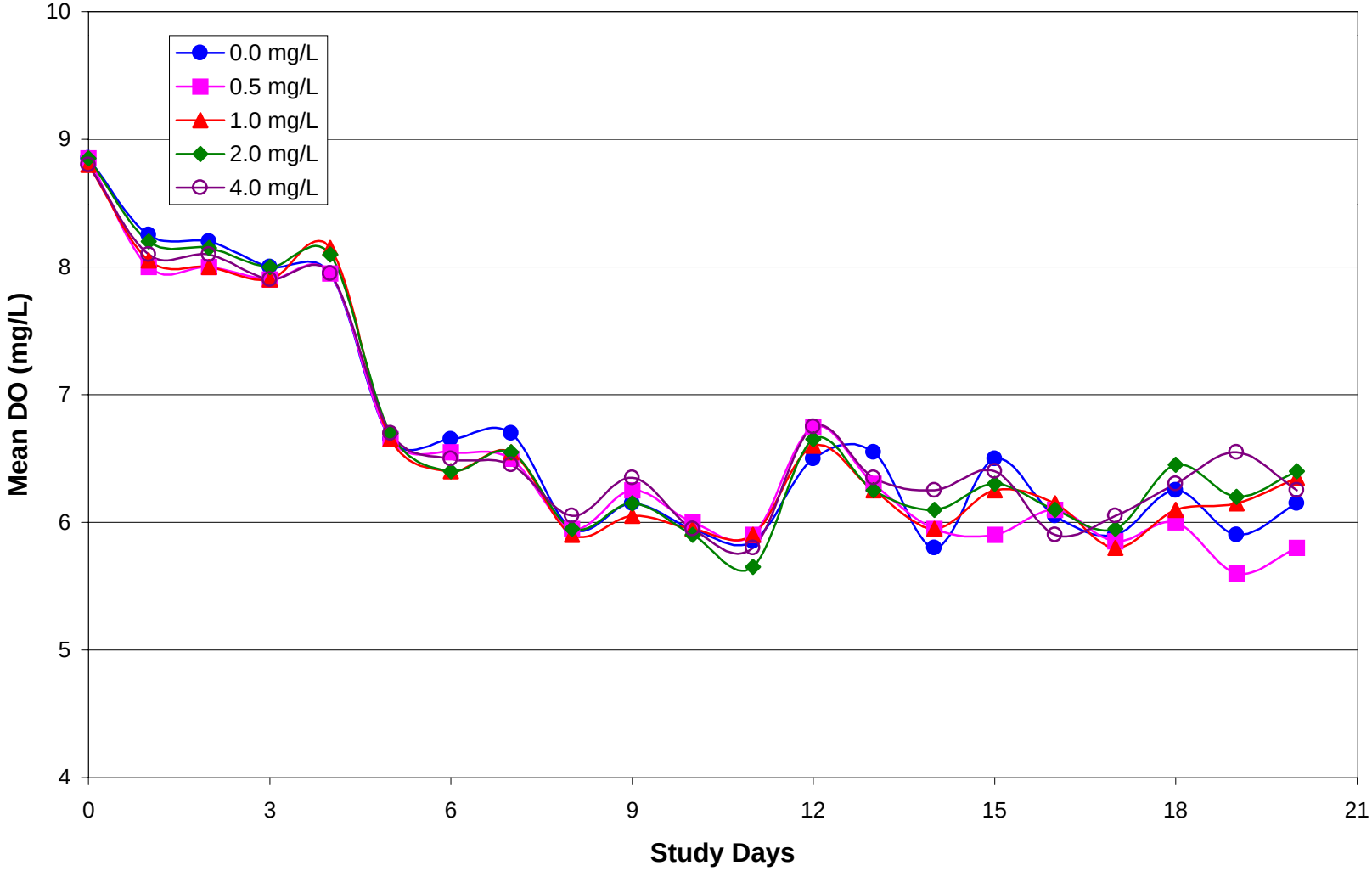
BATT02-00062
L-Thyroxine
Daily Average Tank Temperatures for Initial Stage 51 Test



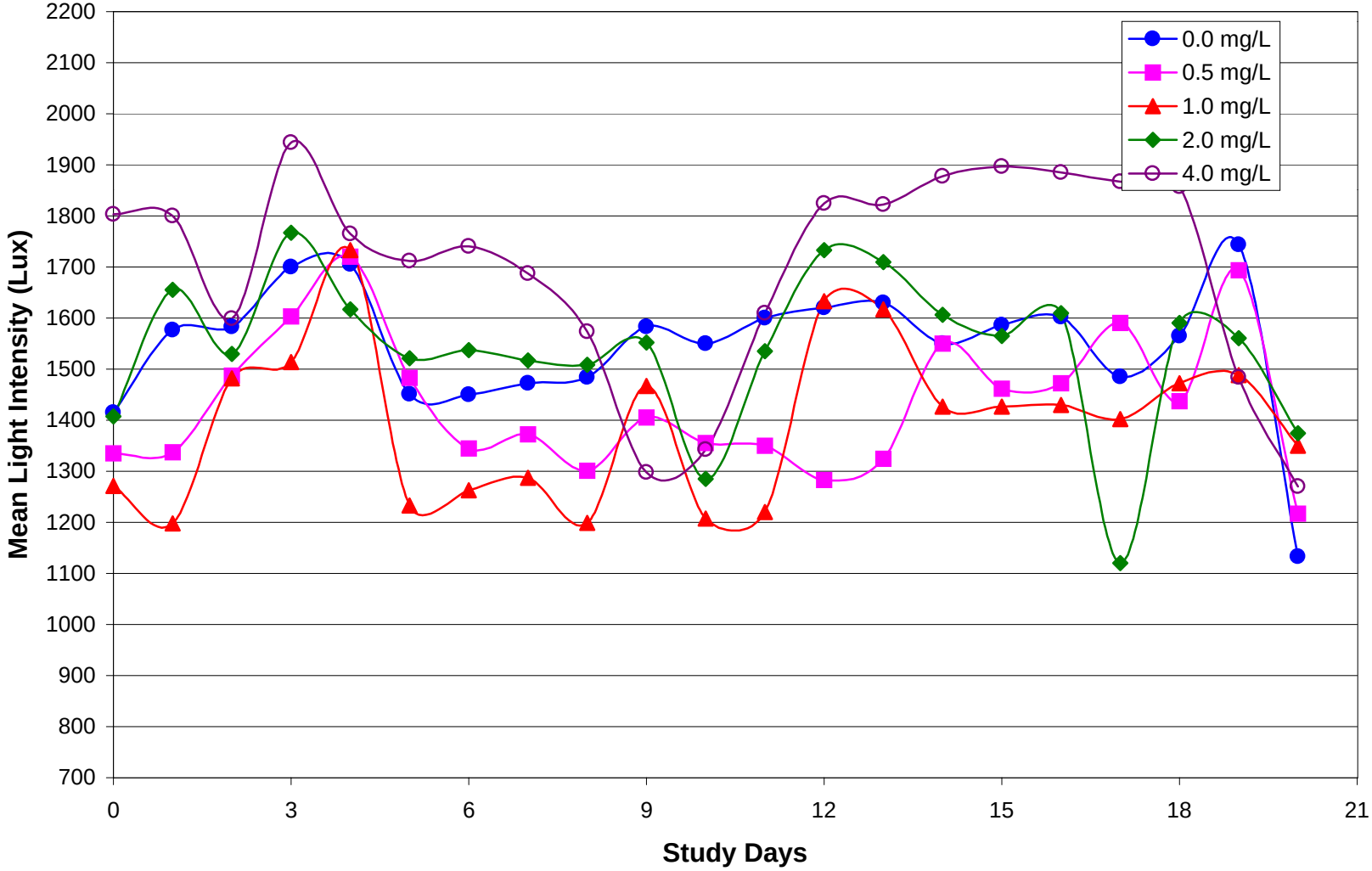
BATT02-00062
L-Thyroxine
Daily Average pH for Initial Stage 51 Test



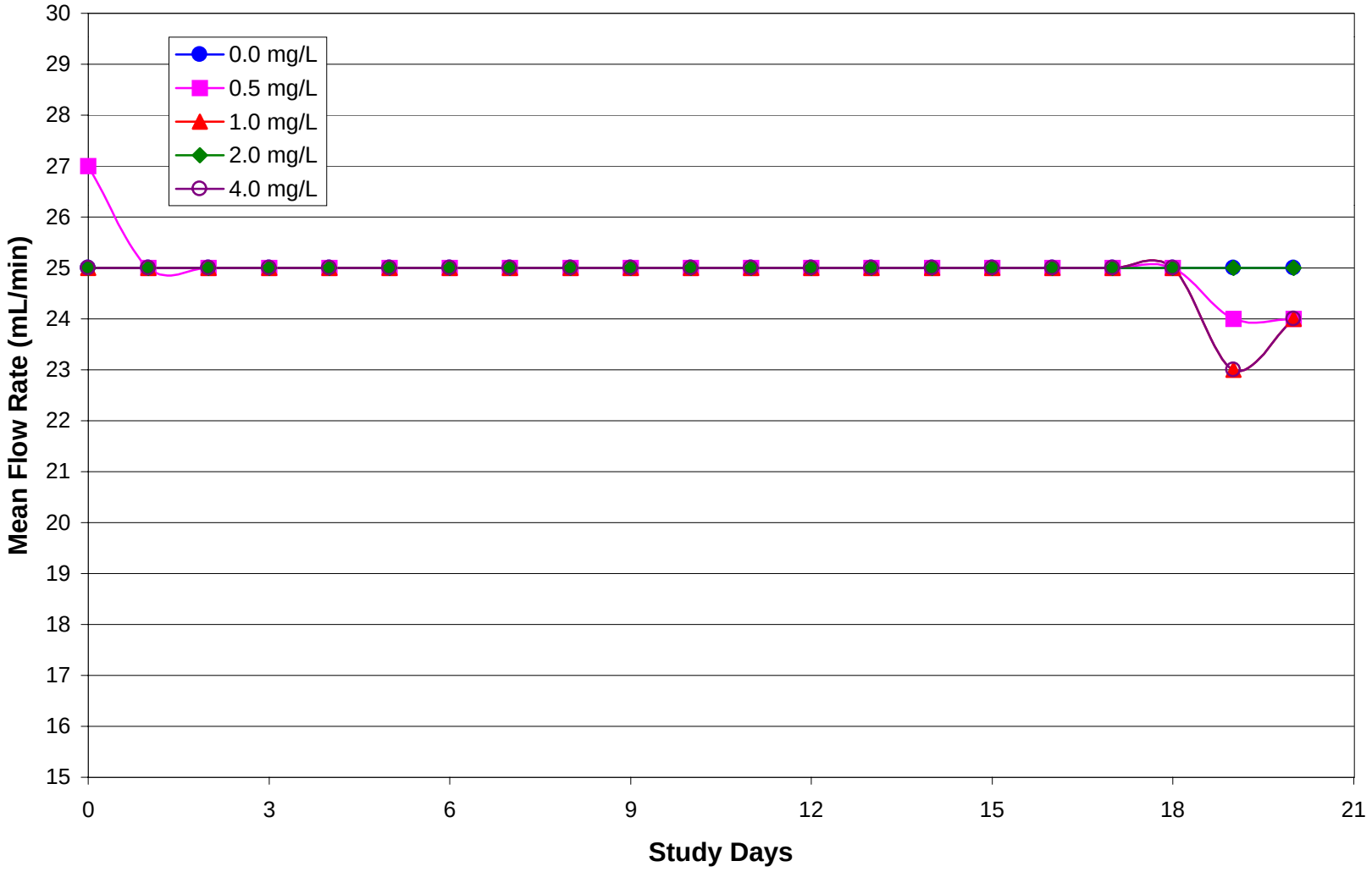
BATT02-00062
L-Thyroxine
Daily Average Dissolved Oxygen (DO) for Initial Stage 51 Test



BATT02-00062
L-Thyroxine
Daily Average Light Intensity for Initial Stage 51 Test



BATT02-00062
L-Thyroxine
Daily Average Flow Rates for Initial Stage 51 Test



WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00062 **Test No: 1**
Test Chemical: L-Thyroxine (T4) **Target Conc: 0.0 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Conc[1] (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	06/17/04			100.0	22.0	7.3	7.2	1130	25			100.0	22.0	7.3	7.3	1200	25	100.0	22.0	7.3	7.3	1165	25	0.05
1	06/18/04		20	100.0	20.5	7.1	6.3	1136	25		20	100.0	20.6	7.2	6.3	1082	25	100.0	20.6	7.2	6.3	1109	25	
2	06/19/04		20	100.0	21.0	7.1	5.9	1485	25		20	100.0	21.0	7.2	6.0	1505	25	100.0	21.0	7.2	6.0	1495	25	
3	06/20/04		20	100.0	21.0	7.2	5.8	1405	25		20	100.0	21.0	7.1	6.1	1520	25	100.0	21.0	7.2	6.0	1463	25	
4	06/21/04		20	100.0	21.8	7.2	5.9	1190	25		20	100.0	21.8	7.1	6.0	1115	25	100.0	21.8	7.2	6.0	1153	25	
5	06/22/04		20	100.0	21.3	7.2	6.8	1166	25		20	100.0	21.4	7.2	6.7	1761	25	100.0	21.4	7.2	6.8	1464	25	
6	06/23/04		20	100.0	21.4	7.1	6.6	1195	25		20	100.0	21.3	7.1	6.4	1705	25	100.0	21.4	7.1	6.5	1450	25	
7	06/24/04		20	100.0	21.7	7.2	6.3	1166	25		20	100.0	21.8	7.3	6.3	1423	25	100.0	21.8	7.3	6.3	1295	25	0.05
8	06/25/04		20	100.0	21.9	7.3	6.3	1090	25		20	100.0	21.9	7.3	6.3	1330	25	100.0	21.9	7.3	6.3	1210	25	
9	06/26/04		20	100.0	21.9	7.4	6.1	1030	25		20	100.0	21.9	7.4	6.1	1340	25	100.0	21.9	7.4	6.1	1185	25	
10	06/27/04		20	100.0	21.8	7.2	5.9	1650	25		20	100.0	21.8	7.2	5.9	1670	25	100.0	21.8	7.2	5.9	1660	25	
11	06/28/04		20	100.0	21.8	7.4	6.1	1075	25		20	100.0	21.9	7.4	6.1	1360	25	100.0	21.9	7.4	6.1	1218	25	
12	06/29/04		20	100.0	21.6	7.1	6.7	1132	25		20	100.0	21.7	7.3	6.4	1281	25	100.0	21.7	7.2	6.6	1207	25	
13	06/30/04		20	100.0	21.7	7.1	6.6	1095	25		20	100.0	21.5	7.2	6.4	1460	25	100.0	21.6	7.2	6.5	1278	25	
14	07/01/04		20	100.0							20	100.0						100.0						1.30
	Median:				**21.7**	**7.2**	**6.3**	**1151**	**25**				**21.8**	**7.2**	**6.3**	**1392**	**25**		**21.7**	**7.2**	**6.3**	**1248**	**25**	
	Mode:				**21.8**	**7.1**	**6.3**	**1166**	**25**				**21.8**	**7.2**	**6.3**	**#N/A**	**25**		**21.0**	**7.2**	**6.3**	**#N/A**	**25**	
	Mean:			**100.0**	**21.5**	**7.2**	**6.3**	**1210**	**25.0**			**100.0**	**21.5**	**7.2**	**6.3**	**1411**	**25.0**	**100.0**	**21.5**	**7.2**	**6.3**	**1311**	**25.0**	**0.467**
	SEM:			**0.00**	**0.12**	**0.03**	**0.11**	**47.34**	**0.00**			**0.00**	**0.11**	**0.03**	**0.10**	**56.00**	**0.00**	**0.00**	**0.11**	**0.03**	**0.10**	**44.20**	**0.00**	**0.42**
	% CV:			**0.00**	**9.39**	**3.99**	**16.38**	**509.19**	**0.00**			**0.00**	**9.21**	**3.75**	**14.18**	**557.84**	**0.00**	**0.00**	**9.25**	**3.55**	**15.02**	**456.82**	**0.00**	**105.64**

[1]All measured test concentrations reported by Battelle as less than method detection limit (MDL) [0.09 µg/L] or none detected (ND) are expressed as 0.05 µg/L (1/2 of MDL).

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00062 **Test No: 1**
Test Chemical: L-Thyroxine (T4) **Target Conc: 0.5 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Conc[1] (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	06/17/04			100.0	22.0	7.4	7.0	1170	25			100.0	22.0	7.5	7.1	1520	25	100.0	22.0	7.5	7.1	1345	25	
1	06/18/04		20	100.0	20.8	7.2	6.1	1496	25		20	100.0	20.8	7.2	5.9	1602	25	100.0	20.8	7.2	6.0	1549	25	
2	06/19/04		20	100.0	21.0	7.2	6.0	1625	25		20	100.0	21.0	7.2	6.1	1475	25	100.0	21.0	7.2	6.1	1550	25	
3	06/20/04		20	100.0	21.0	7.0	6.0	1555	25		20	100.0	21.0	7.1	6.1	1485	25	100.0	21.0	7.1	6.1	1520	25	
4	06/21/04		20	100.0	21.8	7.4	6.1	1525	25		20	100.0	21.8	7.2	6.0	1625	25	100.0	21.8	7.3	6.1	1575	25	
5	06/22/04		20	100.0	21.6	7.3	6.6	1593	25		20	100.0	21.6	7.3	6.5	1640	25	100.0	21.6	7.3	6.6	1617	25	
6	06/23/04		20	100.0	21.4	7.2	6.3	1565	25		20	100.0	21.5	7.2	6.5	1630	25	100.0	21.5	7.2	6.4	1598	25	
7	06/24/04		20	100.0	21.8	7.3	6.0	1477	25		20	100.0	21.8	7.2	5.9	1753	25	100.0	21.8	7.3	6.0	1615	25	
8	06/25/04		20	100.0	22.0	7.3	6.2	1523	25		20	100.0	22.0	7.4	6.2	1530	25	100.0	22.0	7.4	6.2	1527	25	
9	06/26/04		20	100.0	22.0	7.3	6.2	1545	25		20	100.0	21.9	7.3	6.2	1560	25	100.0	22.0	7.3	6.2	1553	25	
10	06/27/04		20	100.0	21.8	7.3	5.8	1250	25		20	100.0	21.9	7.3	6.0	1285	25	100.0	21.9	7.3	5.9	1268	25	
11	06/28/04		20	100.0	21.8	7.3	5.7	1530	25		20	100.0	21.8	7.4	5.9	1515	25	100.0	21.8	7.4	5.8	1523	25	
12	06/29/04		20	100.0	21.9	7.3	6.7	1557	24		20	100.0	21.9	7.4	6.6	1828	24	100.0	21.9	7.4	6.7	1693	24	
13	06/30/04		20	100.0	21.6	7.1	6.4	1250	24		20	100.0	21.6	7.1	6.3	1175	24	100.0	21.6	7.1	6.4	1213	24	
14	07/01/04		20	100.0							20	100.0						100.0						
	Median:				**21.8**	**7.3**	**6.2**	**1528**	**25**				**21.8**	**7.3**	**6.2**	**1545**	**25**		**21.8**	**7.3**	**6.1**	**1550**	**25**	
	Mode:				**21.8**	**7.3**	**6.0**	**1250**	**25**				**21.8**	**7.2**	**5.9**	**#N/A**	**25**		**21.8**	**7.2**	**6.1**	**#N/A**	**25**	
	Mean:			**100.0**	**21.6**	**7.3**	**6.2**	**1476**	**24.9**			**100.0**	**21.6**	**7.3**	**6.2**	**1545**	**24.9**	**100.0**	**21.6**	**7.3**	**6.2**	**1510**	**24.9**	**#DIV/0!**
	SEM:			**0.00**	**0.11**	**0.03**	**0.10**	**38.16**	**0.10**			**0.00**	**0.11**	**0.03**	**0.09**	**44.74**	**0.10**	**0.00**	**0.11**	**0.03**	**0.09**	**36.88**	**0.10**	**#DIV/0!**
	% CV:			**0.00**	**8.71**	**4.04**	**14.26**	**371.72**	**7.28**			**0.00**	**8.60**	**4.47**	**13.66**	**425.91**	**7.28**	**0.00**	**8.64**	**3.95**	**13.69**	**355.10**	**7.28**	**#DIV/0!**

[1]Samples collected for this nominal test concentration were not analyzed per instructions from EPA.

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00062 **Test No: 1**
Test Chemical: L-Thyroxine (T4) **Target Conc: 1.0 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Conc[1] (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	06/17/04			100.0	22.0	7.4	7.2	1315	25			100.0	22.0	7.4	7.1	1165	25	100.0	22.0	7.4	7.2	1240	25	
1	06/18/04		20	100.0	20.7	7.2	6.1	1341	25		20	100.0	20.7	7.2	6.0	1241	25	100.0	20.7	7.2	6.1	1291	25	
2	06/19/04		20	100.0	21.0	7.1	6.0	1642	25		20	100.0	21.0	7.0	6.1	1840	25	100.0	21.0	7.1	6.1	1741	25	
3	06/20/04		20	100.0	21.0	7.0	6.1	1365	25		20	100.0	21.0	7.1	6.0	1375	25	100.0	21.0	7.1	6.1	1370	25	
4	06/21/04		20	100.0	21.8	7.3	6.0	1485	25		20	100.0	21.8	7.3	5.9	1260	25	100.0	21.8	7.3	6.0	1373	25	
5	06/22/04		20	100.0	21.6	7.3	6.6	1482	25		20	100.0	21.6	7.3	6.5	1157	25	100.0	21.6	7.3	6.6	1320	25	
6	06/23/04		20	100.0	21.4	7.2	6.4	1480	25		20	100.0	21.2	7.3	6.4	1175	25	100.0	21.3	7.3	6.4	1328	25	
7	06/24/04		20	100.0	21.8	7.3	6.0	1858	25		20	100.0	21.7	7.3	6.0	1148	25	100.0	21.8	7.3	6.0	1503	25	
8	06/25/04		20	100.0	21.8	7.3	6.0	1861	25		20	100.0	22.0	7.3	6.0	1308	25	100.0	21.9	7.3	6.0	1585	25	
9	06/26/04		20	100.0	21.9	7.4	6.2	1820	25		20	100.0	21.9	7.3	6.1	1330	25	100.0	21.9	7.4	6.2	1575	25	
10	06/27/04		20	100.0	22.0	7.2	6.1	1830	25		20	100.0	21.9	7.1	5.9	1360	25	100.0	22.0	7.2	6.0	1595	25	
11	06/28/04		20	100.0	21.8	7.3	5.8	1810	25		20	100.0	21.7	7.2	6.0	1370	25	100.0	21.8	7.3	5.9	1590	25	
12	06/29/04		20	100.0	21.8	7.3	6.3	1746	23		20	100.0	22.0	7.4	6.3	1836	23	100.0	21.9	7.4	6.3	1791	23	
13	06/30/04		20	100.0	21.6	7.2	6.3	1205	24		20	100.0	21.7	7.4	6.2	1225	24	100.0	21.7	7.3	6.3	1215	24	
14	07/01/04		20	100.0							20	100.0						100.0						
	Median:				**21.8**	**7.3**	**6.1**	**1564**	**25**				**21.7**	**7.3**	**6.1**	**1284**	**25**		**21.8**	**7.3**	**6.1**	**1438**	**25**	
	Mode:				**21.8**	**7.3**	**6.0**	**#N/A**	**25**				**22.0**	**7.3**	**6.0**	**#N/A**	**25**		**21.9**	**7.3**	**6.1**	**#N/A**	**25**	
	Mean:			**100.0**	**21.6**	**7.3**	**6.2**	**1589**	**24.8**			**100.0**	**21.6**	**7.3**	**6.2**	**1342**	**24.8**	**100.0**	**21.6**	**7.3**	**6.2**	**1465**	**24.8**	**#DIV/0!**
	SEM:			**0.00**	**0.11**	**0.03**	**0.09**	**62.13**	**0.15**			**0.00**	**0.12**	**0.03**	**0.09**	**60.09**	**0.15**	**0.00**	**0.11**	**0.03**	**0.09**	**49.38**	**0.15**	**#DIV/0!**
	% CV:			**0.00**	**8.81**	**4.06**	**13.90**	**583.22**	**11.63**			**0.00**	**9.28**	**4.54**	**12.93**	**613.74**	**11.63**	**0.00**	**8.97**	**3.95**	**13.27**	**482.70**	**11.63**	**#DIV/0!**

[1]Samples collected for this nominal test concentration were not analyzed per instructions from EPA.

DAILY DATA

Workorder No: 00062 **Test No: 1**
Test Chemical: L-Thyroxine (T4) **Target Conc: 2.0 ug/L**

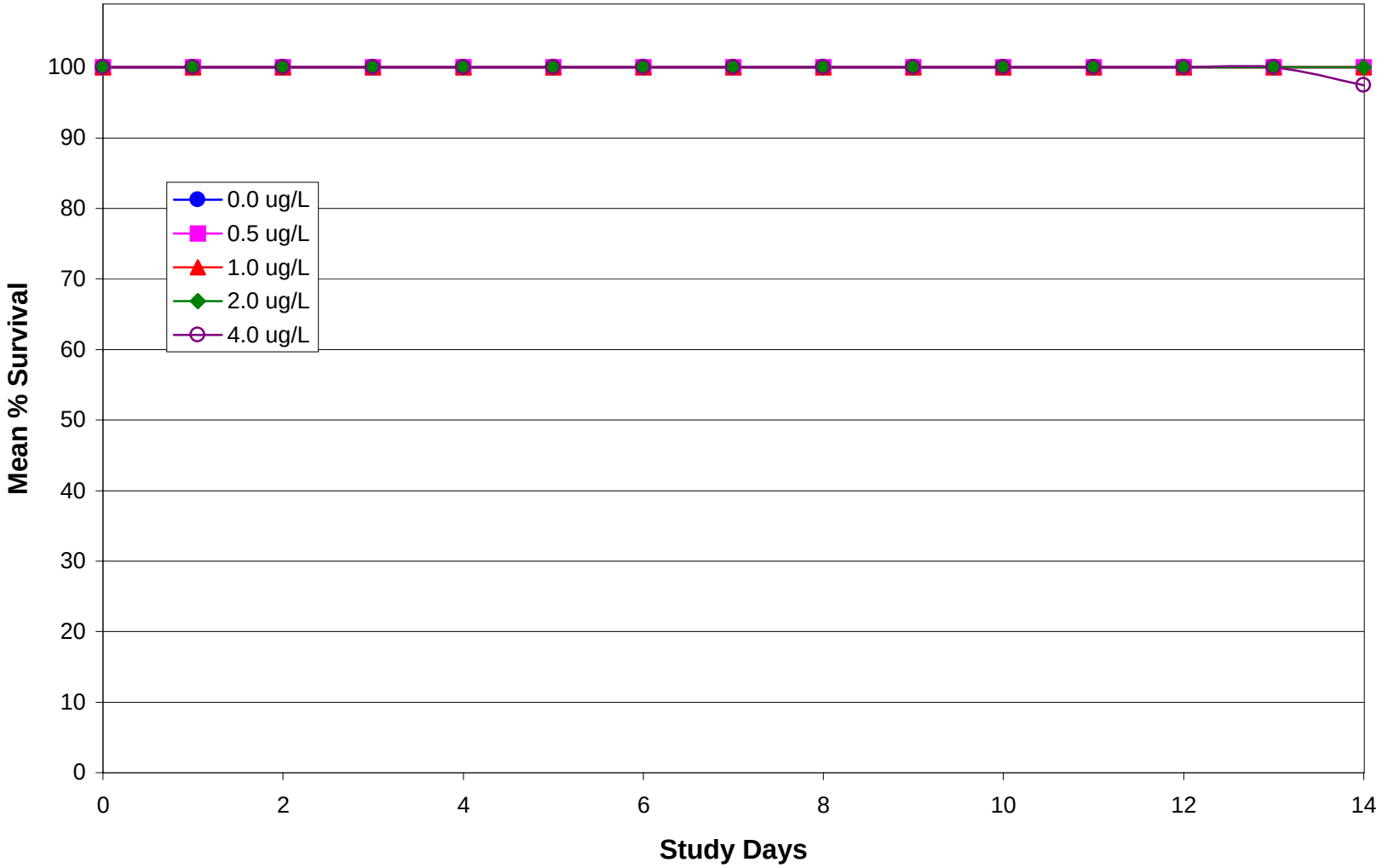
		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	06/17/04			100.0	22.0	7.4	7.4	1245	25			100.0	22.0	7.3	7.1	1685	25	100.0	22.0	7.4	7.3	1465	25	1.75
1	06/18/04		20	100.0	20.8	7.2	5.9	1303	25		20	100.0	20.7	7.2	5.9	1533	25	100.0	20.8	7.2	5.9	1418	25	
2	06/19/04		20	100.0	21.0	7.0	6.2	1715	25		20	100.0	21.0	6.9	5.9	1550	25	100.0	21.0	7.0	6.1	1633	25	
3	06/20/04		20	100.0	21.0	7.1	6.1	1480	25		20	100.0	21.0	7.0	6.0	1620	25	100.0	21.0	7.1	6.1	1550	25	
4	06/21/04		20	100.0	21.8	7.3	6.0	1335	25		20	100.0	21.8	7.4	5.9	1515	25	100.0	21.8	7.4	6.0	1425	25	
5	06/22/04		20	100.0	21.3	7.3	6.3	1391	25		20	100.0	21.4	7.3	6.7	1760	25	100.0	21.4	7.3	6.5	1576	25	
6	06/23/04		20	100.0	21.4	7.3	6.3	1385	25		20	100.0	21.4	7.3	6.2	1705	25	100.0	21.4	7.3	6.3	1545	25	
7	06/24/04		20	100.0	21.6	7.2	5.7	1507	25		20	100.0	21.7	7.3	6.1	1769	25	100.0	21.7	7.3	5.9	1638	25	1.40
8	06/25/04		20	100.0	21.9	7.3	5.9	1356	25		20	100.0	21.9	7.4	6.2	1477	25	100.0	21.9	7.4	6.1	1417	25	
9	06/26/04		20	100.0	21.8	7.3	6.2	1360	25		20	100.0	21.8	7.3	6.2	1455	25	100.0	21.8	7.3	6.2	1408	25	
10	06/27/04		20	100.0	21.8	7.4	5.8	1245	25		20	100.0	21.8	7.4	6.0	1130	25	100.0	21.8	7.4	5.9	1188	25	
11	06/28/04		20	100.0	21.6	7.4	6.0	1380	25		20	100.0	21.8	7.3	6.2	1450	25	100.0	21.7	7.4	6.1	1415	25	
12	06/29/04		20	100.0	21.9	7.3	5.8	1408	25		20	100.0	21.8	7.4	6.4	1747	25	100.0	21.9	7.4	6.1	1578	25	
13	06/30/04		20	100.0	21.7	7.3	6.1	1310	25		20	100.0	21.7	7.3	6.2	1360	25	100.0	21.7	7.3	6.2	1335	25	
14	07/01/04		20	100.0							20	100.0						100.0						1.95
	Median:				**21.7**	**7.3**	**6.1**	**1370**	**25**				**21.8**	**7.3**	**6.2**	**1542**	**25**		**21.7**	**7.3**	**6.1**	**1445**	**25**	
	Mode:				**21.8**	**7.3**	**5.9**	**1245**	**25**				**21.8**	**7.3**	**6.2**	**#N/A**	**25**		**21.8**	**7.4**	**5.9**	**#N/A**	**25**	
	Mean:			**100.0**	**21.5**	**7.3**	**6.1**	**1387**	**25.0**			**100.0**	**21.6**	**7.3**	**6.2**	**1554**	**25.0**	**100.0**	**21.6**	**7.3**	**6.2**	**1471**	**25.0**	**1.700**
	SEM:			**0.00**	**0.10**	**0.03**	**0.11**	**32.21**	**0.00**			**0.00**	**0.11**	**0.04**	**0.09**	**47.82**	**0.00**	**0.00**	**0.10**	**0.03**	**0.09**	**33.31**	**0.00**	**0.16**
	% CV:			**0.00**	**8.27**	**4.22**	**16.72**	**323.61**	**0.00**			**0.00**	**8.56**	**5.53**	**13.43**	**453.87**	**0.00**	**0.00**	**8.38**	**4.70**	**14.14**	**324.99**	**0.00**	**21.35**

DAILY DATA

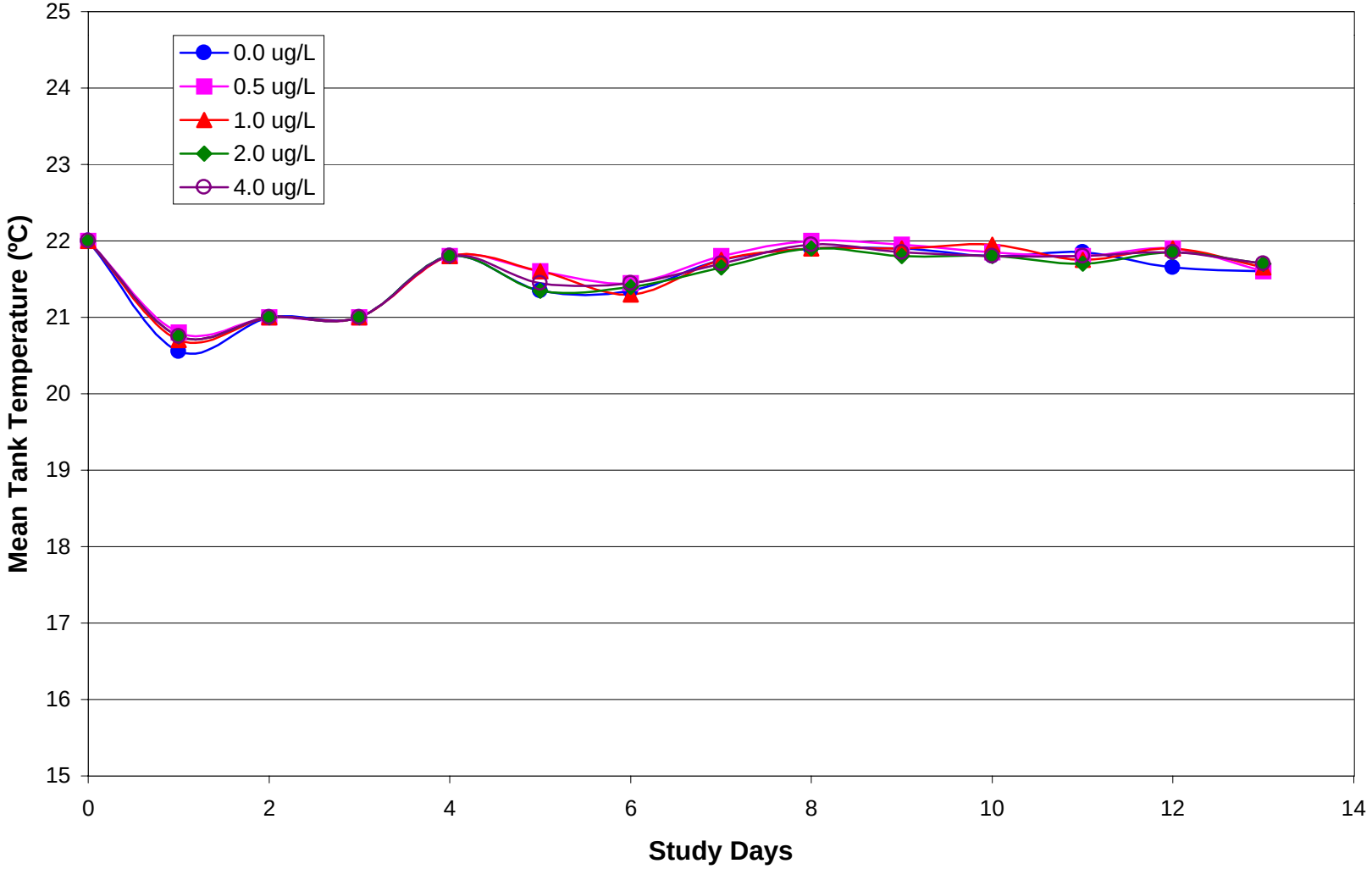
Workorder No: 00062 **Test No: 1**
Test Chemical: L-Thyroxine (T4) **Target Conc: 4.0 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	06/17/04			100.0	22.0	7.4	7.0	1190	25			100.0	22.0	7.4	7.1	1450	25	100.0	22.0	7.4	7.1	1320	25	3.20
1	06/18/04		20	100.0	20.6	7.2	6.0	1461	25		20	100.0	20.9	7.1	5.8	1474	25	100.0	20.8	7.2	5.9	1468	25	
2	06/19/04		20	100.0	21.0	7.1	6.0	1620	25		20	100.0	21.0	7.1	6.0	1485	25	100.0	21.0	7.1	6.0	1553	25	
3	06/20/04		20	100.0	21.0	7.0	5.8	1570	25		20	100.0	21.0	7.0	5.9	1525	25	100.0	21.0	7.0	5.9	1548	25	
4	06/21/04		20	100.0	21.8	7.4	6.2	1475	25		20	100.0	21.8	7.4	6.0	1440	25	100.0	21.8	7.4	6.1	1458	25	
5	06/22/04		20	100.0	21.4	7.3	6.5	1566	25		20	100.0	21.5	7.3	6.3	1727	25	100.0	21.5	7.3	6.4	1647	25	
6	06/23/04		20	100.0	21.4	7.3	6.5	1565	25		20	100.0	21.5	7.3	6.4	1710	25	100.0	21.5	7.3	6.5	1638	25	
7	06/24/04		20	100.0	21.7	7.3	5.8	1883	25		20	100.0	21.7	7.2	5.7	1575	25	100.0	21.7	7.3	5.8	1729	25	3.30
8	06/25/04		20	100.0	21.9	7.3	5.8	1672	25		20	100.0	22.0	7.3	5.7	1707	25	100.0	22.0	7.3	5.8	1690	25	
9	06/26/04		20	100.0	21.9	7.3	6.2	1680	25		20	100.0	21.8	7.3	6.1	1710	25	100.0	21.9	7.3	6.2	1695	25	
10	06/27/04		20	100.0	21.8	7.4	6.1	1280	25		20	100.0	21.8	7.4	6.1	1465	25	100.0	21.8	7.4	6.1	1373	25	
11	06/28/04		20	100.0	21.8	7.3	6.4	1640	25		20	100.0	21.8	7.3	6.1	1765	25	100.0	21.8	7.3	6.3	1703	25	
12	06/29/04		20	100.0	21.9	7.3	5.7	1637	23		20	100.0	21.8	7.4	6.3	1636	23	100.0	21.9	7.4	6.0	1637	23	
13	06/30/04		20	100.0	21.7	7.1	6.5	1430	24		20	100.0	21.7	7.2	6.4	1670	24	100.0	21.7	7.2	6.5	1550	24	
14	07/01/04	1	19	95.0							20	100.0						97.5						4.05
	Median:				**21.8**	**7.3**	**6.2**	**1568**	**25**				**21.8**	**7.3**	**6.1**	**1606**	**25**		**21.8**	**7.3**	**6.1**	**1595**	**25**	
	Mode:				**21.8**	**7.3**	**5.8**	**#N/A**	**25**				**21.8**	**7.3**	**6.1**	**1710**	**25**		**21.8**	**7.3**	**6.0**	**#N/A**	**25**	
	Mean:			**99.7**	**21.6**	**7.3**	**6.2**	**1548**	**24.8**			**100.0**	**21.6**	**7.3**	**6.1**	**1596**	**24.8**	**99.8**	**21.6**	**7.3**	**6.2**	**1572**	**24.8**	**3.517**
	SEM:			**0.33**	**0.11**	**0.03**	**0.10**	**46.56**	**0.15**			**0.00**	**0.10**	**0.03**	**0.10**	**32.02**	**0.15**	**0.17**	**0.11**	**0.03**	**0.09**	**34.24**	**0.15**	**0.27**
	% CV:			**12.93**	**9.16**	**4.51**	**14.82**	**442.79**	**11.63**			**0.00**	**7.94**	**4.74**	**14.65**	**299.93**	**11.63**	**6.46**	**8.50**	**4.51**	**14.07**	**323.19**	**11.63**	**24.77**

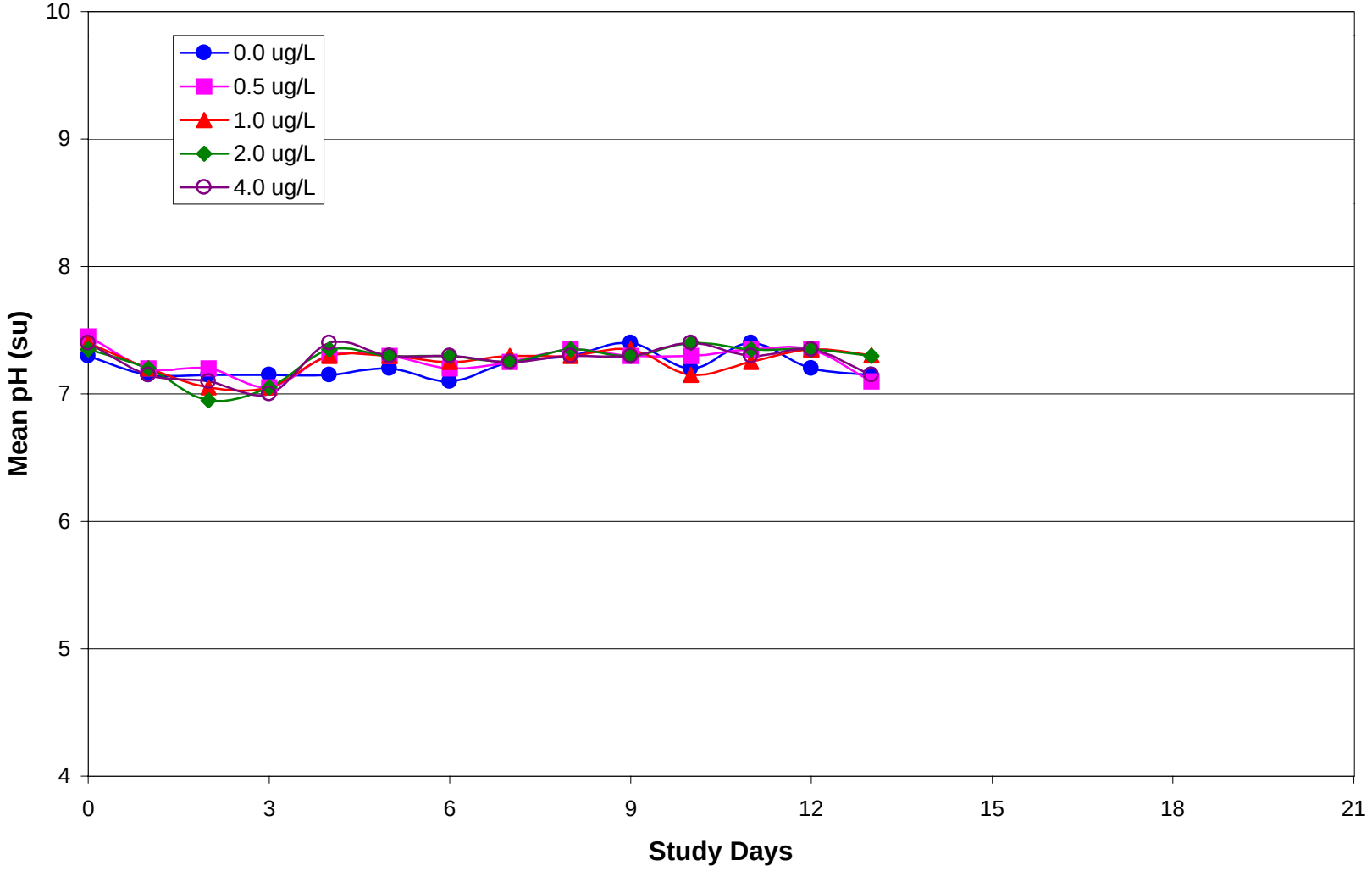
BATT02-00062
L-Thyroxine
Daily Average of Larval Survival for Initial Stage 54 Test



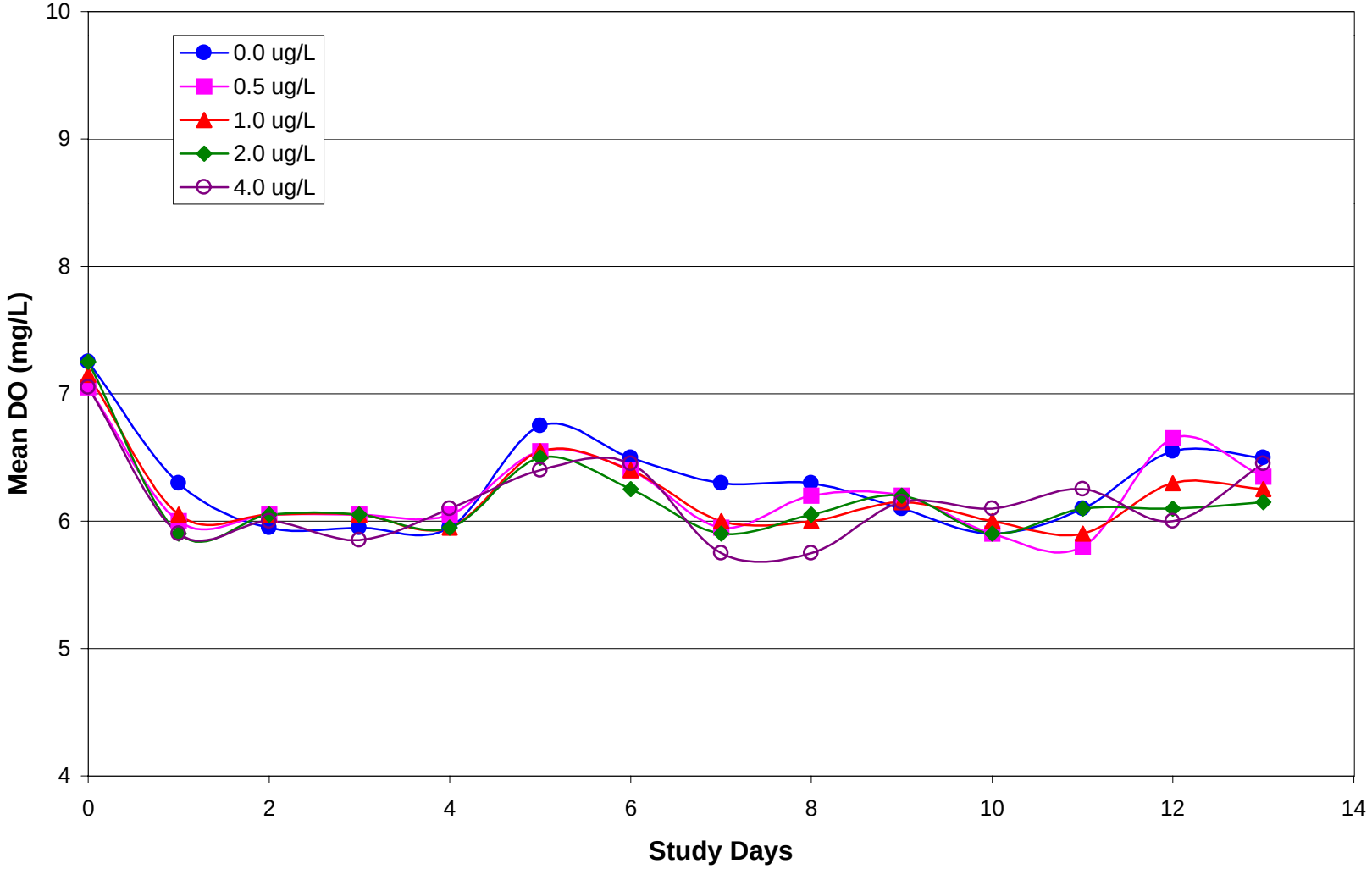
BATT02-00062
L-Thyroxine
Daily Average Tank Temperatures for Initial Stage 54 Test



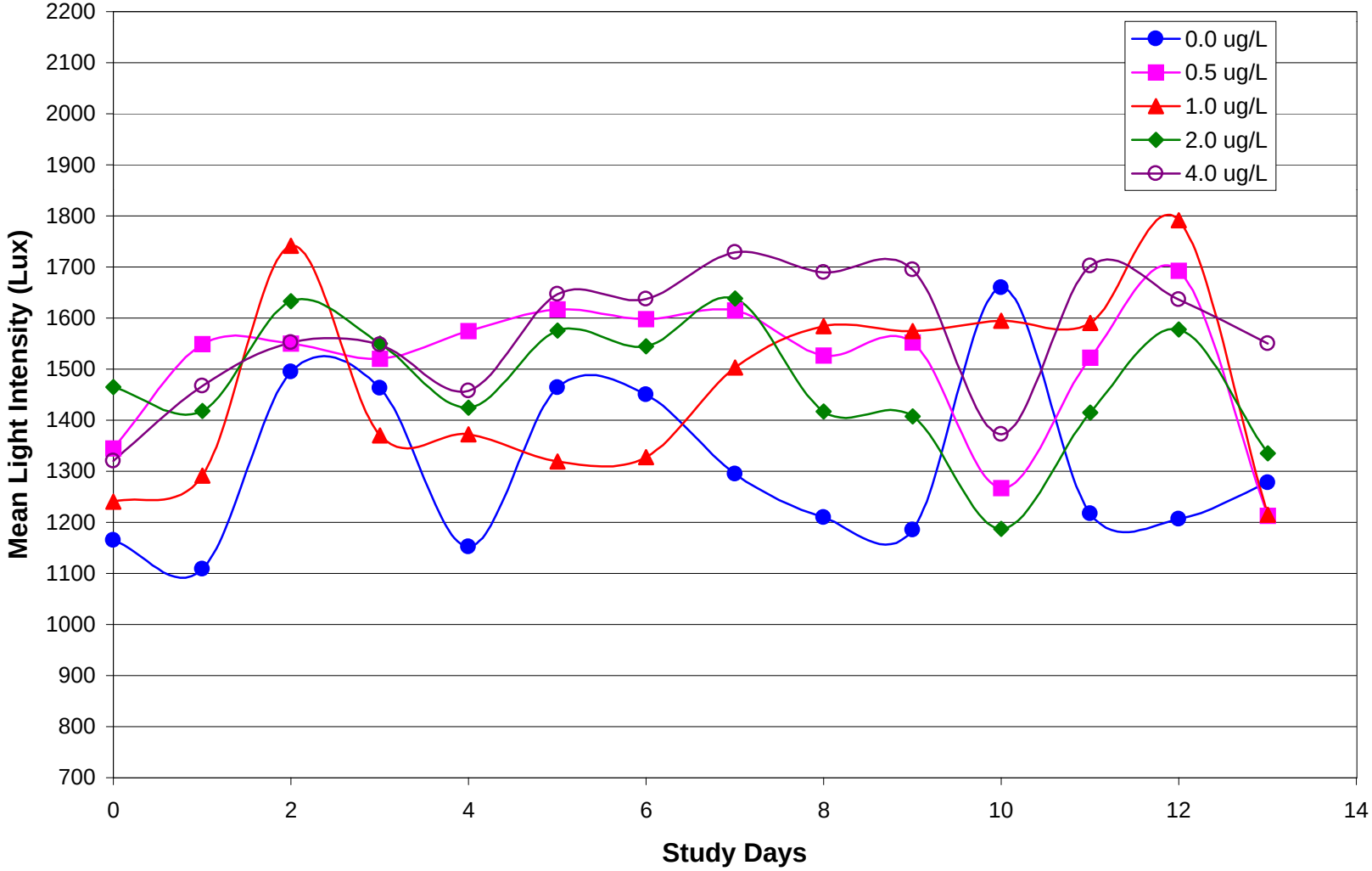
BATT02-00062
L-Thyroxine
Daily Average pH for Initial Stage 54 Test



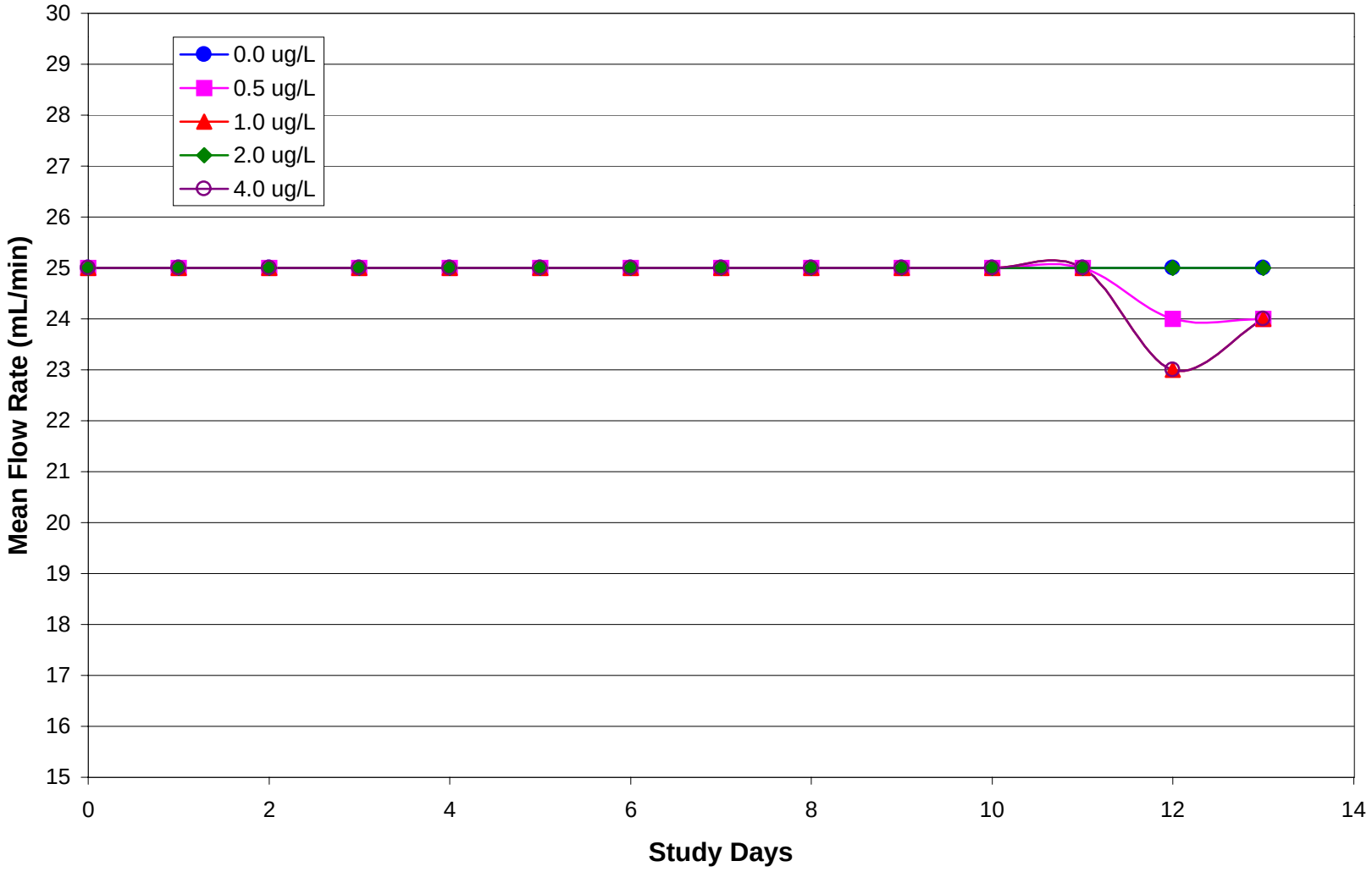
BATT02-00062
L-Thyroxine
Daily Average Dissolved Oxygen (DO) for Initial Stage 54 Test



BATT02-00062
L-Thyroxine
Daily Average Light Intensity for Initial Stage 54 Test



BATT02-00062
L-Thyroxine
Daily Average Flow Rates for Initial Stage 54 Test



L-Thyroxine (T4)
Metamorphosis Assay:
Test Termination Data – Stage and Growth

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00062 **Test No: 1**

Test Chemical: L-Thyroxine (T4) **Target Conc: 0.0 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	001	0.4713	56	
	002	0.6330	59	
	003	0.6159	59	
	004	0.5481	56	
	005	0.5728	59	
	006	0.4250	56	
	007	0.3262	55	
	008	0.6419	57	
	009	0.6008	56	
	010	0.3980	56	
	011	0.6775	58	
	012	0.6258	57	
	013	0.5116	57	
	014	0.3748	55	
	015	0.4338	57	
	016	0.7337	59	
	017	0.5025	57	
	018	0.7891	57	
	019	0.5935	57	
	020	0.4336	56	
	Median:	**0.560**	**57**	
	Mode:		**57**	
	Mean:	**0.545**	**56.950**	
	SEM:	**0.028**	**0.285**	
	%CV:	**16.833**	**16.912**	
B	021	0.4793	56	
	022	0.5478	57	
	023	0.6926	57	
	024	0.6211	57	
	025	0.3219	55	
	026	0.5863	56	
	027	0.5664	57	
	028	0.5631	57	
	029	0.5812	57	
	030	0.5203	56	
	031	0.5378	57	
	032	0.5930	59	
	033	0.4914	56	
	034	0.4328	55	
	035	0.6004	56	
	036	0.4632	56	
	037	0.5294	56	
	038	0.4847	56	
	039	0.5183	56	
	040	0.4883	56	
	Median:	**0.534**	**56**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.531**	**56.400**	
	SEM:	**0.018**	**0.197**	
	%CV:	**10.794**	**11.752**	
	Median:	**0.543**	**57**	
	Mode:		**56**	
	Total Mean:	**0.538**	**56.675**	
	SEM:	**0.016**	**0.177**	
	%CV:	**14.032**	**14.855**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00062 **Test No: 1**

Test Chemical: L-Thyroxine (T4) **Target Conc: 0.5 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	041	0.5683	57	
	042	0.6391	59	
	043	0.5268	57	
	044	0.6829	59	
	045	0.6039	59	
	046	0.5223	57	
	047	0.3530	57	
	048	0.4847	57	
	049	0.3739	57	
	050	0.5127	57	
	051	0.4381	56	
	052	0.5261	57	
	053	0.4578	57	
	054	0.5414	57	
	055	0.6711	57	
	056	0.7885	59	
	057	0.5536	57	
	058	0.5702	57	
	059	0.6332	57	
	060	0.4755	56	
	Median:	**0.534**	**57**	
	Mode:		**57**	
	Mean:	**0.546**	**57.300**	
	SEM:	**0.024**	**0.206**	
	%CV:	**14.274**	**12.198**	
B	061	0.3586	56	
	062	0.4751	56	
	063	0.3802	56	
	064	0.5411	57	
	065	0.6526	57	
	066	0.4500	57	
	067	0.6282	57	
	068	0.6051	59	
	069	0.3964	56	
	070	0.6379	57	
	071	0.7596	59	
	072	0.5234	57	
	073	0.5816	57	
	074	0.6418	59	
	075	0.5086	57	
	076	0.5709	57	
	077	0.4358	57	
	078	0.6479	57	
	079	0.6228	58	
	080	0.6080	57	
	Median:	**0.576**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.551**	**57.150**	
	SEM:	**0.024**	**0.209**	
	%CV:	**14.480**	**12.346**	
	Median:	**0.548**	**57**	
	Mode:		**57**	
	Total Mean:	**0.549**	**57.225**	
	SEM:	**0.017**	**0.145**	
	%CV:	**14.197**	**12.155**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00062 **Test No: 1**

Test Chemical: L-Thyroxine (T4) **Target Conc: 1.0 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	081	0.2890	59	
	082	0.4221	59	
	083	0.4769	59	
	084	0.3962	59	
	085	0.5805	59	
	086	0.5943	59	
	087	0.2946	59	
	088	0.4906	59	
	089	0.6893	59	
	090	0.2818	63	
	091	0.5097	59	
	092	0.4981	59	
	093	0.5057	59	
	094	0.5371	59	
	095	0.4619	59	
	096	0.4307	59	
	097	0.4849	60	
	098	0.4799	59	
	099	0.3277	59	
	Median:	**0.480**	**59**	
	Mode:		**59**	
	Mean:	**0.461**	**59.263**	
	SEM:	**0.025**	**0.214**	
	%CV:	**15.974**	**12.126**	
B	100	0.3450	62	
	101	0.4360	59	
	102	0.3851	59	
	103	0.4144	59	
	104	0.4935	59	
	105	0.4992	59	
	106	0.4569	59	
	107	0.4958	59	
	108	0.4478	61	
	109	0.5760	59	
	110	0.4634	59	
	111	0.6464	59	
	112	0.2971	62	
	113	0.5579	59	
	114	0.2981	59	
	115	0.5462	59	
	116	0.5054	59	
	117	0.4804	59	
	118	0.4267	59	
	119	0.5620	59	
	Median:	**0.472**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.467**	**59.400**	
	SEM:	**0.020**	**0.222**	
	%CV:	**13.293**	**12.907**	
	Median:	**0.480**	**59**	
	Mode:		**59**	
	Total Mean:	**0.464**	**59.333**	
	SEM:	**0.016**	**0.153**	
	%CV:	**14.463**	**12.400**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00062 **Test No: 1**

Test Chemical: L-Thyroxine (T4) **Target Conc: 2.0 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	120	0.2318	63	
	121	0.1511	62	
	122	0.1845	62	
	123	0.1469	62	
	124	0.3387	60	
	125	0.2467	61	
	126	0.2536	61	
	127	0.1746	61	
	128	0.2947	59	
	129	0.4865	59	
	130	0.1957	59	
	131	0.5312	59	
	132	0.3313	59	
	133	0.3499	59	
	134	0.2979	59	
	135	0.2798	59	
	136	0.2992	59	
	137	0.2352	59	
	138	0.4739	59	
	139	0.2680	59	
	Median:	**0.274**	**59**	
	Mode:		**59**	
	Mean:	**0.289**	**60.000**	
	SEM:	**0.024**	**0.308**	
	%CV:	**20.108**	**17.770**	
B	140	0.2336	61	
	141	0.1441	61	
	142	0.2486	61	
	143	0.2122	61	
	144	0.2315	61	
	145	0.3968	59	
	146	0.2824	61	
	147	0.2971	59	
	148	0.2852	62	
	149	0.2428	60	
	150	0.3840	60	
	151	0.2694	59	
	152	0.3756	59	
	153	0.2935	59	
	154	0.1646	60	
	155	0.4488	59	
	156	0.1985	60	
	157	0.4438	59	
	158	0.3948	60	
	159	0.2084	60	
	Median:	**0.276**	**60**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.288**	**60.050**	
	SEM:	**0.020**	**0.211**	
	%CV:	**16.920**	**12.189**	
	Median:	**0.275**	**60**	
	Mode:		**59**	
	Total Mean:	**0.288**	**60.025**	
	SEM:	**0.016**	**0.184**	
	%CV:	**18.345**	**15.043**	

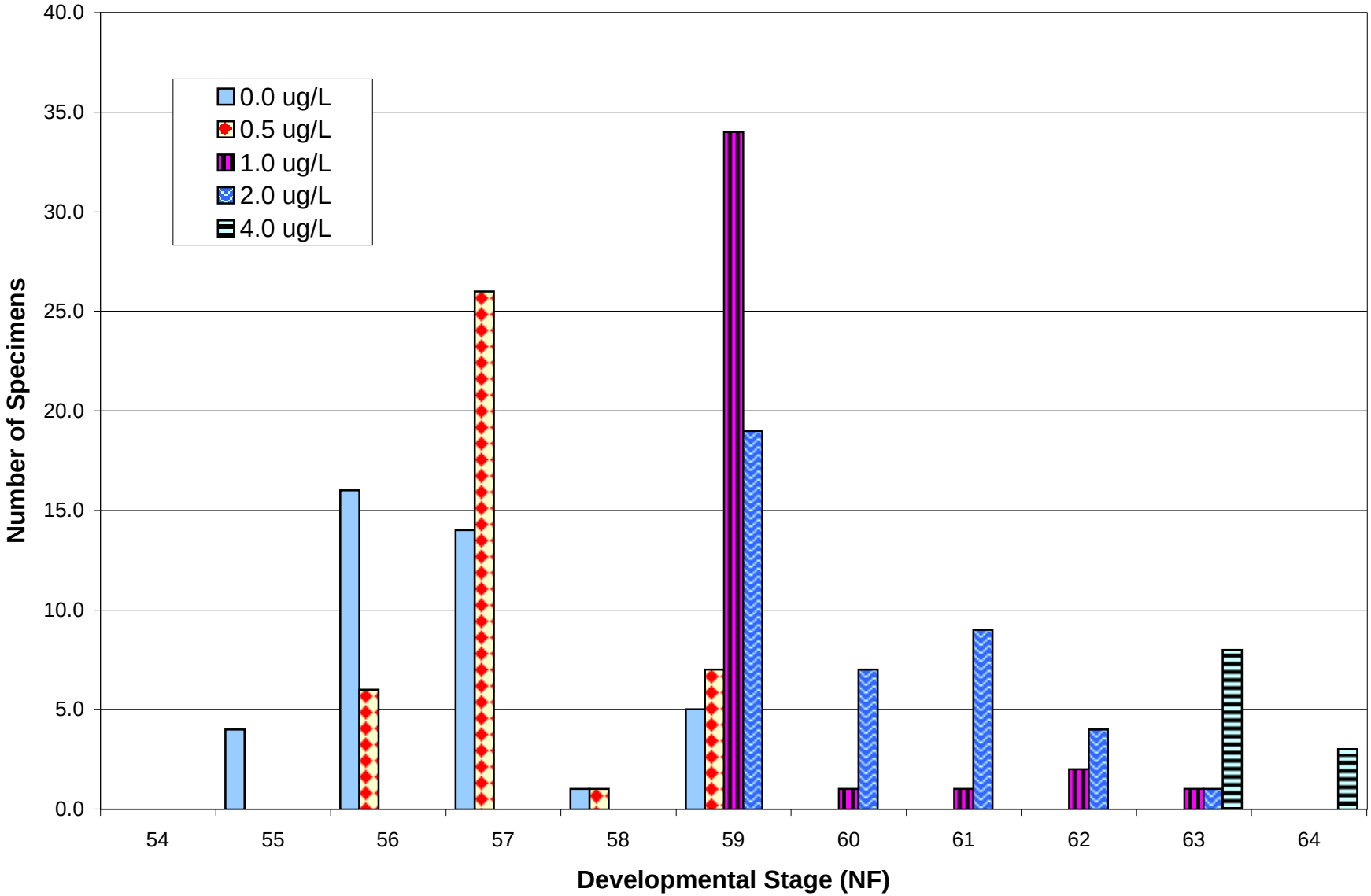
TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00062 **Test No: 1**

Test Chemical: L-Thyroxine (T4) **Target Conc: 4.0 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	160	0.1072	64	
	161	0.1339	64	
	162	0.0796	63	
	163	0.0967	63	
	164	0.0714	63	
	165	0.0910	63	
	166	0.0736	63	
	167	0.0780	63	
	Median:	**0.085**	**63**	
	Mode:		**63**	
	Mean:	**0.091**	**63.250**	
	SEM:	**0.007**	**0.164**	
	%CV:	**6.982**	**5.821**	
B	168	0.1576	64	
	169	0.1088	63	
	170	0.1261	63	
	Median:	**0.126**	**63**	**Comments:**
	Mode:		**63**	
	Rep Mean:	**0.131**	**63.333**	
	SEM:	**0.014**	**0.333**	
	%CV:	**6.840**	**7.255**	
	Median:	**0.097**	**63**	
	Mode:		**63**	
	Total Mean:	**0.102**	**63.273**	
	SEM:	**0.008**	**0.141**	
	%CV:	**8.699**	**5.872**	

BATT02-00062
L-Thyroxine
Stage Distribution for Initial Stage 51 Test



TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00062 **Test No: 1**

Test Chemical: L-Thyroxine (T4) **Target Conc: 0.0 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	171	0.4315	60	
	172	0.6659	59	
	173	0.5340	59	
	174	0.6511	59	
	175	0.5349	57	
	176	0.5549	57	
	177	0.5296	57	
	178	0.7180	59	
	179	0.7572	59	
	180	0.6037	59	
	181	0.5756	59	
	182	0.6618	59	
	183	0.6864	57	
	184	0.5323	57	
	185	0.6145	57	
	186	0.5570	57	
	187	0.4065	59	
	188	0.5388	57	
	189	0.6091	57	
	190	0.6000	57	
	Median:	**0.588**	**58**	
	Mode:		**57**	
	Mean:	**0.588**	**58.050**	
	SEM:	**0.020**	**0.246**	
	%CV:	**11.452**	**14.425**	
D	191	0.4877	59	
	192	0.5301	59	
	193	0.6646	59	
	194	0.5269	57	
	195	0.5360	57	
	196	0.6469	57	
	197	0.5334	59	
	198	0.6885	59	
	199	0.5672	59	
	200	0.6268	59	
	201	0.7418	59	
	202	0.5828	59	
	203	0.5311	59	
	204	0.6302	59	
	205	0.5466	58	
	206	0.6373	57	
	207	0.7619	57	
	208	0.5569	57	
	209	0.5120	57	
	210	0.3908	61	
	Median:	**0.562**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.585**	**58.350**	
	SEM:	**0.020**	**0.254**	
	%CV:	**11.723**	**14.881**	
	Median:	**0.571**	**59**	
	Mode:		**59**	
	Total Mean:	**0.587**	**58.200**	
	SEM:	**0.014**	**0.176**	
	%CV:	**11.440**	**14.603**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00062 **Test No: 1**

Test Chemical: L-Thyroxine (T4) **Target Conc: 0.5 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	211	0.3630	61	
	212	0.3505	61	
	213	0.7007	57	
	214	0.5305	57	
	215	0.6463	59	
	216	0.6141	59	
	217	0.8158	59	
	218	0.5419	57	
	219	0.7058	57	
	220	0.7181	57	
	221	0.6631	59	
	222	0.6761	59	
	223	0.7436	59	
	224	0.5909	59	
	225	0.5396	57	
	226	0.5777	57	
	227	0.4508	57	
	228	0.4647	58	
	229	0.6576	57	
	230	0.8333	57	
	Median:	**0.630**	**58**	
	Mode:		**57**	
	Mean:	**0.609**	**58.150**	
	SEM:	**0.030**	**0.302**	
	%CV:	**17.191**	**17.684**	
D	231	0.7281	58	
	232	0.6396	59	
	233	0.6902	59	
	234	0.8301	59	
	235	0.7068	59	
	236	0.6425	59	
	237	0.6054	57	
	238	0.5709	57	
	239	0.5809	57	
	240	0.6554	57	
	241	0.6703	57	
	242	0.6032	57	
	243	0.4936	57	
	244	0.6450	57	
	245	0.5638	57	
	246	0.7157	59	
	247	0.5431	60	
	248	0.4300	61	
	249	0.4141	61	
	250	0.6445	59	
	Median:	**0.641**	**59**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.619**	**58.300**	
	SEM:	**0.022**	**0.309**	
	%CV:	**12.757**	**18.078**	
	Median:	**0.641**	**58**	
	Mode:		**57**	
	Total Mean:	**0.614**	**58.225**	
	SEM:	**0.019**	**0.213**	
	%CV:	**14.937**	**17.679**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00062 **Test No: 1**

Test Chemical: L-Thyroxine (T4) **Target Conc: 1.0 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	251	0.5050	59	
	252	0.7133	59	
	253	0.7192	59	
	254	0.5759	59	
	255	0.6890	59	
	256	0.4320	59	
	257	0.5258	59	
	258	0.6373	59	
	259	0.5328	57	
	260	0.4337	57	
	261	0.6269	57	
	262	0.6072	59	
	263	0.6384	59	
	264	0.6194	59	
	265	0.4578	58	
	266	0.4566	59	
	267	0.4303	57	
	268	0.3781	57	
	269	0.3960	58	
	270	0.3935	57	
	Median:	**0.529**	**59**	
	Mode:		**59**	
	Mean:	**0.538**	**58.300**	
	SEM:	**0.025**	**0.206**	
	%CV:	**15.371**	**12.093**	
D	271	0.7361	59	
	272	0.7263	59	
	273	0.5275	59	
	274	0.7012	59	
	275	0.5626	59	
	276	0.5652	59	
	277	0.5520	59	
	278	0.6279	58	
	279	0.5286	58	
	280	0.6275	58	
	281	0.5253	58	
	282	0.5094	59	
	283	0.5632	59	
	284	0.5988	59	
	285	0.5496	58	
	286	0.5868	58	
	287	0.5626	59	
	288	0.5256	59	
	289	0.4496	61	
	290	0.4353	58	
	Median:	**0.563**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.573**	**58.750**	
	SEM:	**0.018**	**0.160**	
	%CV:	**10.565**	**9.346**	
	Median:	**0.557**	**59**	
	Mode:		**59**	
	Total Mean:	**0.556**	**58.525**	
	SEM:	**0.016**	**0.134**	
	%CV:	**13.158**	**11.071**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00062 **Test No: 1**

Test Chemical: L-Thyroxine (T4) **Target Conc: 2.0 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	291	0.5850	59	
	292	0.5428	59	
	293	0.5631	59	
	294	0.3689	59	
	295	0.4111	59	
	296	0.3974	59	
	297	0.5562	59	
	298	0.5500	59	
	299	0.4768	59	
	300	0.7283	59	
	301	0.4674	60	
	302	0.5402	60	
	303	0.4311	61	
	304	0.4877	61	
	305	0.3466	61	
	306	0.5242	61	
	307	0.3948	61	
	308	0.4008	59	
	309	0.4764	59	
	310	0.5112	59	
	Median:	**0.482**	**59**	
	Mode:		**59**	
	Mean:	**0.488**	**59.600**	
	SEM:	**0.020**	**0.197**	
	%CV:	**13.028**	**11.432**	
D	311	0.5789	59	
	312	0.5410	59	
	313	0.6960	59	
	314	0.5898	59	
	315	0.7599	59	
	316	0.6430	59	
	317	0.4751	59	
	318	0.3805	59	
	319	0.5015	59	
	320	0.6843	59	
	321	0.4967	59	
	322	0.4540	59	
	323	0.3286	61	
	324	0.4008	61	
	325	0.2764	61	
	326	0.5083	60	
	327	0.3106	61	
	328	0.3702	61	
	329	0.4131	61	
	330	0.4498	61	
	Median:	**0.486**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.493**	**59.750**	
	SEM:	**0.030**	**0.216**	
	%CV:	**19.178**	**12.504**	
	Median:	**0.482**	**59**	
	Mode:		**59**	
	Total Mean:	**0.490**	**59.675**	
	SEM:	**0.018**	**0.145**	
	%CV:	**16.201**	**11.867**	

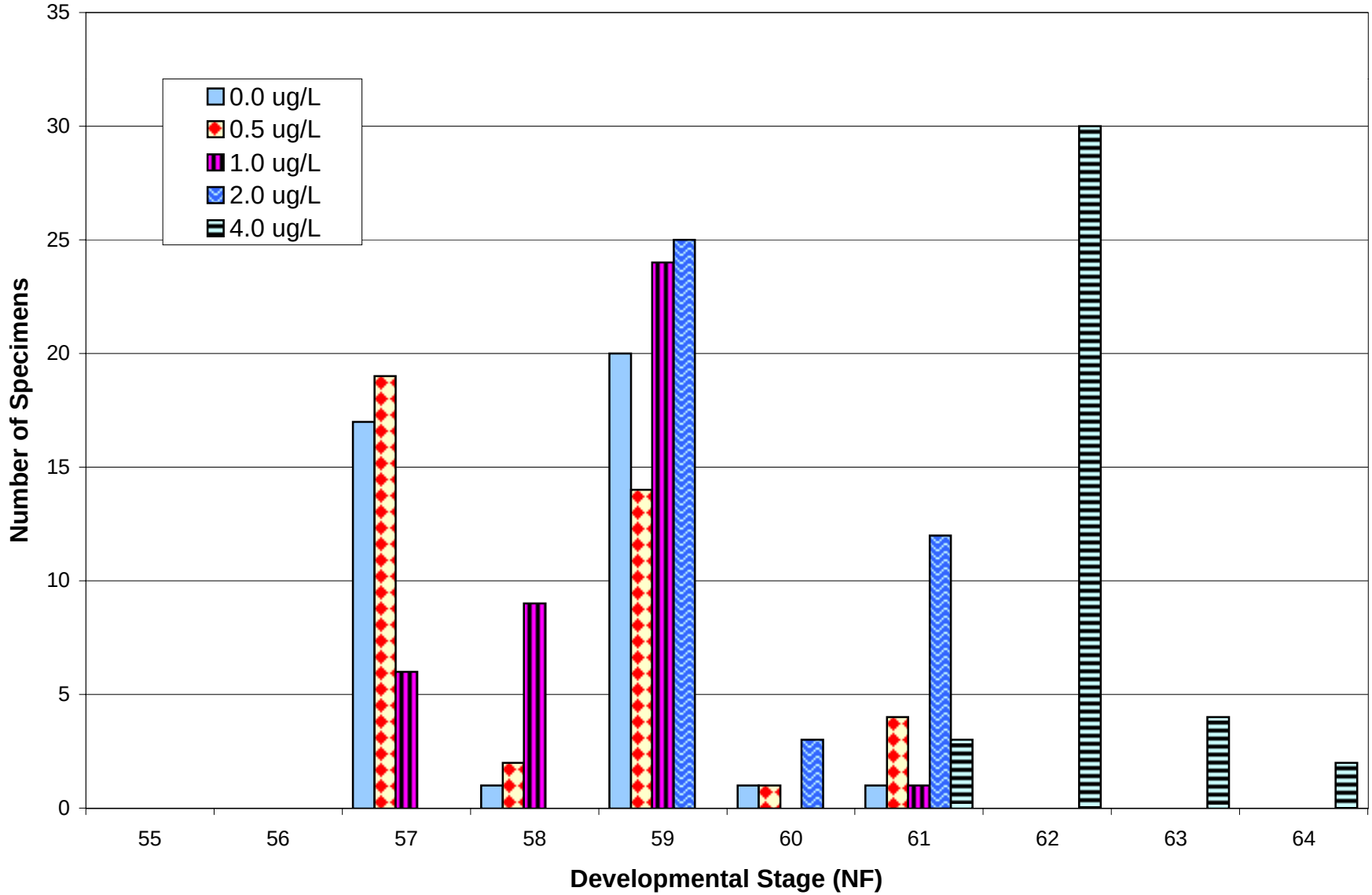
TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00062 **Test No: 1**

Test Chemical: L-Thyroxine (T4) **Target Conc: 4.0 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	331	0.2080	64	
	332	0.2782	61	
	333	0.3853	61	
	334	0.1644	63	
	335	0.2465	61	
	336	0.2350	63	
	337	0.2733	62	
	338	0.2743	62	
	339	0.1181	62	
	340	0.1933	62	
	341	0.2173	62	
	342	0.1965	62	
	343	0.2466	62	
	344	0.1538	62	
	345	0.2624	62	
	346	0.2318	62	
	347	0.2336	62	
	348	0.2964	62	
	349	0.3635	62	
	Median:	**0.235**	**62**	
	Mode:		**62**	
	Mean:	**0.241**	**62.053**	
	SEM:	**0.015**	**0.162**	
	%CV:	**13.377**	**8.950**	
D	350	0.3139	62	
	351	0.1307	63	
	352	0.2707	64	
	353	0.2890	62	
	354	0.2194	62	
	355	0.2015	62	
	356	0.2484	62	
	357	0.1612	62	
	358	0.2060	62	
	359	0.3235	62	
	360	0.2789	62	
	361	0.3667	62	
	362	0.1780	62	
	363	0.1992	63	
	364	0.2919	62	
	365	0.2062	62	
	366	0.2120	62	
	367	0.2314	62	
	368	0.2133	62	
	369	0.1882	62	
	Median:	**0.216**	**62**	**Comments:**
	Mode:		**62**	
	Rep Mean:	**0.237**	**62.200**	
	SEM:	**0.013**	**0.117**	
	%CV:	**12.300**	**6.633**	
	Median:	**0.232**	**62**	
	Mode:		**62**	
	Total Mean:	**0.239**	**62.128**	
	SEM:	**0.010**	**0.098**	
	%CV:	**12.679**	**7.799**	

BATT02-00062
L-Thyroxine
Stage Distribution for Initial Stage 54 Test



L-Thyroxine (T4)
Metamorphosis Assay:
Statistical Data – Stage and Growth

One Way Analysis of Variance

Data source: BATT02-00062_T4_Stage 51_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0ug/L	40	0	56.5	56	57
0.5ug/L	40	0	57	57	57
1.0ug/L	39	0	59	59	59
2.0ug/L	40	0	60	59	61
4.0ug/L	11	0	63	63	63.75

H = 127.592 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
4.0ug/L vs 0.0ug/L	127.235	7.593	Yes	
2.0ug/L vs 0.0ug/L	87.475	7.948	Yes	
1.0ug/L vs 0.0ug/L	70.386	6.355	Yes	**LOEC**
0.5ug/L vs 0.0ug/L	14.875	1.352	No	**NOEC**

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00062_T4_Stage 51_Growth

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.087)

Group Name	N	Missing	Mean	Std Dev	SEM
0.0ug/L	40	0	0.538	0.103	0.0163
0.5ug/L	40	0	0.549	0.105	0.0166
1.0ug/L	39	0	0.464	0.0985	0.0158
2.0ug/L	40	0	0.288	0.0985	0.0156
4.0ug/L	11	0	0.102	0.0278	0.00838

Source of Variation	DF	SS	MS	F	P
Between Groups	4	3.056	0.764	78.826	<0.001
Residual	165	1.599	0.00969		
Total	169	4.655			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Bonferroni t-test):

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050	
0.0ug/L vs. 4.0ug/L	0.436	13.009	<0.001	Yes	
0.0ug/L vs. 2.0ug/L	0.25	11.358	<0.001	Yes	
0.0ug/L vs. 1.0ug/L	0.0745	3.363	0.004	Yes	**LOEC**
0.0ug/L vs. 0.5ug/L	0.0105	0.478	1	No	**NOEC**

One Way Analysis of Variance

Data source: BATT02-00062_T4_Stage 54_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0ug/L	40	0	59	57	59
0.5ug/L	40	0	58	57	59
1.0ug/L	40	0	59	58	59
2.0ug/L	40	0	59	59	61
4.0ug/L	39	0	62	62	62

H = 124.129 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
4.0ug/L vs 0.0ug/L	113.633	8.768	Yes	
2.0ug/L vs 0.0ug/L	50.725	3.939	Yes	**LOEC**
1.0ug/L vs 0.0ug/L	9.888	0.768	No	**NOEC**
0.5ug/L vs 0.0ug/L	0.637	0.0495	Do Not Test	

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00062_T4_Stage 54_Growth

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.015)

Group Name	N	Missing	Mean	Std Dev	SEM
0.0ug/L	40	0	0.587	0.0876	0.0139
0.5ug/L	40	0	0.614	0.117	0.0185
1.0ug/L	40	0	0.556	0.0981	0.0155
2.0ug/L	40	0	0.49	0.113	0.0179
4.0ug/L	39	0	0.239	0.0619	0.00992

Source of Variation	DF	SS	MS	F	P
Between Groups	4	3.61	0.902	94.285	<0.001
Residual	194	1.857	0.00957		
Total	198	5.466			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Bonferroni t-test):

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050	
0.0ug/L vs. 4.0ug/L	0.348	15.802	<0.001	Yes	
0.0ug/L vs. 2.0ug/L	0.0961	4.393	<0.001	Yes	**LOEC**
0.0ug/L vs. 1.0ug/L	0.0308	1.409	0.642	No	**NOEC**
0.0ug/L vs. 0.5ug/L	0.0274	1.251	0.849	Do Not Test	

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Tests should be treated as if there is no significant difference between the means, even though one may appear to exist.

L-Thyroxine (T4)
Test Chemical Analytical Data from Battelle:
Purity / Stability Report

Chemical Repository Services for the EDSP

EPA Contract No. 68-W-01-023

1.0 TITLE AND APPROVAL

Chemistry Report for WA 3-11 L-Thyroxine in well water

January 25, 2005

Prepared By:

Tim Fortman
Chemical Repository Analyst

Date

Approved By:

Richard M. Ecker
Director, Marine Sciences Laboratory

Date

Michael E Cobb
Chemical Repository Study Director

Date

Battelle Marine Sciences Laboratory

1529 West Sequim Bay Road

Sequim, WA 98382

Submitted to:

Doug Fort

Fort Environmental Laboratories

Stillwater, Oklahoma 74074

2.0 QA REVIEWER AND CONTRIBUTOR PAGE

Chemistry Report for WA 3-11
L-Thyroxine in Well Water

Reviewed by: _____

Mary Lynn, EDSP Quality Assurance Representative
Windward Company, Gig Harbor

Date: _____

Personnel participating in this study:

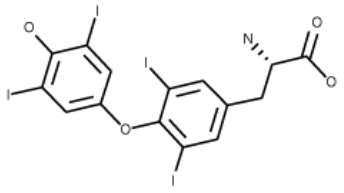
Analysts: Margaret O'Neill, Tim Fortman, Rebecca Wood

Chemical Repository Study Director: Michael Cobb

3.0 EXECUTIVE SUMMARY

Chemistry Report for WA 3-11
L-Thyroxine in Well Water

Chemical Procurement Information

Parameter	Chemical	Chemical Structure
Compound Name	L-Thyroxine (T4)	
CAS #	51-48-9	
Central File No.	2109	
Initial Receipt Date	10/09/03	
Expiration Date	09/2005	
Manufacturer	Sigma	
Lot Number	092K1061	
Battelle Study #	WA 3-11	
Method	EDSP.I8-003	

Executive Summary
Work Assignment (WA) 3-11 of the Environmental Protection Agency's Endocrine Disruptor Screening Program (EDSP) describes a reproductive and developmental study on frogs raised in water spiked with one of six study chemicals. The Chemical Repository (Repository) has the responsibility for carrying out the purity, stability, and in-life concentration determinations of the selected study chemicals for EDSP studies.

The chemical of interest for this portion of the study is L-Thyroxine (T4). The analytical method developed in-house for the T4 purity, stability study, and in-life samples utilized Ion Chromatography-Inductively Coupled Plasma/Mass Spectrometry IC-ICP/MS. This method was selected based on two factors: first, a preliminary evaluation of the viability of employing High Performance Liquid Chromatography (HPLC) as the analytical method was unsuccessful due to insufficient system sensitivity. In order to use HPLC, samples would have required concentration prior to analysis, a workable but less than desirable approach; second, from inquiries made to the EPA lab in Duluth, we learned they had successfully used IC-ICP/MS for T4 analysis. It was decided to use IC-ICP/MS as the method of choice. As will be discussed throughout this document, a series of equipment problems in the IC-ICP/MS were experienced (initially very subtle and intermittent) that resulted in excessive performance variability. The problem eventually caused a serious instrument breakdown during the in-life sample analysis phase. Despite the problems, the study was continued and the data were analyzed and when the preliminary data were presented to the client, decisions were made to allow completion of the studies and initiation and completion of the in-life studies at Fort Laboratories.

The chemical purity of T4, as determined by the manufacturer, was 99.4%. The Battelle-Sequim purity result by IC-ICP/MS was 93.01%.

The stability of the water dosed with T4 was analyzed at two[1] concentrations, one representing the stock feed water (4.0 mg/L) the other representing one of the amphibian exposure

[1] The protocol specified the preparation and analysis of two concentrations (1.0 µg/L and 4.0 mg/L) of T4 for the stability testing. Additional levels were evaluated during the study and will be discussed in the body of the text.

concentrations (1.0 µg/L). These concentrations were to be tested over twenty-one[2] days. Based on the specification of less than 10% degradation over the study period, the L-Thyroxine in the stock feed water was demonstrated to remain greater than or equal to 90% of the target concentration for up to an estimated 7 days. The measurement of L-Thyroxine in the diluted water (I.0 µg/L) was problematic on day zero due to an analytical problem (described in the body of the text) but the problem was resolved for the day two analysis and results remained at 100%+/-1% through day seven. The levels dropped precipitously from day ten on through day 23 and were at zero at day 51.

Notes:
The slow deterioration (instability) of the stock T4 solution after an initial 10 days of meeting stability specs is suspect due to a number of instrument problems experienced during the study. An additional measurement of the two stability samples (beyond the time period specified in the protocol) was carried out following servicing of the instrumentation on day 51. The 4.0 mg/L sample showed a recovery of 104% at the 51 day sampling with good precision for the triplicate values. The 1.0 µg/L sample was variable for most of the study and the level dropped to 0% recovery on day 51. We are clearly in close proximity of the sensitivity limits of the analytical system in measuring the 1.0 µg/L sample. Combining that factor with the technical problems experienced with the instrumentation, one has to look at the trending of the low end data more than the individual points to draw any conclusions from the efforts. Over the study period, the 1.0 µg/L sample was near 100% recovery for the first 7 days and then rapidly dropped to nearly zero at end of four weeks. Though there is more variability in the data than the other 5 chemicals evaluated for the WA 3-11, the data trending strongly supports that the low level sample is deteriorating over time. With this trend noted (also corroborated with a similar trend seen in data from 4 µg/L sample added to the study to help assess the low results), a decision was made with the In-Life Study Director to proceed with the exposure study with the recommendation that the in-life samples be collected and shipped immediately via overnight courier to the Repository lab. Analysis of the In-Life samples was planned to be carried out within 5 days of collection.

[2] The protocol specified a 21 day study but due to the ongoing instrument problems throughout the study, several additional data points were measured with a final measurement carried out at 51 days, after the instrumentation had been through another significant service call.

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5.0 INTRODUCTION

The goal of the Battelle-Sequim, Marine Sciences Laboratory (MSL) Repository for the Endocrine Disruptor Screening Program is to provide the participating laboratory with requested chemicals of documented quality at required concentrations and in a matrix appropriate for different toxicological tests. The EDSP Chemical Repository supplies the manufacturer's information regarding purity and stability, the material safety data sheet (MSDS) chemical information, and independent analysis of purity and stability in a matrix specified by the Study Protocol: Purity and Stability of Test Substances for Work Assignment 3-11 [EDSP Study Number: EDSP.311-01], made in collaboration with the requesting Study Director.
Under Work Assignment (WA) 3-11, Fort Environmental Laboratories requested purity and stability testing of L-Thyroxine (figure 1).

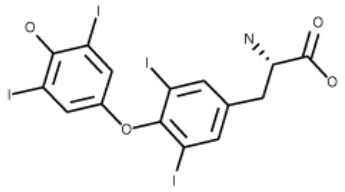
6.0 GENERAL METHODS

Methods of standard operation of the Repository are currently addressed in MSL SOPs numbered R-001 through R-017. These procedures address chemical procurement including procurement of controlled substances, when applicable, which have unique permitting, ordering, handling, inventory, and storage requirements; chemical receipt and chain of custody, chemical log-in and labeling, inventory, chemical storage; stock solution preparation, documentation and archiving; test solution preparation, documentation and shipping; chemical disposal, and repository maintenance over time. The quality assurance (QA) requirements for procurement of chemicals for use in the Repository are addressed in Version 2 of the Quality Assurance Project Plan (QAPP) for EDSP Chemical Repository.

6.1 CHEMICAL PROCUREMENT

As requested (Figure 1) by Doug Fort, the Study Director for WA 3-11, L-Thyroxine, (CAS No. 51-48-9), was purchased for purity and stability analysis and used as the test chemical, in a one generational study of frogs. The L-Thyroxine was purchased from Sigma/Aldrich and the first shipment of lot number 092K1061 was received on 10/09/03 with an expiration date of 09/2005 (Table 1). The chemical was logged in to the Chemical Management System (CMS) and given a CMS barcode and unique log-in (central file) number (CF-2109) as per the QAPP for the EDSP Chemical Repository. The chemical was stored in the Repository in a clean, dry location at room temperature, away from direct sunlight.

Table 1. Chemical Procurement Information

Parameter	Chemical	Chemical Structure
Compound Name	L-Thyroxine (T4)	
CAS #	51-48-9	
Central File No.	2109	
Initial Receipt Date	10/09/03	
Expiration Date	09/2005	
Manufacturer	Sigma	
Lot Number	092K1061	
Battelle Study #	WA 3-11	
Method	EDSP.I8-003	

EDSP Chemical Request Form

For EPA WA: 3-11

Study Director:
Name: Douglas J. Fort, Ph.D.
Affiliation: Fort Environmental Laboratories
Location: Stillwater, Oklahoma 74074
Telephone number: 405-624-6771

Bioassay Information:

Proposed Bioassay: Frog Metamorphosis Assay Demonstration

Test Chemical: L-Thyroxine (T4)

Carrier(s): NA

Concentrations/Dilution Series: 4.0,8.0,16.0,32.0 μg/L

*Consider if analysis method detection limit which may be determined in Purity analysis is above or below desired test concentrations?

In vitro or *in vivo* tests? *In vivo*

Organism to be tested: *Xenopus laevis* (South African Clawed Frog)

Method of test solution administration: Flow-through exposure/diluter system

Planned/proposed test duration: 14 and 21 days

Chemical Information:

Chemical Name: L-Thyroxine (T4)

CAS: 51-48-9

Any known purity information: may refer to attached documentation

Any known stability information: Contact Dr. Joe Tietge, EPA, Duluth, MN

Desired purity (%) for test? ≥98%

Is there a known calibration standard available?
Name:
CAS:
Manufacturer:

Figure 1. EDSP Chemical Request Form for L-Thyroxine

6.2 CHEMICAL PURITY

Chemical purity was verified by Ion Chromatography-Inductively Coupled Plasma/Mass Spectrometry (IC-ICP/MS) analysis to determine areas under peaks other than the principal peak and compared to the manufacturer's certificate of analysis/purity (Appendix A). No statistical analyses were performed for the verification of chemical purity. Purity verification was conducted by making a solution of L-Thyroxine in 50:50 ethanol:10mM phosphate buffer (pH 3.0) of about 110 µg/mL. This matrix was then run on an IC-ICP/MS. An ethanol:10mM phosphate buffer blank was also analyzed on the system. The purity was determined by first identifying the peaks in the L-Thyroxine chromatogram that were the same as the peaks in the analysis of the blank. The areas associated with these common peaks were then eliminated and the remaining peaks were reported as a percentage of the total peak area. The percentage associated with the largest peak represented the purity of L-Thyroxine. Three replicates were analyzed.

6.3 STABILITY TESTING PLAN DESIGN AND DETAIL

A general study plan for stability testing based on the WA 3-11 Chemical Request from Doug Fort (Figure 1) was developed as the ***Purity and Stability Testing Protocol*** and is presented in Appendix B. A stock solution and two diluter solutions were prepared and covered the chemical concentrations requested for stability analysis (Table 2). All samples were analyzed in triplicate for calculation of a mean concentration and relative standard deviation (RSD).

L-Thyroxine water matrices were prepared on 5/4/04 (4.04 mg/L and 1 µg/L) and 5/11/04 (4 µg/L) for testing as described in Table 2. Briefly, for the 4 mg/L, L-Thyroxine, 0.0146 g was weighed into a 4 liter amber bottle and 3.7 L of well water was added using a class A graduated cylinder. This solution was then poured into 2 separate amber bottles, one stored in the refrigerator at 2-8° C and the other stored at room temperature. For the 1 µg/L solution, 0.125 mL of the above 4 mg/L stock was measured into another amber bottle and diluted with 500 ml of dechlorinated tap water received from Fort Environmental. For the 4 µg/L solution, 0.5 mL of the above 4 mg/L stock was measured into another amber bottle and diluted with 500 ml of well water. All measurements were done with Class A graduated cylinders. The solution was agitated to insure uniformity. One and 4 µg/L solutions were stored on the counter at room temperature for the duration of the test. Note: A 5 mg/L standard was also prepared on 5/26/05 by weighing 0.0173 g of the L-Thyroxine into a 4 L amber bottle with 3.5 liters of house DI water. This solution was prepared to test the solubility of the T-4 in deionized water.

Table 2. Stock Matrix Composition for Stability Testing

Study, Duration	Test Chemical	Target Conc.	Sample ID	Actual Conc.	Stock Matrix
WA 3-11, 21 days	L-Thyroxine	4.04 mg/L	Stock	3.946 mg/L	50mM NaOH
		1 µg/L	Diluter	0.9865 µg/L	0.125 ml of 3.946 mg/L in 500mL tap water
		4 µg/L	Diluter	3.946 µg/L	0.5 ml of 3.946 mg/L in 500mL eluent

6.4 ANALYTICAL CHEMISTRY FOR STABILITY TESTING

Chemical stability was evaluated under storage conditions and matrix specifications as requested by the participating laboratory. At initiation and at each time period throughout the duration of the test, the chemical concentration was determined by chromatographic analysis.

Generally, triplicate aliquots were tested. The frequency of determinations and the duration of testing were determined by the requesting Study Director and the chemists based on *a priori* knowledge about chemical stability. The duration and frequency of testing deviated from the original testing protocol due to instrument malfunction.

L-Thyroxine stock solution was sampled by taking 0.01 mL of the stock using an appropriate pipette and diluting it to 10 mL with 9.99 ml diluent (except on 06/24/04 when 0.01 mL of the stock solution was diluted up to 5 mL with diluent).

L-Thyroxine diluter solution was sampled by taking 4.5 mL of the diluter sample using an appropriate pipette and diluting it to 10 mL with 5 mL of Methanol and 0.5mL of Phosphate Buffer. Note the sampling of the diluter at T=0 was not prepared with the buffer solution. Low recovery at T=0 resulted from trying to elute sample diluted in water instead of sample diluted in eluent. Water blanks were prepared by diluting water to 10 mL with 5 mL of Methanol and 0.5mL of Phosphate Buffer. Spike solutions were prepared at 1.09 µg/L and 54.5 µg/L from stock solutions stored at 2-8° C. These solutions were then analyzed by the IC/ICPMS for quantification. Initial calibration verification and continuing calibration verification (ICV/CCV) samples were analyzed to demonstrate on-going calibration accuracy. The ICV sample was prepared from a separate source of T4, lot number 411874/1, obtained Fluka Production GmbH.

Table 3. IC/ICPMS Conditions

Detector	6100 ICPMS
Flow Rate	0.25 mL/min
Injection Volume	1.0mL
Eluent; flow pattern	Gradient (see method)
Column	Phenominex Synergi 4µ Hydro RP 80A 50x2mm
Guard Column	C18 (ODS octadecyl) 4mm Lx2.0mm ID

7.0 RESULTS

7.1 CHEMICAL PURITY

Battelle-Sequim conducted a purity scan on L-Thyroxine as described in section 6.2 above. Three replicates of a 110 µg/L standard were analyzed. The chromatograms, showed one large peak that had the appropriate retention time for L-Thyroxine with several smaller peaks. The area of the L-Thyroxine peak was 93.24%, 92.5% and 93.28% of the total area of all peaks in the chromatograms for each of the three replicates. Chemical purity of L-Thyroxine determined by the manufacturer via HPLC was 99.4% (Appendix A).

7.2 ANALYTICAL CHEMISTRY FOR STABILITY TESTING

Multiple problems were encountered during the stability testing for T-4. The initial analysis of the 1 ug/L stability solution failed because standard make-up was different from the mobile phase. This was rectified in subsequent samplings. Another more serious problem occurred with the detector used, the ICP-MS. The ICP-MS is cooled several ways, one component of the cooling system is provided indirectly by the laboratory ventilation system. Overloading of the cooling demands on the laboratory ventilation system caused excessively high temperatures within the machine which in turn caused electronic fluctuations, eventually resulting in a failure

of the electronics controlling the ICP-MS. These fluctuations and eventual failure, were not successfully diagnosed by factory service personnel for a number of visits over several weeks time, resulting in erratic performance of the ICP-MS. Another problem occurred during the stability studies which ultimately affected the quality of the results[3]. Deterioration of the chromatography column caused peak splitting and retention time shifts. The summation of these problems is that the system was not performing well enough to meet our strict QA/QC requirements.

7.2.1 Stock Solution Stability

Chemical stability testing was initiated on 05/4/04 for the 4 mg/L stock. Chemical concentrations were determined 6 times for the room temperature bottle and 5 times for the bottle stored at 4 deg C. Tests lasted 51 days for the room temperature sample and 38 days for the refrigerated sample. Average concentrations measured in these tests ranged from 80% to 110% of the target concentration for the room temperature test and 88% to 114% for the refrigerated test. An additional concentration of a 5 mg/L stock standard was also run and was initiated on 5/26/04. 3 samplings were done over the 29 day test. Average recoveries ranged from 92% to 173% of the target concentration. This 5 mg/L test was run to evaluate a theory that indications of instability were caused by precipitation of the T-4 to the walls of the container resulting from the hardness of Battelle's well water. The 171% recovery is likely due to the inclusion of an outlying result. Eliminating this outlier reduces the 171% recovery to 95% and the overall range of this particular study to 91% to 106%.

7.2.2 Diluter Stability

Stability testing for the 1 ug/L test was initiated on 05/4/04. This 1st sampling showed low recovery and was due to a mismatch between the mobile phase and the sample. While the diluter sample was made on 5/4/04 a true initiation date would be 5/6/04 when the test was rerun with the sample constitution matching the mobile phase. Chemical concentrations were determined 5 times over the 49 days. Average concentrations measured in these tests ranged from 101.7% to 0.0% of the target concentration. An additional concentration of a 4 ug/L diluter stability was also run and was initiated on 5/11/04. Four samplings were done over the 44 day test. Average recoveries ranged from 113% at day one of the study to 40% of the target concentration at day 44. This 4 ug/L test was run due to the concern that the 1 ug/L test was at or near the detection limit of the method.

7.2.3 Quality Control Samples

The analytical and quality control (QC) results are presented in Appendix C. Three to 6 point calibration curves all met the R^2 criteria of ≥0.995, see table 4. Blanks and spikes were analyzed with most batches for QC purposes. All blanks were less than 3 times the detection limit of 0.09 µg/L of L-Thyroxine. Spikes were generally within the acceptance criteria of +/- 25% with the exception of one at 67%, this analysis had the retention time shift due to column or flow problems. ICV/CCV results were generally within the acceptance criteria and eliminating 3 of the outliers of 1.94%, 279% and 144%, the recoveries ranged from 88% to 109%. The recovery of 1.94% is attributed to a mistake in the preparation of that standard and the recovery of the 279% is attributed to a 144% retention time shift possibly caused by column or flow problems. The QA/QC samples outside the acceptance criteria have a variety of reasons for exceeding the criteria, one is that occasionally the retention time would shift, causing poor performance. Also,

[3] Copies of service reports and maintenance logs are attached at the end of this document as Appendix D.

integration was difficult at times due to noise, caused by the machine behaving erratically. Acceptance of a few outliers was considered the best choice to continue the stability study. The MDL was determined to be 0.09 µg/L.

Table 4. Calibration Acceptance

Calibration Date	R2 Value (≥ .995)	Curve Fit	# Standards Included
4/23/04	0.997391	Linear	6
5/4/04	0.998817	Linear	6
5/6/04	0.999987	Linear	3
5/11/04	0.997820	Linear	5
5/14/04	0.999510	Linear	6
5/26/04	0.999579	Linear	6
6/11/04	0.996330	Linear	3
6/24/04	0.999919	Linear	5

8.0 CONCLUSIONS

Initial stability analyses indicated that the T-4 was not stable for the 21 day period for both the stock solution and the diluter solution. With further analyses and taking into account the problems encountered with the equipment, data suggests the 4 mg/L stock solution is actually stable for the course of the stability study and possibly beyond. The 1 ug/L stability data was extremely noisy, most likely due to marginally performing equipment and the proximity of the concentration to the limits of detection. While the data is variable, it can be interpreted to show a degradation of the 1 ug/L diluter solution. A data point at 51 days shows a recovery of this 1 ug/L solution at 242%, but this is probably an anomaly especially when the 4 ug/L data is analyzed. Both show a downward trend.

It is concluded that the L-Thyroxine stock solution of 4 mg/L remained stable for the duration of the study based upon analysis of the results. The stability trend for the diluter samplings showed the L-Thyroxine to be stable only for the initial 7 days of the study, assuming the 1st data point is inaccurate. Stability of the 4 ug/L stock solution of L-Thyroxine remained within 90% of the day zero concentration on day three of the study. Though the diluted concentration dropped to 80% of the target value by day seven in the 1 ug/L study and day 3 of the 4 ug/L study, it was decided to proceed with the in-life portion of the study with the caveat that a new stock solution would be prepared every 5 days over the duration of the in-life studies, and samples would be collected immediately and shipped via overnight courier to the lab in Sequim for analysis. All analyses were intended to be carried out within 24 hours of sample receipt.

Several anomalous QA results and stability recoveries point to difficulties during the analysis of Thyroxine rather than instability of the chemical. A comparison between results of the 4 mg/L stability stored at room temperature with results from the same standard refrigerated at 2-8° C, demonstrates little effect of refrigeration upon stability of Thyroxine. This result suggests that L-Thyroxine is equally stable when stored at 2-8° C. or at room temperature.

This study was conducted under GLP regulations, but due to instrument and chromatography problems and several procedural anomalies, certain aspects of the study justify the filing of deviations. Appendix D is a list indicating the specific deviations that occurred during the study.

APPENDIX A

MANUFACTURER'S CERTIFICATE OF ANALYSIS/PURITY

SIGMA-ALDRICH

Certificate of Analysis

Product Name	L-Thyroxine
Product Number	T2376
Product Brand	SIGMA
CAS Number	51-48-9
Molecular Formula	$C_{15}H_{11}I_4NO_4$
Molecular Weight	776.87

TEST	SPECIFICATION	LOT 092K1061 RESULTS
APPEARANCE	WHITE TO LIGHT YELLOW WITH A TAN CAST POWDER	LIGHT YELLOW POWDER
SOLUBILITY	CLEAR TO SLIGHTLY HAZY YELLOW SOLUTION AT 50MG/ML IN 4M AMMONIUM HYDROXIDE IN METHANOL	SLIGHTLY HAZY DARK YELLOW
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONFORMS
CARBON	22.7 TO 23.7%	23.1%
NITROGEN	1.5 TO 2.1%	1.8%
SPECIFIC ROTATION	CONSISTENT WITH OPTICAL ISOMER	+20.0 DEG (C = 1 IN ETHANOL:1 M HYDRO- CHLORIC ACID (2:1) AT 22 DEG CENTIGRADE)
PURITY BY HPLC	NLT 98%	99.4%
SHELF LIFE SOP QC-12-006	3 YEARS	SEPTEMBER 2005
QC ACCEPTANCE DATE		SEPTEMBER 2002
PRODUCT CROSS REFERENCE INFORMATION		REPLACEMENT FOR ALDRICH #234702

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

APPENDIX B

PURITY AND STABILITY TESTING PROTOCOL

Study Protocol:
Purity and Stability of Test Substances for Work Assignment 3-11
EDSP Study Number: EDSP.311-01

Study Objective: •

To verify the purity of 6 test substances (listed in Table 1), the solubility, (at the concentrations specified in Table 2), and to determine the stability of same dissolved in well water over a 21 day period. This study is in support of EPA contract number 68-W-01-023, MSL Work Assignment Number 3-11, Task 3, Amphibian Metamorphosis Assay Demonstration Study.

Address of Testing Facility and Sponsor's Representative:

Battelle – Marine Science Laboratory
1529 West Sequim Bay Road
Sequim, Washington

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propyl-2-thiouracil	PTU
L-Thyroxine	T4
Phenobarbital	ΦBarb
Pregnenolone-16-Alpha-carbonitrile	P16αc
Dexamethosone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	SOP MSL-O-	TBA	TBA	TBA	TBA	TBA

TABLE 3
Reference Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	TCI America	Fluka	TBA	TBA	TBA	Aldrich
CAS #	51-52-5	51-48-9	TBA	TBA	TBA	60-56-0
Lot #	0630144	411874/1	TBA	TBA	TBA	06109DP
Purity req.	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%
Mfg. Purity claim	> 99%	99.6%	TBA	TBA	TBA	99.7%

Proposed experimental start and termination dates:

Start Date – November 1, 2003
Termination Date – June 22, 2004

Experimental Design:

- Solubility of the substances will be determined as per SOP EDSP.C-0XX-01 (EDSP Chemical Characterization-TBA) in the diluent (well water) at a concentration approximately 10% above the stock solution concentration (see Table 2).
- If the solubility of the test substance is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP EDSP.C-0XX-01. Note: exposure concentrations won't change, so stability of an exposure solution will be carried out at the target concentration.
- Substance purity will be verified using HPLC, GC/MS or IC/ICP/MS as appropriate, and should be 98% or greater and be within ±2% of the value provided on the Certificate of Analysis by the manufacturer
- Stock solution – made up to concentrations delineated in Table 2 (approximate levels) in well water (obtained from MSL).
- Stability test solutions – Stability testing will be carried out on stock solutions and at a second level near or within the in-life exposure dose range. Nominal concentrations to be tested are delineated in Table 2 in well water (obtained from MSL).
- Storage and Labeling Requirements of Test Solution – stored in the dark (i.e., same storage conditions of solutions employed in the in-life studies of WA 3-11) at room temperature (20-25˚C). Minimally, containers will be labeled with the name of the test substance, the date of preparation, the solution concentration, and the study number.
- Test Schedule – Samples will be analyzed the day of collection from the test solution
- Replicates – 3 per sample tested at each analysis time point
- Sampling Timing – Sampling will be carried out on the first day and then as appropriate for statistical analysis for duration of the 21 day study
- For details of the analytical methods see the appropriate SOP associated with the analytical method employed for the specific substance: MSL-O-018-00 (HPLC), MSL-X-XXX-XX (GC/MS)-TBA, MSL-X-XXX-XX (ICP/MS)-TBA.

Statistical Analysis:

Log linear degradation curves will be fit to the stability data to describe the trend of the chemical concentration over time. Lack of fit and residual plots will be evaluated to determine the form of

the regression. Power calculations based on the observed variability will be used to determine the sensitivity of the test to detect degraded concentrations.

Regulatory requirements:

This study will be conducted in compliance with EPA FIFRA Good Laboratory Practices (40CFR, Part 160).

Records to be maintained:

All records and the final report generated as a result of this study will be transported to and maintained in the EDSP Data Coordination Center for archival purposes at the following address:

EDSP Data Coordination Center
Battelle Columbus Operations (PNL)
505 King Ave
Columbus, OH 43201

Sponsor Representative_____ _____
Michael Blanton **Date**

Study Director_____ _____
Michael Cobb **Date**

PROTOCOL AMENDMENT
STUDY NUMBER: EDSP.311-01
AMENDMENT NUMBER: A-1
DATE: FEBRUARY 4, 2004
Page 1 of 2

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01			DATE: February 4, 2004		
AMENDMENT NUMBER: A-1			WAL/STUDY DIRECTOR:		
NOTEBOOK NUMBER: N/A			Michael Blanton/Michael Cobb		
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

1. Solubility of the substances will be determined as per SOP EDSP.C-0XX-01 (EDSP Chemical Characterization-TBA)…
   If the solubility of the test substance is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP EDSP.C-0XX-01.
2. For details of the analytical methods see the appropriate SOP associated with the analytical method employed for the specific substance: MSL-O-018-00 (HPLC), MSL-X-XXX-XX (GC/MS)-TBA, MSL-X-XXX-XX (ICP/MS)-TBA.
3. All records and the final report generated as a result of this study will be transported to and maintained in the EDSP Data Coordination Center for archival purposes at the following address:
   EDSP Data Coordination Center
   Battelle Columbus Operations (PNL)
   505 King Ave
   Columbus, OH 43201

4. Sponsor Representative_____ _____
   Michael Blanton Date

AMENDMENT:

1. Sentences amended to read: “Solubility of the substances will be determined as per SOP MSL-R-009 (Chemical Characterization)”…
   “If the solubility of the test substances is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP MSL-R-009.”
   Last several SOPs identified in #2 below.
   Sentence amended to read: “For details of the analytical methods, see the method identified in Table 2 and provided in each specific chemical characterization notebook. These methods are supported by specific operation and maintenance SOPs appropriate to the analytical method employed for the particular substance under study: MSL-R-012 (Operation and Maintenance of HPLC Systems), MSL-O-008-02 (Operation and Maintenance of Gas Chromatographs (GC) and Gas Chromatograph/Mass
2. Spectrometer (GC/MS) Systems), MSL-R-014 (Operation & Maintenance of IC ICP/MS Systems – currently being drafted).

PROTOCOL AMENDMENT
STUDY NUMBER: EDSP.311-01
AMENDMENT NUMBER: A-1
DATE: FEBRUARY 4, 2004
Page 2 of 2

3. Sentence amended to read: "All records and the final report generated as a result of this study will be maintained and archived per SOP MSL-D-003 (Archiving of Records, Data and Retired SOPs). In addition, the documentation will be transported in electronic form to the EDSP Data Coordination Center for archival purposes at the following address:

   EDSP Data Coordination Center
   Battelle Columbus Operations
   505 King Ave
   Columbus, OH 43201

4. Sponsor Representative_____ _____
   David Houchens Date

REASON FOR CHANGE:

1. The SOP numbers were not available (in the "Experimental Design" section) when the protocol was written. These SOP numbers are now available and are provided in this amendment.
2. Our new documentation approach involves generation of a specific "Analytical Method" document developed for the chemical characterization and provided in the final report. Each specific "Method" will correspond to a generic SOP.
3. Original document section in "Records to be maintained" section requires clarification. Battelle Columbus Operations incorrectly labeled as PNL.
4. Correct Sponsor Representative is EDSP Program Manager.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01				DATE: February 24, 2004	
AMENDMENT NUMBER: A-2				WAL/STUDY DIRECTOR:	
NOTEBOOK NUMBER: N/A				Michael Blanton/Michael Cobb	
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

Table entries that are earmarked for change in this amendment are highlighted in **Yellow**

TABLE 2
Test Substance Specifications:

Chemical Name	**PTU**	**T4**	**ΦBarb**	**P16αc**	**DxM**	**Mtmz**
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	**SOP MSL-O-018-00 (HPLC)**	**TBA**	**TBA**	**TBA**	**TBA**	**TBA**

AMENDMENT:
New table entries are highlighted in **Orange**

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

REASON FOR CHANGE:

Information was not available when protocol was originally generated.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01				DATE: March 30, 2004	
AMENDMENT NUMBER: A-3				WAL/STUDY DIRECTOR:	
NOTEBOOK NUMBER: N/A				Michael Blanton/Michael Cobb	
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

Table entries that are earmarked for change in this amendment are presented underlined and italicized in ***Georgia Font***

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propyl-***2***-thiouracil	PTU
L-Thyroxine	T4
Phenobarbital	ΦBarb
Pregnenolone-16-Alpha-carbonitrile	P16αc
Dexameth***o***sone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	***0,*** 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50***,*** ***100*** mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

TBA = To Be Amended

AMENDMENT:

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propylthiouracil	PTU
L-Thyroxine	T4
Phenobarbital	ΦBarb
Pregnenolone-16-Alpha-carbonitrile	P16αc
Dexamethasone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

REASON FOR CHANGE:

Information was not available when protocol was originally generated.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01			DATE: August 3, 2004		
AMENDMENT NUMBER: A-4			WAL/STUDY DIRECTOR:		
NOTEBOOK NUMBER: N/A			Michael Blanton/Michael Cobb		
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

Table entries that are earmarked for change in this amendment are presented as bold, underlined, and italicized in ***Georgia Font***

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	***TBA***	TBA	***TBA***	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	***0630144***	092K1061	***TBA***	TBA	***TBA***	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	***TBA***	TBA	***TBA***	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	***TBA***	TBA	***TBA***	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	***TBA***	TBA	***TBA***	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	***TBA***	TBA	***TBA***	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	*0,* 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	***TBA***	TBA	***TBA***	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	**EDSP.H4-001-00**	**EDSP.I8-003-00**	**EDSP.H4-004-00**	**EDSP.H4-005-00**	**EDSP.H4-006-00**	**EDSP.H4-002-00**

TABLE 3
Reference Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	TCI America	Fluka	***TBA***	TBA	***TBA***	Aldrich
CAS #	51-52-5	51-48-9	***TBA***	TBA	***TBA***	60-56-0
Lot #	***0630144***	411874/1	***TBA***	TBA	***TBA***	06109DP
Purity req.	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%
Mfg. Purity claim	***> 99%***	99.6%	***TBA***	TBA	***TBA***	99.7%

AMENDMENT:

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	Sigma	TBA	Biomol Intl.	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	063K0144	092K1061	081K2620	TBA	P4311	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	≥ 99%	TBA	99%	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	≥ 8000 mg/L (pH adjusted)	TBA	≥ 10.0 mg/L	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	8000 mg/L	TBA	10.0 mg/L	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	8000 mg/L & 125 mg/L	TBA	10.0 mg/L & 2.0 µg/L	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	1500,1000, 500, 250,125 mg/L	TBA	1.0, 3.9, 15.6 62.5, 250.0 µg/L	6.25, 12.5, 25, 50 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

TABLE 3
Reference Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	TCI America	Fluka	Sigma	TBA	Fluka	Aldrich
CAS #	51-52-5	51-48-9	50-06-6	TBA	50-02-2	60-56-0
Lot #	GF01	411874/1	62K8804	TBA	41405/1	06109DP
Purity req.	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%
Mfg. Purity claim	99.6%	99.6%	≥ 98%	TBA	99.5%	99.7%

REASON FOR CHANGE:

For all changes to Tables 2 and 3 for Phenobarbital and Dexamethasone, the information was not available (TBA) when protocol was originally generated.

The lot number and purity changes in Tables 2 and 3 for 6-n-Propylthiouracil were due to transcription errors in putting the original protocol document together. This error was documented on an EDSP Deviation form – deviation number EDSP.311-01-D2.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01				DATE: September 27, 2004	
AMENDMENT NUMBER: A-5				WAL/STUDY DIRECTOR:	
NOTEBOOK NUMBER: N/A				Michael Blanton/Michael Cobb	
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:
1. Address of Testing Facility and Sponsor's Representative:

Battelle – Marine Science Laboratory
1529 West Sequim Bay Road
Sequim, Washington

2. Table entries that are earmarked for change in this amendment are presented as bold, underlined, and italicized in ***Georgia Font***

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	**PTU**	**T4**	**ΦBarb**	**P16αc**	**DxM**	**Mtmz**
Manufacturer	Sigma	Sigma	Sigma	***TBA***	Biomol Intl.	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	063K0144	092K1061	081K2620	***TBA***	P4311	073K3519
Purity req.	***≥ 97%***	***≥ 97%***	***≥ 97%***	***≥ 97%***	***≥ 97%***	***≥ 97%***
Mfg. Purity Claim	100%	99.4%	≥ 99%	***TBA***	99%	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	≥ 8000 mg/L (pH adjusted)	***TBA***	≥ 10.0 mg/L	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	8000 mg/L	***TBA***	10.0 mg/L	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	8000 mg/L & 125 mg/L	***TBA***	10.0 mg/L & 2.0 µg/L	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	1500,1000, 500, 250,125 mg/L	***TBA***	1.0, 3.9, 15.6 62.5, 250.0 µg/L	6.25, 12.5, 25, 50 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	**EDSP.H4-001-00**	**EDSP.I8-003-00**	**EDSP.H4-004-00**	**EDSP.H4-005-00**	**EDSP.H4-006-00**	**EDSP.H4-002-00**

TABLE 3
Reference Substance Specifications:

Chemical Name	**PTU**	**T4**	**ΦBarb**	**P16αc**	**DxM**	**Mtmz**
Manufacturer	TCI America	Fluka	Sigma	***TBA***	Fluka	Aldrich
CAS #	51-52-5	51-48-9	50-06-6	***TBA***	50-02-2	60-56-0
Lot #	GF01	411874/1	62K8804	***TBA***	41405/1	06109DP
Purity req.	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%
Mfg. Purity claim	99.6%	99.6%	≥ 98%	***TBA***	99.5%	99.7%

AMENDMENT:

1. Address of Testing Facility and Sponsor's Representative
ADD:
For the analysis of Pregnenolone-16-Alpha-carbonitrile, all analyses for solubility, purity, stability, and In-Life testing will be carried out under the supervision and in the labs of Steve Graves at:
Battelle Columbus
Chemistry Technical Center
505 King Avenue
Columbus, Ohio
43201-2693

2.

TABLE 2

TBA = To Be Amended

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	Sigma	Biomol	Biomol Intl.	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	063K0144	092K1061	081K2620	P5507	P4311	073K3519
Purity req.	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%
Mfg. Purity Claim	100%	99.4%	≥ 99%	99%	99%	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	≥ 8000 mg/L (pH adjusted)	≥220 µg/L	≥ 10.0 mg/L	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	8000 mg/L	200 µg/L	10.0 mg/L	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	8000 mg/L & 125 mg/L	7.4 µg/L & 200 µg/L	10.0 mg/L & 2.0 µg/L	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	1500,1000, 500, 250,125 mg/L	7.4, 22,2, 66,7, 200 µg/L	1.0, 3.9, 15.6 62.5, 250.0 µg/L	6.25, 12.5, 25, 50 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

TABLE 3
Reference Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	TCI America	Fluka	Sigma	Sigma	Fluka	Aldrich
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	GF01	411874/1	62K8804	023K0824	41405/1	06109DP
Purity req.	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%
Mfg. Purity claim	99.6%	99.6%	≥ 98%	99%	99.5%	99.7%

REASON FOR CHANGE:

1. The Chemical Repository in Sequim does not have the appropriate analytical instrumentation to run the analysis of P16ac, therefore the task was transferred to the backup lab for the Repository at Battelle, Columbus.

2. For all changes to Tables 2 and 3 for Pregnenolone-16-alpha-carbonitrile, the information was not available (TBA) when protocol was originally generated.

The purity requirement changes in Tables 2 for all six compounds was due to a transcription error in putting the original protocol document together. The text in the protocol specified a minimum purity of 98% but it was documented as 97% in the table. All compounds used in testing were ≥ 98% in purity.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

NOTE: A signed copy of this protocol and all relevant Amendments are available in the files of the Chemical Repository Study Director in MSL 5.

Protocol Deviations

Deviation EDSP.311-01-D4 was implemented to address the change from the statistical evaluation of stability using Log linear degradation curves to comparing the percent deviation of stability test samples (mean values) from the mean day zero results.

A number of protocol deviations that occurred during this study are being drafted to include in the final signed version of this document.

APPENDIX C

ANALYTICAL RESULTS OF STABILITY TESTING

Table C1. L-Thyroxine concentration in Well Water 4 mg/L Room Temperature Stock

Target Conc. (mg/L)	Sample ID	Date	L-Thyroxine (mg/L)	Average (mg/L)	Recovery	RSD
3.946	4ppm t4 rmtempr1	5/4/04	3.7040			
3.946	4ppm t4 rmtempr2	5/4/04	3.8000	4.2180	106.89%	19.17%
3.946	4ppm t4 rmtempr3	5/4/04	5.1500			
3.946	4ppm t4 rmtempr1	5/11/04	4.3177			
3.946	4ppm t4 rmtempr2	5/11/04	4.2830	4.3238	109.57%	1.02%
3.946	4ppm t4 rmtempr3	5/11/04	4.3707			
3.946	4ppm t4 rmtempr1	5/14/04	3.5021			
3.946	4ppm t4 rmtempr2	5/14/04	3.6510	3.5731	90.55%	2.09%
3.946	4ppm t4 rmtempr3	5/14/04	3.5661			
3.946	4ppm t4 rmtempr1	5/26-27/04	3.2335			
3.946	4ppm t4 rmtempr2	5/26-27/04	3.1243	3.1575	80.02%	2.09%
3.946	4ppm t4 rmtempr3	5/26-27/04	3.1148			
3.946	4ppm t4 rmtempr1	6/11/04	3.6812			
3.946	4ppm t4 rmtempr2	6/11/04	3.8705	3.7519	95.08%	2.75%
3.946	4ppm t4 rmtempr3	6/11/04	3.7040			
3.946	4ppm rm temp t4 X500r1	6/24/04	4.0063			
3.946	4ppm rm temp t4 X500r2	6/24/04	4.1531	4.0899	103.65%	1.85%
3.946	4ppm rm temp t4 X500r3	6/24/04	4.1103			

Table C2. L-Thyroxine concentration in Well Water 4 mg/L Refrigerated Stock

Target Conc. (mg/L)	Sample ID	Date	L-Thyroxine (mg/L)	Average (mg/L)	Recovery	RSD
3.946	4ppm t4 refrig r1	5/4/04	3.5370			
3.946	4ppm t4 refrig r2	5/4/04	3.9090	3.6627	92.82%	5.82%
3.946	4ppm t4 refrig r3	5/4/04	3.5420			
3.946	4ppm t4 refrig r1	5/11004	4.3084			
3.946	4ppm t4 refrig r2	5/11/04	4.2782	4.4866	113.70%	7.47%
3.946	4ppm t4 refrig r3	5/11/04	4.8732			
3.946	4ppm t4 refrig r1	5/14/04	3.3835			
3.946	4ppm t4 refrig r2	5/14/04	3.5078	3.4659	87.83%	2.06%
3.946	4ppm t4 refrig r3	5/14/04	3.5065			
3.946	4ppm t4 refrig r1	5/26-27/04	3.3143			
3.946	4ppm t4 refrig r2	5/26-27/04	3.0996	4.3297	109.72%	44.98%
3.946	4ppm t4 refrig r3	5/26-27/04	6.5751*			
3.946	4ppm t4 refrig r1	6/11/04	4.0100			
3.946	4ppm t4 refrig r2	6/11/04	3.9874	3.9987	101.33%	0.40%

*Retention time shift

Table C3. L-Thyroxine concentration in Well Water 5 mg/L Stock

Target Conc. (mg/L)	Sample ID	Date	L-Thyroxine (mg/L)	Average (mg/L)	Recovery	RSD
4.943	5ppm t4 r1	5/26-27/04	4.6043			
4.943	5ppm t4 r2	5/26-27/04	4.5621	4.5342	91.73%	1.93%
4.943	5ppm t4 r3	5/26-27/04	4.4363			
4.943	5ppm t4 r1	6/11/04	4.7607			
4.943	5ppm t4 r2	6/11/04	12.3439*	8.5523	173.02%	62.70%
4.943	5ppm t4 r1	6/24/04	5.3563			
4.943	5ppm t4 r2	6/24/04	5.2043	5.2848	106.91%	1.45%
4.943	5ppm t4 r3	6/24/04	5.2939			

*Retention time shift

Table C4. L-Thyroxine concentration in Well Water 1 ug/L diluter

Target Conc. (ug/L)	Sample ID	Date	L-Thyroxine (ug/L)	Average (ug/L)	Recovery	RSD
0.9865	1ppb t4 r1*	5/4/04	0.2500			
0.9865	1ppb t4 r2*	5/4/04	0.1300	0.1700	17.23%	40.75%
0.9865	1ppb t4 r3*	5/4/04	0.1300			
0.9865	1ppb t4 r1	5/6/04	1.1333			
0.9865	1ppb t4 r2	5/6/04	0.9111	0.9926	100.62%	12.33%
0.9865	1ppb t4 r3	5/6/04	0.9333			
0.9865	1ppb t4 r1	5/11/04	0.9798			
0.9865	1ppb t4 r2	5/11/04	0.9240	1.0033	101.70%	9.29%
0.9865	1ppb t4 r3	5/11/04	1.1060			
0.9865	1ppb t4 r1	5/14/04	0.3313			
0.9865	1ppb t4 r2	5/14/04	0.2749	0.2813	28.52%	16.75%
0.9865	1ppb t4 r3	5/14/04	0.2378			
0.9865	1ppb t4 r1	5/26-27/04	0.0847			
0.9865	1ppb t4 r2	5/26-27/04	0.0153	0.0469	4.75%	74.82%
0.9865	1ppb t4 r3	5/26-27/04	0.0407			
0.9865	1ppb t4 r1	6/24/04	0.0000			
0.9865	1ppb t4 r2	6/24/04	0.0000	0.0000	0.0%	--------
0.9865	1ppb t4 r3	6/24/04	0.0000			

*Preparation dilution error, restarted on 5/6

Table C5. L-Thyroxine concentration in Well Water 4 ug/L diluter

Target Conc. (ug/L)	Sample ID	Date	L-Thyroxine (ug/L)	Average (ug/L)	Recovery	RSD
3.946	4 ppb r1	5/11/04	4.8284			
3.946	4 ppb r2	5/11/04	4.3133	4.4566	112.94%	7.29%
3.946	4 ppb r3	5/11/04	4.2280			
3.946	4 ppb r1	5/14/04	3.8096			
3.946	4 ppb r2	5/14/04	3.6487	3.6417	92.29%	4.71%
3.946	4 ppb r3	5/14/04	3.4669			
3.946	4 ppb r1	5/26-27/04	2.9018			
3.946	4 ppb r2	5/26-27/04	2.9496	3.0462	77.20%	6.90%
3.946	4 ppb r3	5/26-27/04	3.2873			
3.946	4 ppb r1	6/24/04	1.5684			
3.946	4 ppb r2	6/24/04	1.5024	1.5785	40.00%	5.17%
3.946	4 ppb r3	6/24/04	1.6647			

Table C6. ICV/CCV Data for L-Thyroxine in Well Water

Sample Name	Date	Expected L-Thyroxine (µg/L)	Measured L-Thyroxine (µg/L)	Recovery
icv 10.9ppb	5/4/04	10.9	10.2900	94.40%
ccv 11ppb t4	5/4/04	11	9.7500	88.64%
ccv 11ppb t4	5/4/04	11	10.1720	92.47%
icv 10.9ppb t4	5/6/04	10.9	11.8900	109.08%
ccv 11ppb t4	5/6/04	11	11.4000	103.64%
ccv 11ppb t4	5/6/04	11	11.6400	105.82%
icv 10.9ppb	5/11/04	10.9	0.2118	1.94%
ccv 11ppb t4	5/11/04	11	10.3308	93.92%
ccv 11ppb t4	5/11/04	11	11.3899	103.54%
ccv 11ppb t4	5/11/04	11	11.8461	107.69%
icv 10.9ppb	5/14/04	10.9	11.5427	105.90%
ccv 11ppb t4	5/14/04	11	10.6945	97.22%
ccv 11ppb t4	5/14/04	11	10.0059	90.96%
ccv 11ppb t4	5/14/04	11	9.8002	89.09%
icv 10.9ppb	5/26-27/04	10.9	10.0036	91.78%
ccv 11ppb t4	5/26-27/04	11	10.3324	93.93%
ccv 11ppb t4	5/26-27/04	11	9.9016	90.01%
ccv 11ppb t4	5/27/04	11	15.8907	144.46%*
ICV 10.9ppb	6/11/04	10.9	30.4172	279.06%*
ccv 10ppb t4	6/11/04	10	9.7765	97.77%
ccv 10ppb t4	6/11/04	10	10.6651	106.65%
10ppb ccv	6/11/04	10	10.8216	108.22%
ccv 10ppb t4	6/11/04	10	10.0348	100.35%
ICV 10.9ppb	6/24/04	10.9	10.0017	91.76%
ccv 10.1ppb t4	6/24/04	10.1	9.3684	92.76%
ccv 10.1ppb	6/24/04	10.1	10.1300	100.30%
ccv 10.1ppb	6/24/04	10.1	9.9507	98.52%

*Retention time shift

Table C7. Spike Recovery Data for L-Thyroxine in Well Water

Target Conc. (µg/L)	Sample ID	Date	L-Thyroxine (µg/L)	Recovery
1.09	1.09ppb bs	5/4/04	1.0250	94.04%
54.5	54.5ppb bs	5/4/04	53.5800	98.31%
1.09	1.09ppb bs	5/11/04	1.3167	120.80%
54.5	54.5ppb bs	5/11/04	67.6742	124.17%
1.09	1.09ppb bs	5/14/04	0.9188	84.29%
54.5	54.5ppb bs	5/14/04	47.2872	86.77%
1.09	1.09ppb bs	5/26-27/04	1.1245	103.17%
54.5	54.5ppb bs	5/26-27/04	46.3985	85.13%
0.545	0.545ppb from pp1279	5/27/04	0.4960	91.01%
0.545	0.55ppb from pp1269	5/27/04	0.4308	79.05%
5.4500	5.45ppb from pp1279	5/27/04	4.1863	76.81%
5.5	5.5ppb from pp1269	5/27/04	3.6921	67.13%
5	5.0ppb from 4-20-04 100ppm t4 stk	5/27/04	4.1281	82.56%
5.5	5.5ppb from 4-40-04 100ppm t4 stk	5/27/04	4.4967	81.76%
1.09	1.09ppb t4 bs	6/11/04	0.8731	80.10%
54.5	54.5ppb t4 bs	6/11/04	53.7507	98.63%*
1.09	1.09ppb t4 bs	6/23/04	1.0400	95.41%
1.09	1.09ppb t4 bs	6/23/04	0.9228	84.66%
1.09	1.09ppb t4 bs	6/24/04	1.2476	114.46%
1.09	1.09ppb t4 bs	6/24/04	1.1411	104.69%

*above range of standard curve – extrapolated value

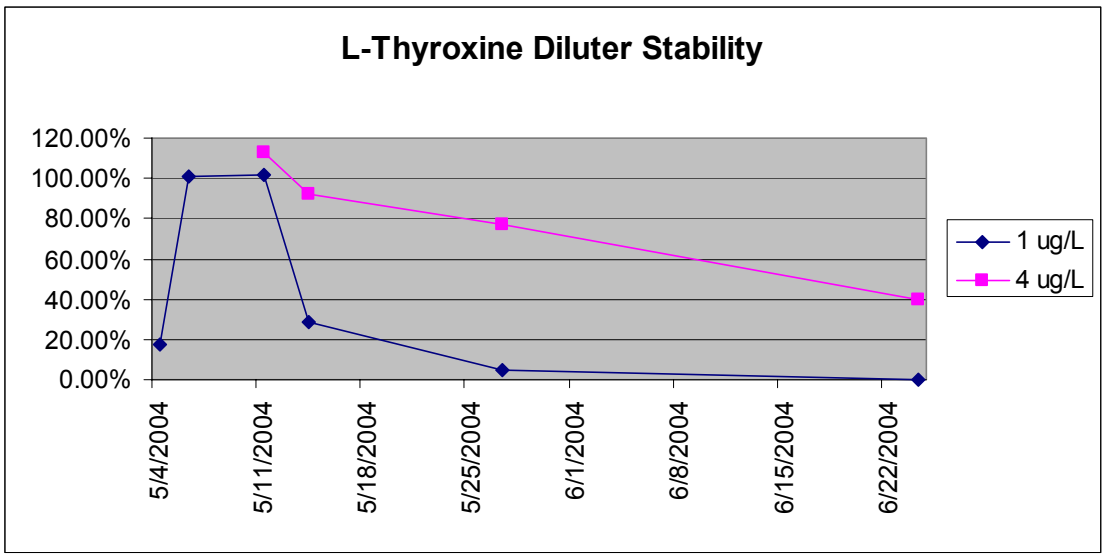


Figure 2. Observed concentration of L-Thyroxine with a target concentration of 1 ug/L and 4 ug/L plotted against time

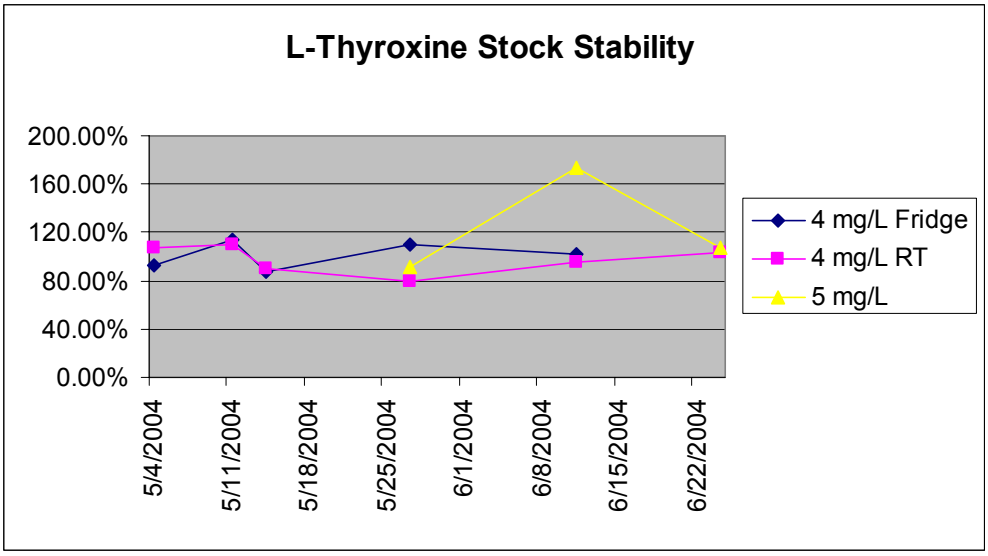


Figure 3. Observed concentration of L-Thyroxine with a target concentration of 4 mg/L and 5 mg/L plotted against time

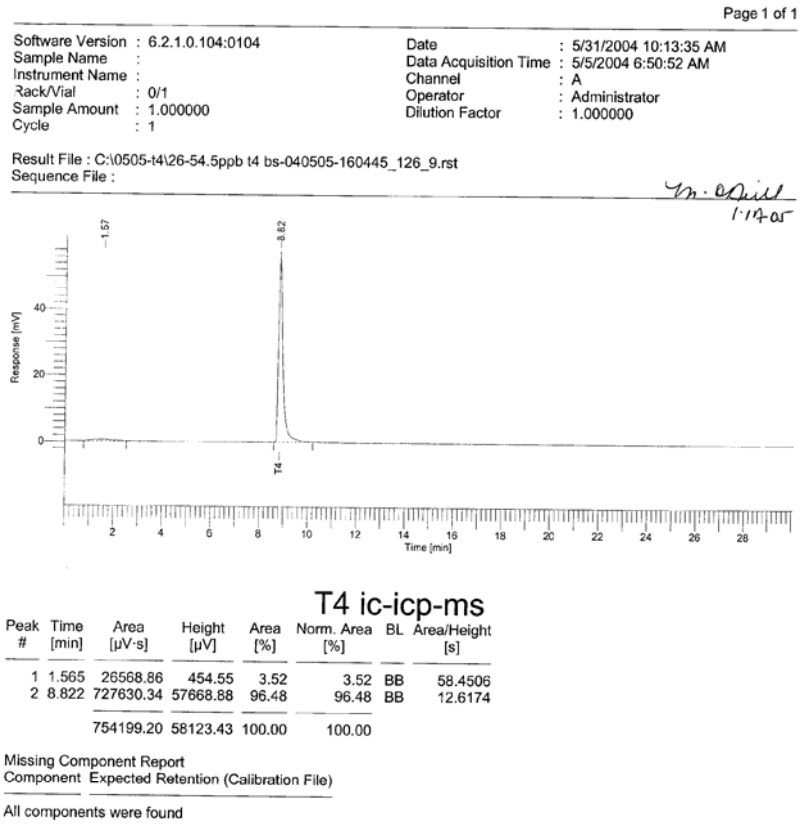


Figure 4. Typical Chromatogram, 54.5 ug/L L-Thyroxine

L-Thyroxine (T4)
Test Chemical Analytical Data from Battelle:
In-Life Test Chemical Analysis Report

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Nolf
P.O. #:	

Lab Use Only	
Client	BAT102
	BAT102-00062

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only		
002	DeCl$_2$ Tap H$_2$O	6/10/04 1600	1	6	Blue Ice	L-Thyroxine (T-4)			
003	2.0 mg/L L-Thyroxine (T-4)	↓	1	↓	↓	↓			
004	4.0 mg/L L-Thyroxine (T-4)		1						
005	4.04 mg/L L-Thyroxine (T-4)		2						

DATE:
INITIALS:

SPECIAL INSTRUCTIONS:
Please send back ice chest

Sampled by (print name)	Date/Time	Received by (print name)	Date/Time
Andrea Nolf	6/10/04 ~~1600~~		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

① Mistake. Should be 1700-AN-6/10/04

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andria Nall / [illegible]
P.O. #:	

BATT02-00062

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	pH	Temp	Cl2
006	4.04 mg/L L-Thyroxine (T-4)	6/15/04 1600	2	G	Blue ice	L-Thyroxine (T-4)			
007	Dech Tap H_2O	6/17/04 1600	1	↓	↓	↓			
008	2.0 mg/L L-Thyroxine (T-4)	↓ ↓	1	↓	↓	↓			
009	4.0 mg/L L-Thyroxine (T-4)	↓ ↓	1	↓	↓	↓			

SPECIAL INSTRUCTIONS:

DATE:
INITIALS:

Relinquished By	Date/Time	Received By	Date/Time
Andria Nall	6/17/04 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client: Battelle
Submit Report To: Doug Fort
Sampler's Signature: Andra Noll
P.O. #:

BATT02 - 00042

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	ID#	Temp	pH
010	DeCl$_2$ Tap H$_2$O	6/24/04 1600	1	G	Blue Ice	L-Thyroxine (T-4)			
011	2.0 mg/L L-Thyroxine (T-4)	↓	1	↓	↓	↓			
012	4.0 mg/L L-Thyroxine (T-4)		2						
013	4.04 mg/L L-Thyroxine (T-4)		1						
014	4.04 mg/L L-Thyroxine (T-4)	6/21/04 1000	1	G					

SPECIAL INSTRUCTIONS:
Please send back ice chest

DATE:
INITIALS:

Sampled/Relinquished By	Date/Time	Received By	Date/Time
Andra Noll	6/24/04 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Noll / [illegible]
P.O. #:	

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road

Stillwater, OK 74074

405-624-6771 Fax 405-377-3388

mail@fortlabs.com

Lab Use Only

Lab Project # / WO #

BATT02-00062

Lab Use Only

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Temp	pH
015	DeCl$_2$ Tap H_2O	7/1/04 1600	1	G	Blue Ice	L-Thyroxine		
016	2.0 mg/L L-Thyroxine (T-4)	↓ ↓	1	↓	↓	↓		
017	4.0 mg/L L-Thyroxine (T-4)	↓ ↓	1	↓	↓	↓		
018	4.04 mg/L L-Thyroxine (T-4)	6/28/04 1600	1	↓	↓	↓		

SPECIAL INSTRUCTIONS:

Please send back ice chest

DATE:

INITIALS:

Printed Name	Date	Time	Signature
Andrea Noll	7/1/04	1700	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

L-Thyroxine (T4)

Thyroid Histology Data from EPL:

Sample Identification Key
Thyroid Pathology Report

EPL Sample Key for L-Thyroxine (T4)

FEL Sample Number	Individual Specimen Number[1]	Conc. Replicate Number	Initial Specimen Stage	Chemical Conc. (μg/L)
019	008, 012, 015, 017, 019 022, 024, 027, 029, 031	A B	51	0
020	041, 046, 050, 053, 058 064, 067, 070, 073, 077	A B	51	0.5
021	082, 086, 089, 092, 098 102, 105, 111, 114, 117	A B	51	1.0
022	124, 128, 133, 136, 139 145, 149, 151, 154, 157	A B	51	2.0
	No specimens sent[2]	A B	51	4.0
023	175, 177, 183, 188, 190 194, 195, 206, 208, 209	C D	54	0
024	213, 218, 225, 227, 229 237, 239, 242, 244, 245	C D	54	0.5
025	253, 255, 258, 264, 266 271, 274, 277, 283, 287	C D	54	1.0
026	292, 294, 299, 308, 310 311, 315, 318, 320, 322	C D	54	2.0
027	337, 341, 344, 346, 348 350, 354, 356, 364, 368	C D	54	4.0

[1] Individual specimens for thyroid histology were randomly selected using *Principles and Procedures of Statistics, A Biometrical Approach*, 2nd Ed., by Robert G.D. Steel and James H. Torrie, McGraw-Hill Book Co., USA, 1980.

[2] Due to high lethality, thyroid dysfunction was not an issue. Histopathology was not required, per EPA directive.

EPL®

EXPERIMENTAL PATHOLOGY LABORATORIES, INC.

BATTELLE
STUDY NUMBER BATT02-00062
EPL PROJECT NUMBER 237-013

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

DRAFT PATHOLOGY REPORT

Submitted by:

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DRAFT PATHOLOGY SUMMARY

BATTELLE
STUDY NUMBER BATT02-00062
EPL PROJECT NUMBER 237-013

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

DRAFT PATHOLOGY SUMMARY

A histomorphologic evaluation was performed on the thyroid glands of *Xenopus laevis* (South African clawed frog) that were exposed to various concentrations of L-thyroxine (T_4) to determine the differential sensitivity of exposing Stage 51 and Stage 54 larvae and to establish the necessary duration of exposure. Stage 51 larval frogs were exposed to T_4 for 21 days and Stage 54 larval frogs were exposed to T_4 for 14 days. The experimental designs were as follows:

Stage 51 Frog Larvae Exposed for 21 Days

Group/ Replicate	L-Thyroxine (T_4) (μg/L)	No. of Frogs/Group	Individual Frog Numbers
019A	0	5	008, 012, 015, 017, 019
019B	0	5	022, 024, 027, 029, 031
020A	0.5	5	041, 046, 050, 053, 058
020B	0.5	5	064, 067, 070, 073, 077
021A	1.0	5	082, 086, 089, 092, 098
021B	1.0	5	102, 105, 111, 114, 117
022A	2.0	5	124, 128, 133, 136, 139
022B	2.0	5	145, 149, 151, 154, 157

Stage 54 Frog Larvae Exposed for 14 Days

Group/ Replicate	L-Thyroxine (T_4) (μg/L)	No. of Frogs/Group	Individual Frog Numbers
023C	0	5	175, 177, 183, 188, 190
023D	0	5	194, 195, 206, 208, 209
024C	0.5	5	213, 218, 225, 227, 229
024D	0.5	5	237, 239, 242, 244, 245
025C	1.0	5	253, 255, 258, 264, 266
025D	1.0	5	271, 274, 277, 283, 287
026C	2.0	5	292, 294, 299, 308, 310
026D	2.0	5	311, 315, 318, 320, 322
027C	4.0	5	337, 341, 344, 346, 348
027D	4.0	5	350, 354, 356, 364, 368

No samples were submitted for Stage 51 larvae exposed to 4.0 μg/L of L-thyroxine due to high mortality in this group.

MATERIALS AND METHODS

Slides were prepared by Experimental Pathology Laboratories, Inc. (EPL®). Head samples were trimmed from the frog larvae from just behind the eyes. The heads were placed in cassettes with the cut side down so that the heads were sectioned in a transverse plane from caudal to rostral. Five step sections (30 microns apart) were taken from each block at the level of the thyroid glands and were mounted on one slide. A duplicate slide was made of the five steps. The slides were examined by a technician to assure that a sufficient amount of thyroid tissue was present bilaterally in the sections. If necessary to meet these criteria, additional step sections were cut from the block. One slide was stained with hematoxylin and eosin (H&E) and the second slide remained unstained.

Microscopic findings for thyroid glands from each frog are listed in the Histopathology Incidence Tables. The changes are graded from 1 to 5 depending upon severity (1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high). All lesions are summarized by group on the Summary Incidence Tables. The sexes of the frogs were not reported. Among the Stage 51 frogs exposed to the highest concentration of T_4 (2.0 μg/L) there were three in which there were no thyroid glands in the sections. These were frogs numbered 51-124, 51-149, and 51-154. Among the Stage 54 frogs, no thyroid glands could be located in the sections of the frogs exposed to 4.0 μg/L. An attempt was made to section tissue caudal to the eye in three of these frogs in the event that the thyroid had migrated caudally as occurs in metamorphosis, but thyroid glands were not located in these additional sections.

RESULTS

Stage 51 X*enopus laevis* exposed to T_4 for 21 days

There were exposure-related changes in the thyroid gland which included a decrease in size of the thyroid follicles and a decrease in the amount of colloid present within the follicles. Occasionally the colloid within follicles had a minimal light eosinophilic foamy appearance in contrast to the usual eosinophilic homogeneous appearance of colloid. One or more of these changes in the thyroid glands occurred in all but four of the frogs that were exposed to concentrations of T_4 from 0.5 μg/L to 2.0 μg/L. The prevalence of these three changes in the thyroid glands was highest among the frogs exposed to 2.0 μg/L of T_4 when the replicate groups for each exposure concentration were combined. All of the frogs in Group 020A (0.5 μg/L T_4) had both decreased follicle size and decreased colloid. With replicate groups combined the severity of decreased colloid varied from minimal to moderately severe among the affected frogs. With replicate groups combined, decreased follicular size varied from minimal to moderate among affected frogs in groups exposed to 0.5 and 1.0 μg/L L-thyroxine and from mild to moderately severe in groups exposed to 2.0 μg/L.

Interfollicular pigment was present in the majority of the thyroid glands at all exposure concentrations and varied in severity from minimal to moderate. There was no relationship of the prevalence or severity of this change to exposure concentrations of T_4. See Table 1 below for a tabulation of the prevalence of changes in the thyroid gland described above in Stage 51 frogs.

Table 1: Prevalence with Replicate Groups Combined of Selected Changes in Thyroid Glands of Stage 51 Frogs after Exposure to Various Concentrations of T_4 for 21 Days

Group	19A&B	20A&B	21A&B	22A&B
Exposure Conc. L-Thyroxine	0 μg/L	0.5 μg/L	1.0 μg/L	2.0 μg/L
Thyroid (No. examined)	(10)	(10)	(10)	(7)
Colloid, Decreased	0	7 (70%)	7 (70%)	6 (86%)
Colloid, Light Eosinophilic Foamy	0	1 (10%)	2 (20%)	5 (71%)
Thyroid Follicles, Decreased Size	0	8 (80%)	7 (70%)	6 (86%)
Interfollicular Pigment	7 (70%)	7 (70%)	10 (100%)	5 (71%)

See Appendix A for photographs of thyroid glands in frogs from each of the exposure concentrations. Each frog is illustrated by photographs taken at low magnification (1.25x) and high magnification (10x). The photographs are annotated to illustrate various features of thyroid glands and thyroid gland changes.

Stage 54 *Xenopus laevis* exposed to T_4 for 14 days

There were exposure-related changes in the thyroid gland which included a decrease in the amount of colloid in the thyroid follicles and the presence of minimal light eosinophilic foamy colloid in the follicles of a few frogs in contrast to the usual eosinophilic homogeneous colloid. The prevalence of decreased colloid was greatest in the frogs exposed to 2.0 μg/L of T_4 and varied in severity from minimal to moderate with replicate groups combined. At exposure concentrations of 0.5 and 1.0 μg/L the severity of decreased colloid was usually minimal. No thyroid glands could be located in the ten frogs that were exposed to 4.0 μg/L of T_4.

Interfollicular pigment was present in the majority of the thyroid glands at all exposure concentrations and varied in severity from minimal to moderate. There was no relationship of the prevalence or severity of this change to exposure

concentrations of T_4. See Table 2 below for a tabulation of the prevalence of changes in the thyroid gland described above in Stage 54 frogs.

Table 2: Prevalence with Replicate Groups combined of Selected Changes in Thyroid Glands of Stage 54 *Xenopus laevis* Exposed to Various Concentrations of T_4 for 14 Days

Group/Replicates	23C&D	24C&D	25C&D	26C&D
Exposure Conc. L-Thyroxine	0 µg/L	0.5 µg/L	1.0 µg/L	2.0 µg/L
Thyroid (No. examined)	(10)	(10)	(10)	(10)
Colloid, Decreased	1 (10%)	4 (40%)	3 (30%)	8 (80%)
Colloid, Light Eosinophilic Foamy	0	0	2 (20%)	2 (20%)
Interfollicular Pigment	8 (80%)	7 (70%)	10 (100%)	6 (60%)

See Appendix B for photographs of thyroid glands in frogs from each of the exposure concentrations. Each frog is illustrated by a photograph taken at low magnification (1.25x) and high magnification (10x). The photographs are annotated to illustrate various features of thyroid glands and thyroid gland changes.

DISCUSSION

Thyroxine is an iodinated hormone secreted by the thyroid gland that functions to increase the rate of cell metabolism and is the primary hormone involved in the process of amphibian metamorphosis.[1] Secretion of thyroxine by the thyroid gland is regulated by thyroid stimulating hormone (TSH) which is released by the pituitary gland in response to the level of thyroxine in the circulating blood.[2] A low thyroxine level in the blood is a signal for the release of TSH which, in turn, stimulates the synthesis and release of thyroxine from the thyroid gland. Conversely, TSH would not be released under conditions of higher than normal levels of thyroxine in the blood.

High circulating levels of thyroxine in the blood would slow or halt the synthesis of thyroxine by the follicular cells of the thyroid gland. Colloid, which consists of thyroglobulin, the precursor of thyroxine, would be gradually utilized

but not replaced. As a result and as occurred in the Stage 51 larval frogs in the study described above, the amount of colloid would be decreased with a corresponding decrease in the size of the thyroid follicles. These changes occurred at all exposure concentration levels in Stage 51 frogs, but in Stage 54 frogs, the amount of colloid in follicles was decreased without a substantial change in follicular size. The prevalence of decreased colloid was greater in Stage 51 frogs than in Stage 54 frogs at all exposure concentrations of thyroxine. The differences in the changes between Stage 51 and 54 frogs could be related to the differences in exposure time of the two stages. It is also possible that the less mature Stage 51 frogs were more sensitive to the effects of exogenous thyroxine.

Thyroid glands could not be located in three of ten Stage 51 frogs exposed to 2.0 μg/L thyroxine for 21 days or in ten of ten Stage 54 frogs exposed to 4.0 μg/L thyroxine for 14 days. The heads of the frogs in these two groups were noticeably smaller in transverse section on the slide than were the heads of frogs at other exposure concentrations. A high concentration of thyroxine could promote early metamorphosis resulting in an animal that was of diminutive size.[3] Also, the thyroid glands shift caudally in position during metamorphosis.[3] It is possible that the thyroid glands were not present in the head section as trimmed in these frogs exposed to the highest concentration of thyroxine.

Minimal light eosinophilic foamy colloid was a feature of thyroid glands in a small number of Stage 51 and Stage 54 frogs. The prevalence of this change was highest in Stage 51 frogs exposed to 2.0 μg/L thyroxine. This appearance of the colloid may reflect a loss of thyroglobulin which would result in a decreased density of the colloid.

CONCLUSIONS

Exposure of *Xenopus laevis* to thyroxine at Stage 51 for 21 days resulted in changes in the thyroid gland that included mainly a decrease in the amount of colloid present and a decreased size of the thyroid follicles. These changes occurred in the majority of frogs at exposure concentrations of 0.5 to 2.0 µg/L, but were most prevalent at 2.0 µg/L thyroxine. Exposure of *Xenopus laevis* to thyroxine at Stage 54 for 14 days resulted in decreased colloid in the thyroid follicles in less than half of the frogs at exposure concentrations of 0.5 µg/L and 1.0 µg/L and in 80% of the frogs at 2.0 µg/L thyroxine. Thyroid glands could not be located in three of 10 Stage 51 frogs at the 2.0 µg/L exposure concentration or in ten of ten Stage 54 frogs at the 4.0 µg/L exposure concentration.

MARILYN J. WOLFE, DVM, PhD, Diplomate, ACVP Date
Veterinary Pathologist

MJW/cb

REFERENCES

[1] Denver, Robert J., Glennemeier, Karen A., Boorse, Graham C. "Endocrinology of Complex Life Cycles: Amphibians," Chapter 28 in v. 2 Non-Mammalian Hormone-Behavior Systems of *Hormones, Brain and Behavior* (Donald W. Pfaff, Arthur P. Arnold, Anne M. Etgen, Susan E. Fahrbach, and Robert T. Rubin) Elsevier Science, 2002, pp. 475-476.

[2] Capen, C. C., DeLellis, R. A., and Yarrington, J. T. (1991). Endocrine System. In: Handbook of Toxicologic Pathology, (W.M. Haschek and C. G. Rousseaux, eds.), pp. 733-734. Academic Press, San Diego.

[3] Andrew, W. and Hickman, C. P. (1974). Histology of the Vertebrates: a Comparative Text, pp. 330-332. The C.V. Mosby Company, Saint Louis.

Battelle Study Number BATT02-00062

3/4/05 DRAFT

COMPLIANCE STATEMENT

Client Name	Battelle	EPL Project Coordinator	Marilyn J. Wolfe
Client Study	BATT02-00062	EPL Pathologist	Marilyn J. Wolfe
Species	*Xenopus laevis* (South African clawed frog)	EPL Project Number	237-013
Study Title	Amphibian Metamorphosis Assay Demonstration Study		
Test Article	L-Thyroxine (T_4)		

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 160.

EPL Project Coordinator

Date

3/4/05 DRAFT

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Amphibian Metamorphosis Assay Demonstration Study

Client Study: BATT02-00062 EPL Project Coordinator: Marilyn J. Wolfe

EPL Project Number: 237-013 EPL Pathologist: Marilyn J. Wolfe

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 Project Coordinator and Management are indicated below.

	Dates	
Area Inspected	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management XXX

Date of last quarterly facility inspection 1/05

EPL Quality Assurance Unit Date

SUMMARY INCIDENCE TABLES

STAGE 51 FROGS

SUMMARY INCIDENCE TABLE

BATT02-00062
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 019A	GROUP 019B	GROUP 020A	GROUP 020B	GROUP 021A	GROUP 021B
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Colloid, Decreased			5	2	4	3
Colloid, Light Eosinophilic						
Foamy			1		2	
Interfollicular Pigment	4	3	3	4	5	5
Thyroid Follicles, Decreased						
Size			5	3	4	3

SUMMARY INCIDENCE TABLE

BATT02-00062
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 022A	GROUP 022B				
THYROID (NO. EXAMINED)	(4)	(3)				
Colloid, Decreased	4	2				
Colloid, Light Eosinophilic Foamy	3	2				
Interfollicular Pigment	3	2				
Thyroid Follicles, Decreased Size	4	2				

HISTOPATHOLOGY INCIDENCE TABLES

STAGE 51 FROGS

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00062
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 019A						GROUP 019B						GROUP 020A							
ANIMAL	51-008	51-012	51-015	51-017	51-019		51-022	51-024	51-027	51-029	51-031		51-041	51-046	51-050	51-053	51-058			
THYROID					X			X			X									
Colloid, Decreased													2	3	1	4	2			
Colloid, Light Eosinophilic																				
Foamy														1						
Interfollicular Pigment	2	2	2	3			1		2	3			3	2	2					
Thyroid Follicles, Decreased																				
Size													2	3	1	3	3			

EPL
Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00062
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 020B						GROUP 021A						GROUP 021B							
ANIMAL	51-064	51-067	51-070	51-073	51-077		51-082	51-086	51-089	51-092	51-098		51-102	51-105	51-111	51-114	51-117			
THYROID																				
Colloid, Decreased				1	1		2	1		2	3		4	1		3				
Colloid, Light Eosinophilic Foamy									1		1									
Interfollicular Pigment	3	1	2		1		2	2	2	2	1		2	1	1	1	2			
Thyroid Follicles, Decreased Size		1		1	2		2	1		1	3		3	1		3				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00062
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 022A						GROUP 022B														
ANIMAL	51-124	51-128	51-133	51-136	51-139		51-145	51-149	51-151	51-154	51-157										
THYROID	N							N		N											
Colloid, Decreased		4	2	3	1				4		3										
Colloid, Light Eosinophilic																					
Foamy			1	1	1		1				1										
Interfollicular Pigment		1		1	2				2		1										
Thyroid Follicles, Decreased																					
Size		3	2	2	2				4		2										

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Experimental Pathology Laboratories, Inc.

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1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

SUMMARY INCIDENCE TABLES

STAGE 54 FROGS

SUMMARY INCIDENCE TABLE

BATT02-00062
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 023C	GROUP 023D	GROUP 024C	GROUP 024D	GROUP 025C	GROUP 025D
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Colloid, Decreased	1		2	2	1	2
Colloid, Light Eosinophilic						
Foamy						2
Interfollicular Pigment	4	4	4	3	5	5

SUMMARY INCIDENCE TABLE

BATT02-00062
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 026C	GROUP 026D	GROUP 027C	GROUP 027D		
THYROID (NO. EXAMINED)	(5)	(5)				
Colloid, Decreased	4	4				
Colloid, Light Eosinophilic Foamy	1	1				
Interfollicular Pigment	3	3				

HISTOPATHOLOGY INCIDENCE TABLES

STAGE 54 FROGS

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00062
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 023C						GROUP 023D						GROUP 024C								
ANIMAL	54-175	54-177	54-183	54-188	54-190		54-194	54-195	54-206	54-208	54-209		54-213	54-218	54-225	54-227	54-229				
THYROID				X			X										X				
Colloid, Decreased					1								1		1						
Colloid, Light Eosinophilic Foamy																					
Interfollicular Pigment	3	2	2		2			2	3	2	2		2	2	3	3					

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1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00062
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 024D						GROUP 025C						GROUP 025D						
ANIMAL	54-223	54-239	54-242	54-244	54-245		54-253	54-255	54-258	54-264	54-266		54-271	54-274	54-277	54-283	54-287		
THYROID				X															
Colloid, Decreased			2		1			1							1		1		
Colloid, Light Eosinophilic Foamy															1		1		
Interfollicular Pigment	2	3	2				2	2	3	3	3		2	2	2	3	1		

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P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00062
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 026C						GROUP 026D						GROUP 027C							
ANIMAL	54-292	54-294	54-299	54-308	54-310		54-311	54-315	54-318	54-320	54-322		54-337	54-341	54-344	54-346	54-348			
THYROID								X					N	N	N	N	N			
Colloid, Decreased	3	2		3	3		3		2	1	1									
Colloid, Light Eosinophilic Foamy				1							1									
Interfollicular Pigment		3	2	2			2		2	2										

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1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00062
Stage 54/14 Day Sacrifice
Xenopus laevis

GROUP
027D

ANIMAL	54-350	54-354	54-356	54-364	54-368															
THYROID	N	N	N	N	N															
Colloid, Decreased																				
Colloid, Light Eosinophilic																				
Foamy																				
Interfollicular Pigment																				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

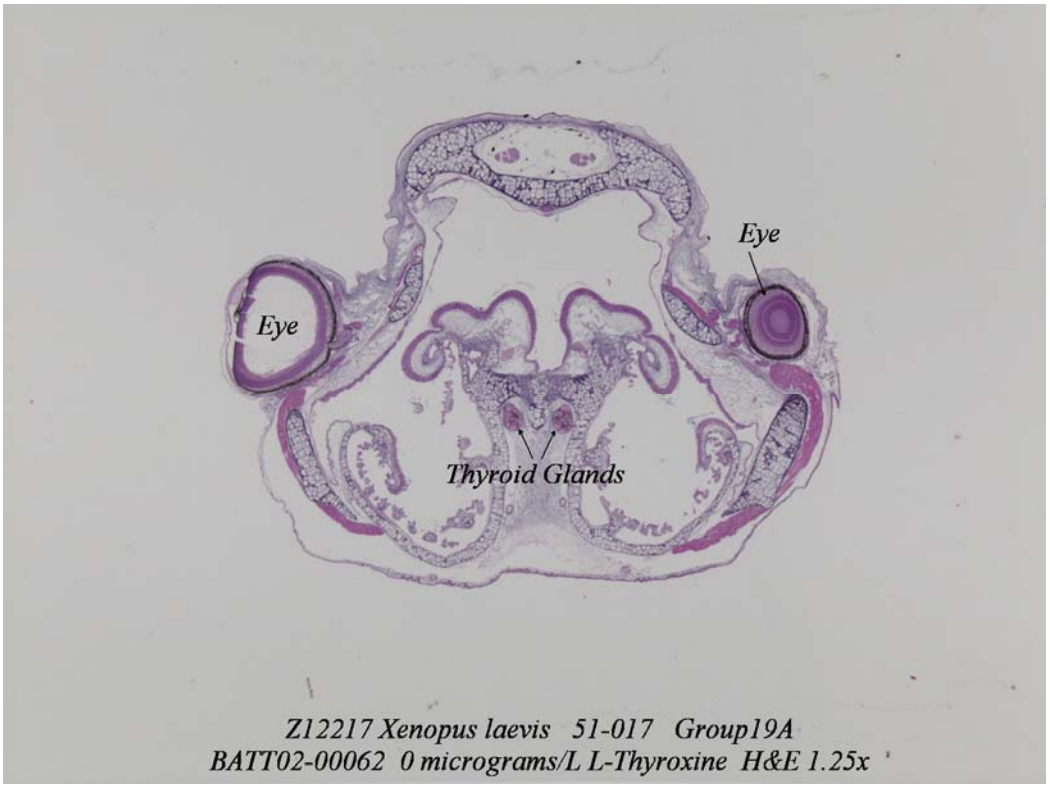
Appendix A:

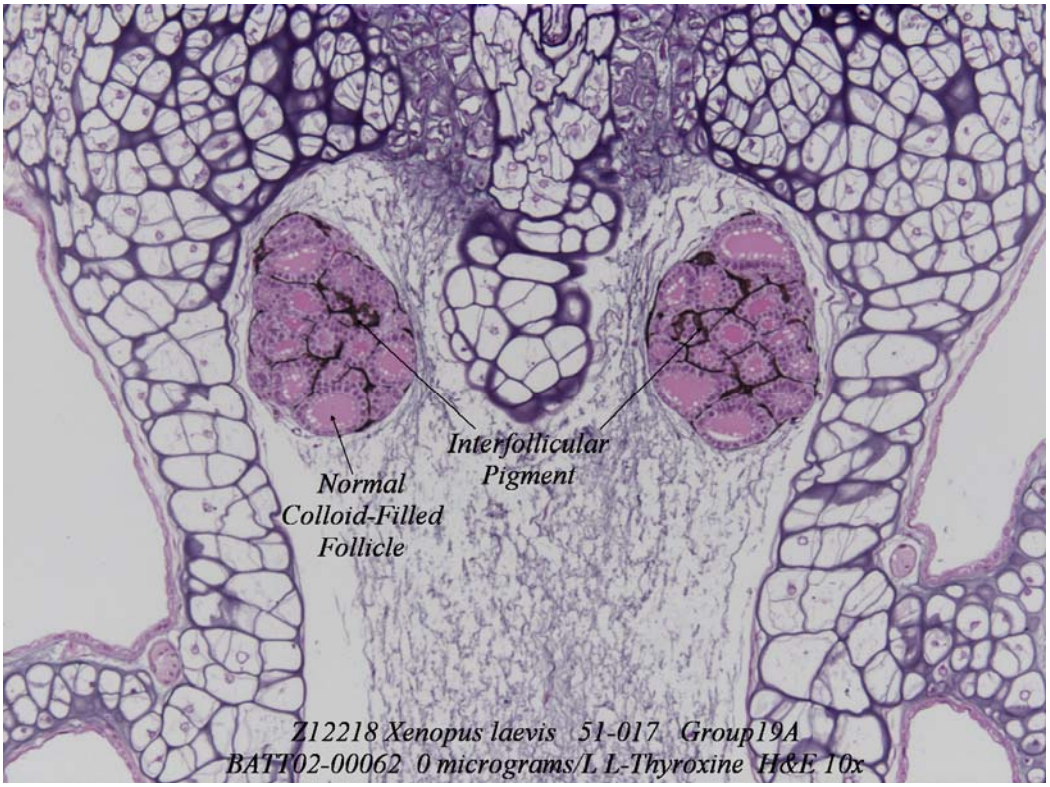
Stage 51 Photo Legends



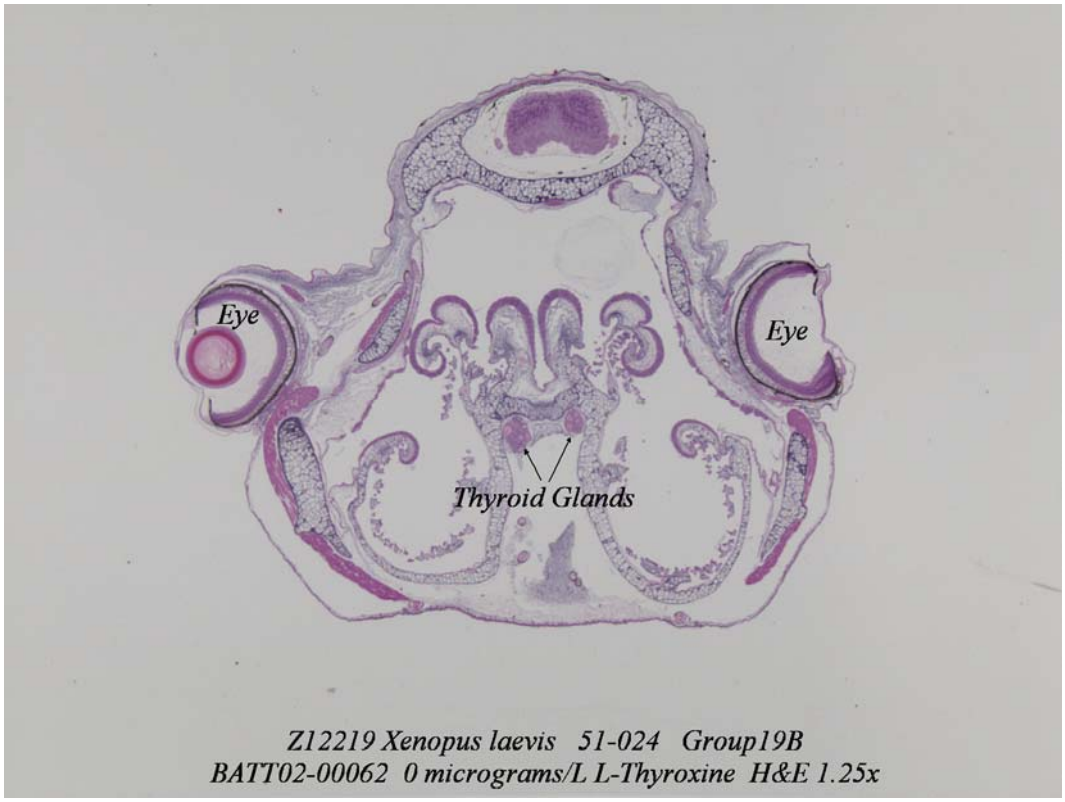


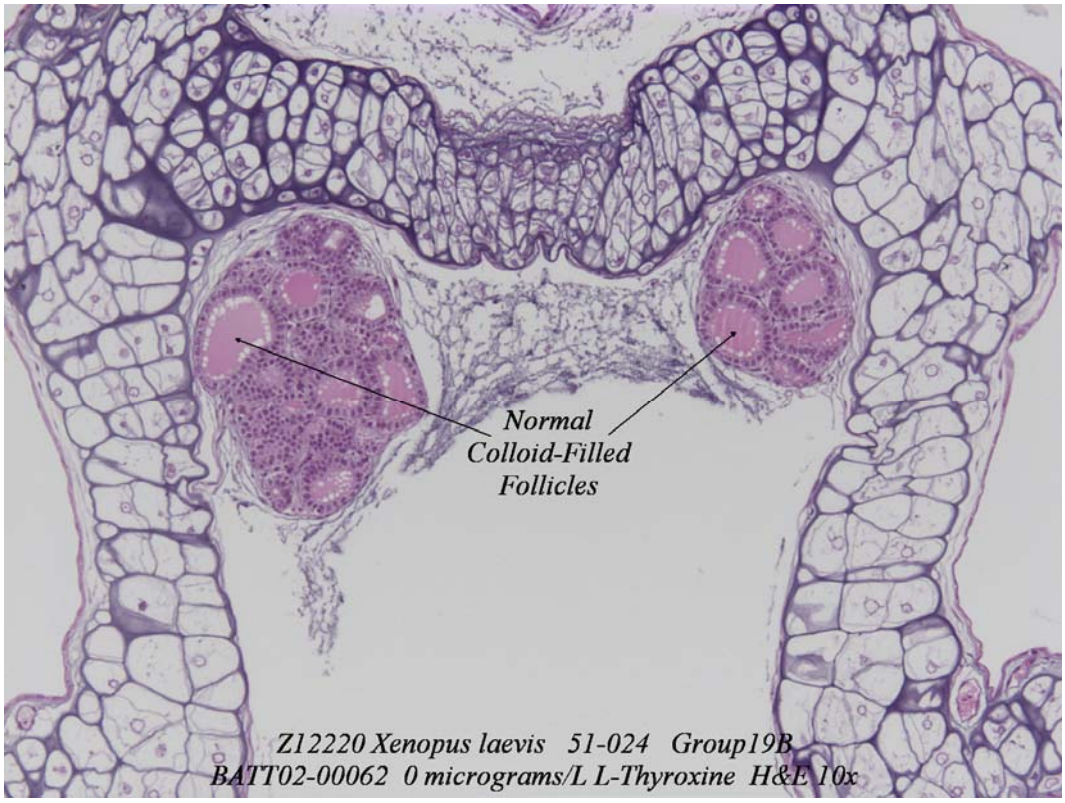
Z12215 (1.25x) and Z12216 (10x). Thyroid glands of control frog 51-012. There is a mild quantity of interfollicular pigment. There is a variation in the size of the normal colloid-filled thyroid follicles.



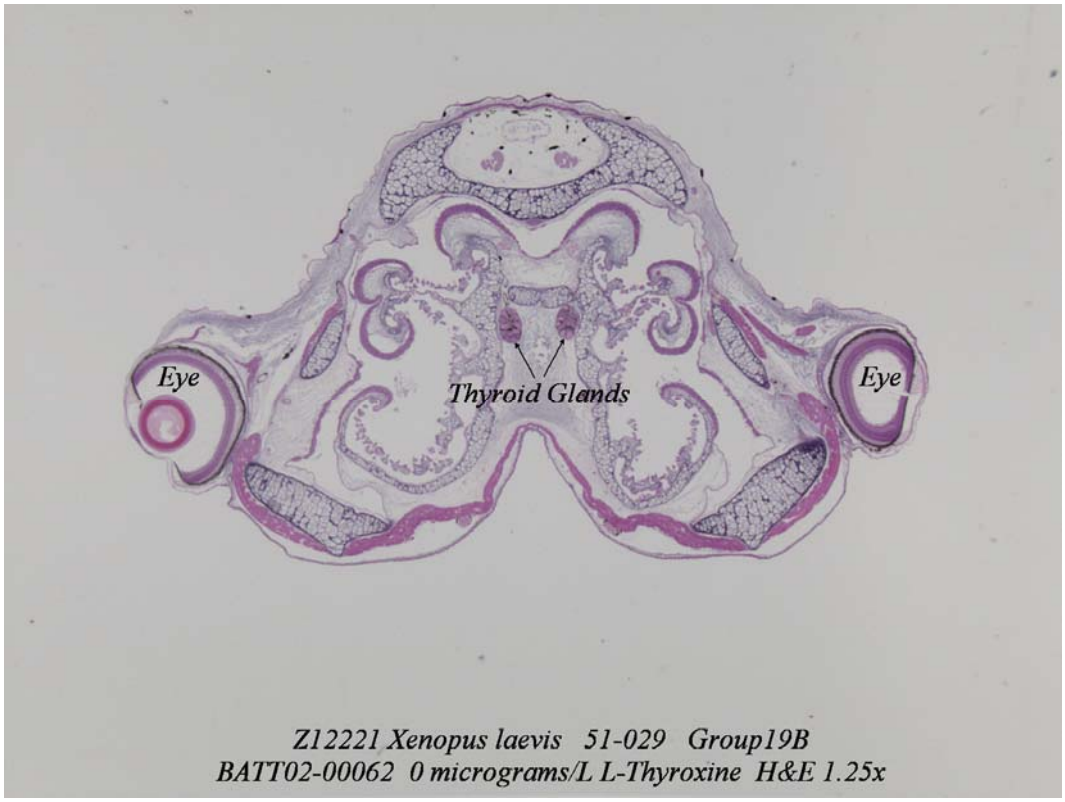


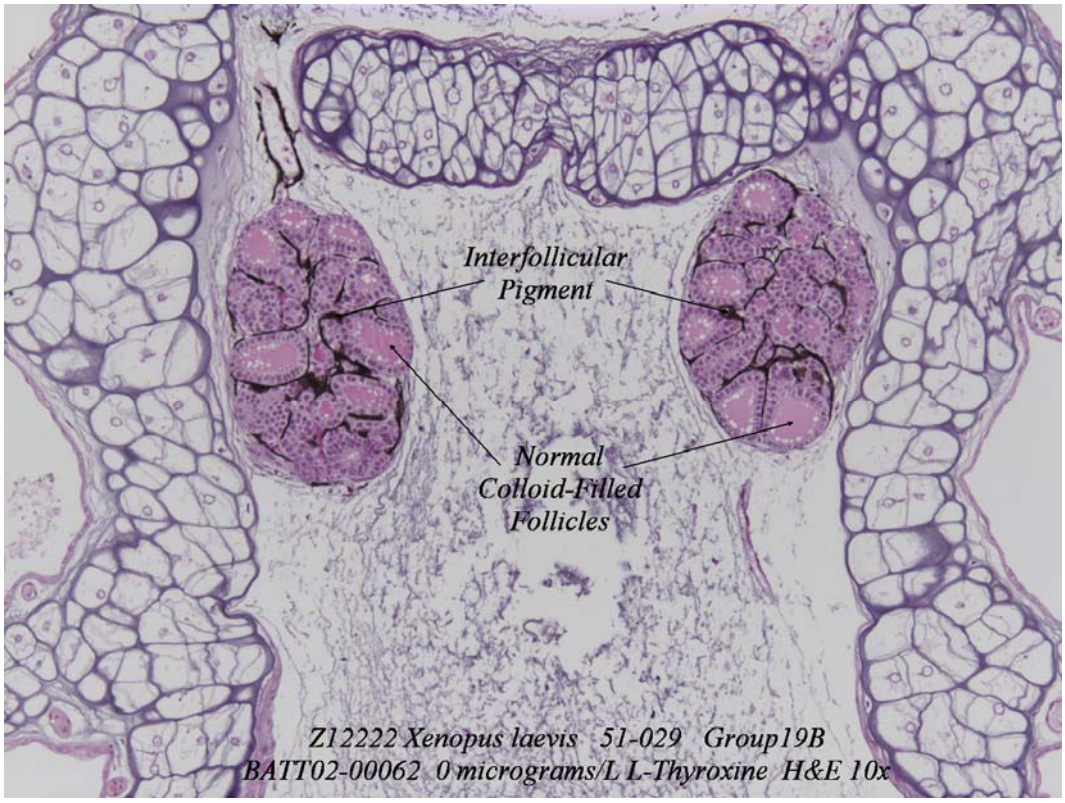
Z12217 (1.25x) and Z12218 (10x). Thyroid glands of control frog 51-017. There is a moderate quantity of interfollicular pigment. There is a variation in size of the normal colloid-filled thyroid follicles.





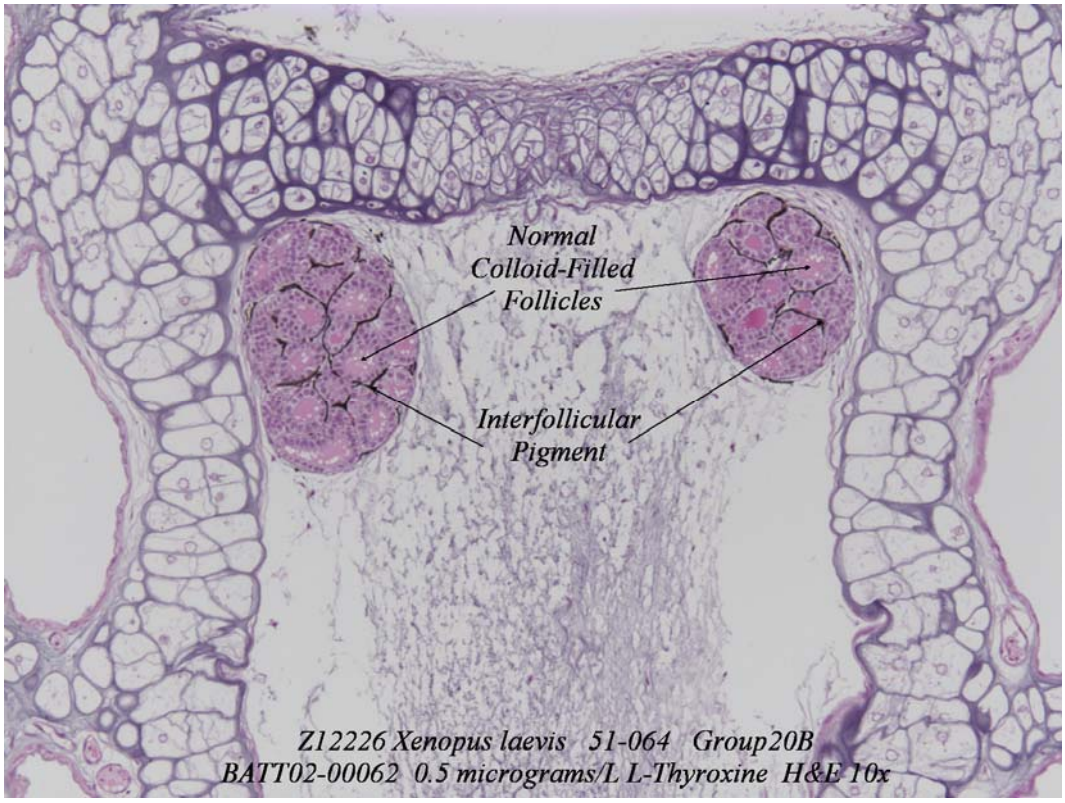
Z12219 (1.25x) and Z12220 (10x). Thyroid glands of control frog 51-024. There is no interfollicular pigment. There is a variation in size of the normal colloid-filled thyroid follicles.



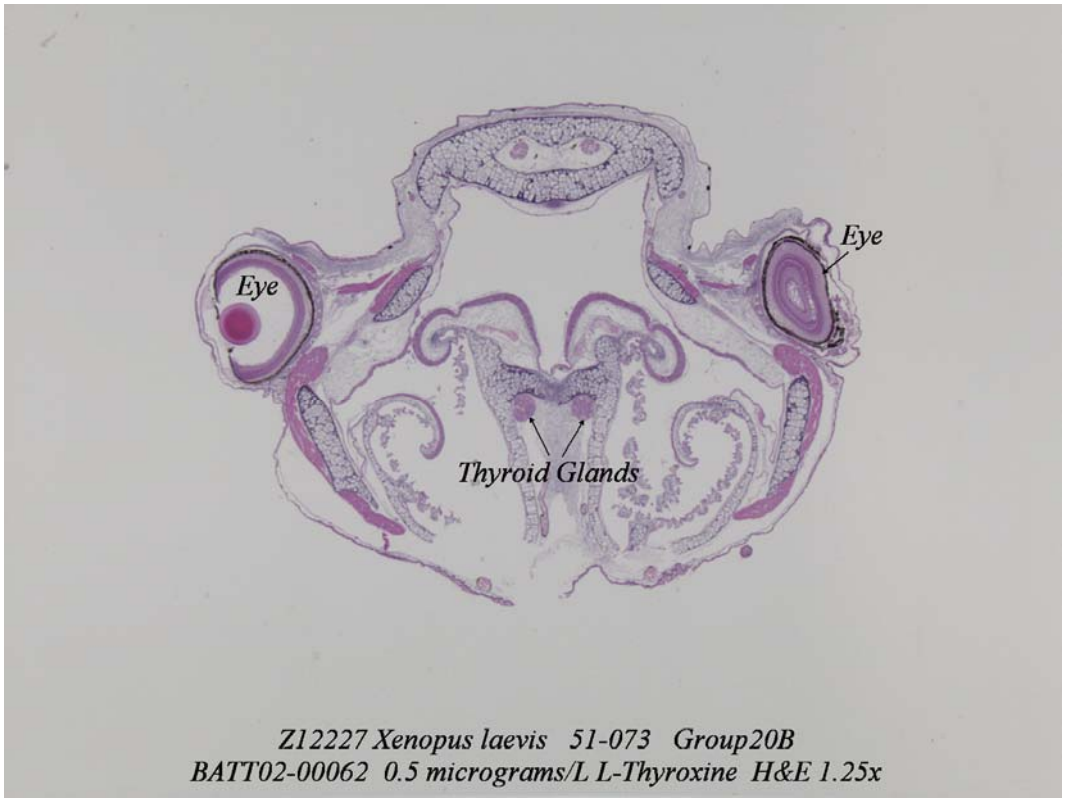


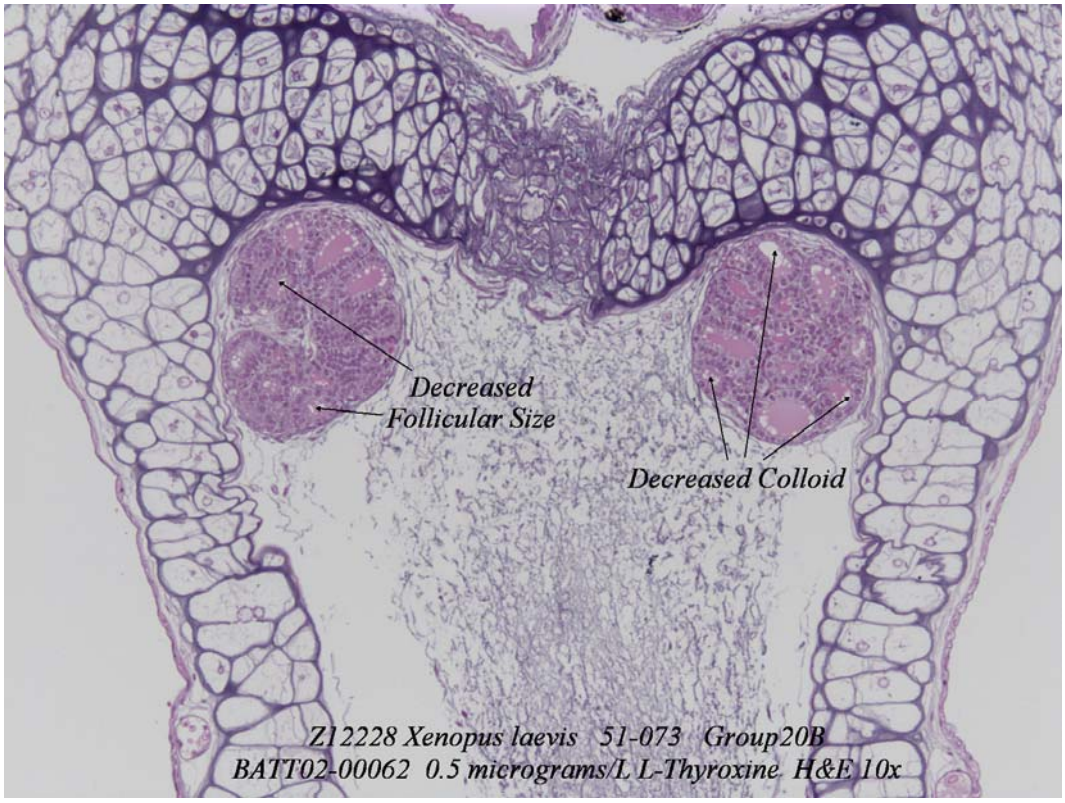
Z12221 (1.25x) and Z12222 (10x). Thyroid glands of control frog 51-029. There is a moderate quantity of interfollicular pigment. There is a variation in size of the normal colloid-filled thyroid follicles.



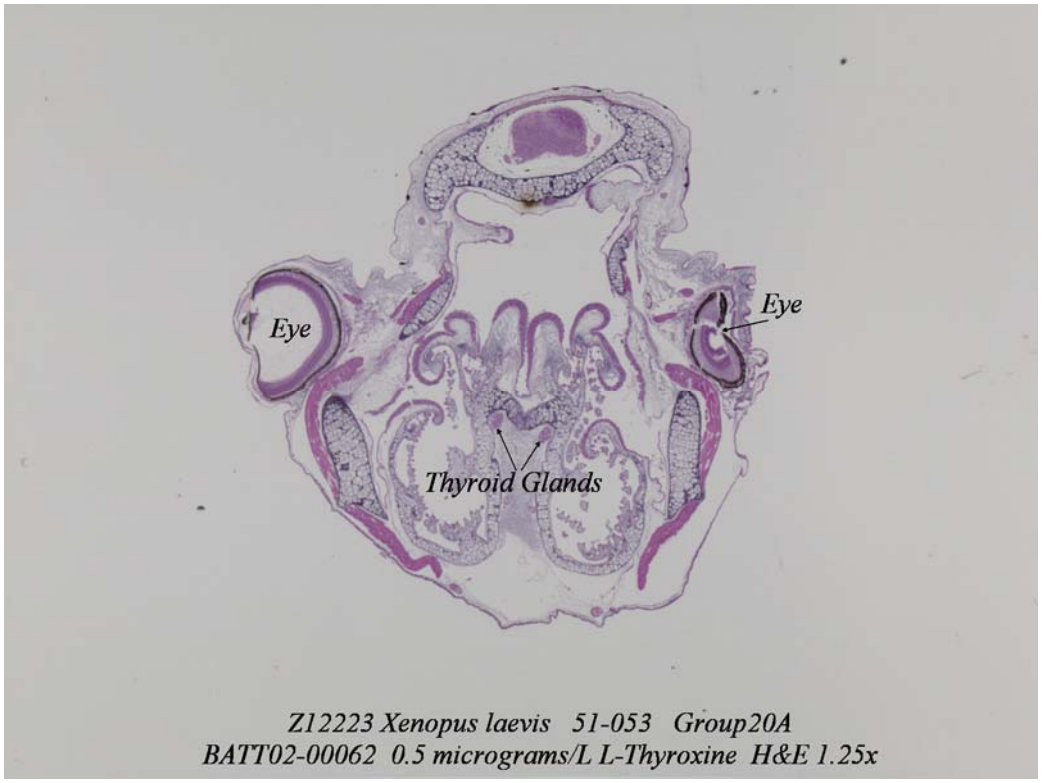


Z12225 (1.25x) and Z12226 (10x). Thyroid glands of frog 51-064 exposed to 0.5 µg/L L-thyroxine. There is a moderate quantity of interfollicular pigment. The thyroid follicles are normal and colloid-filled.



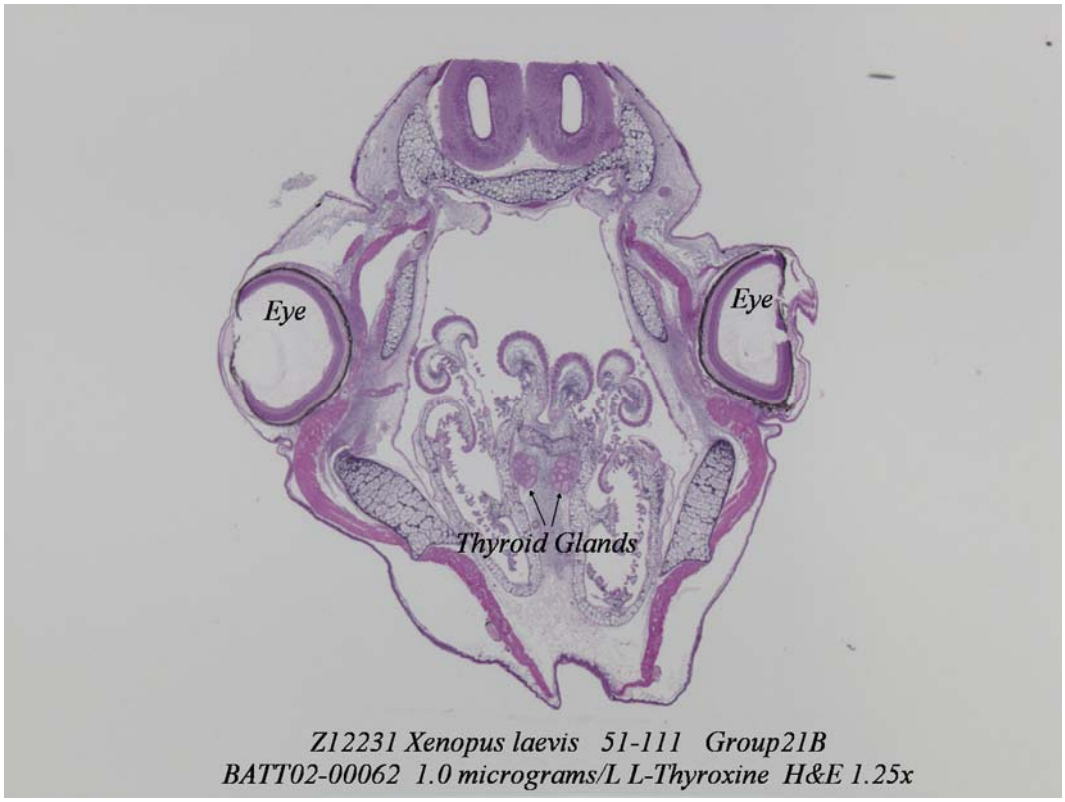


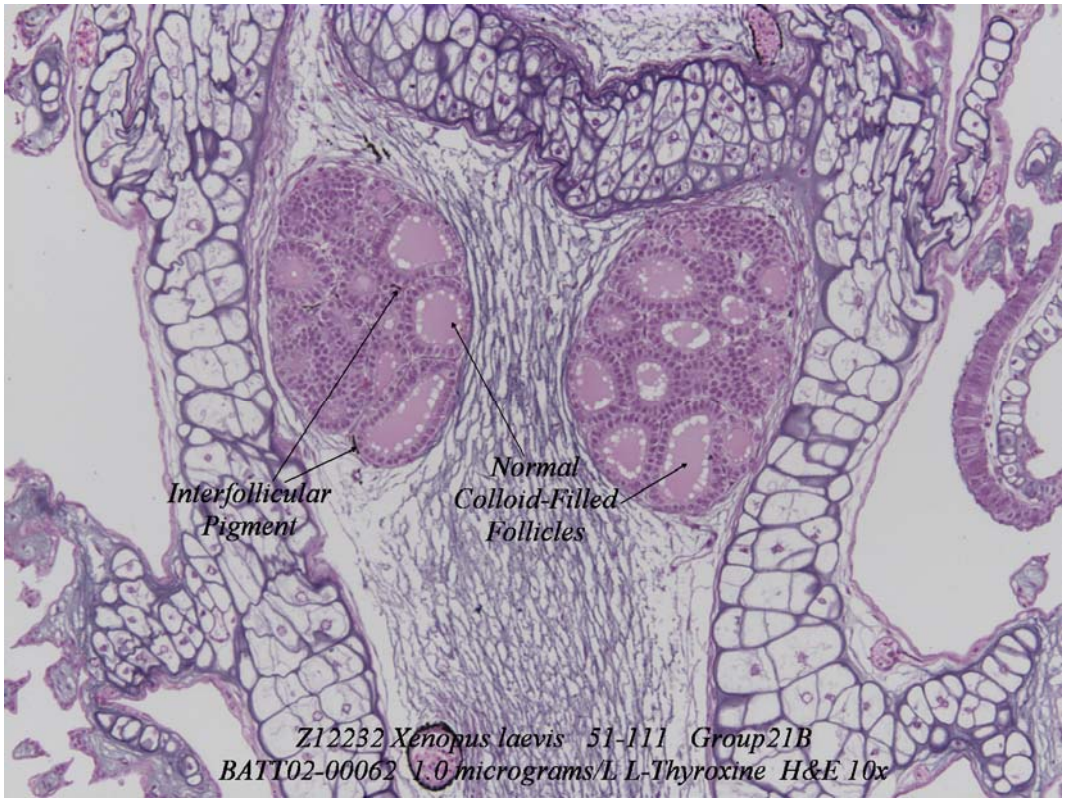
Z12227 (1.25x) and Z12228 (10x). Thyroid glands of frog 51-073 exposed to 0.5 µg/L L-thyroxine. There is minimal decreased quantity of colloid and a minimal decrease in the size of thyroid follicles as compared to control frogs. There is no interfollicular pigment.



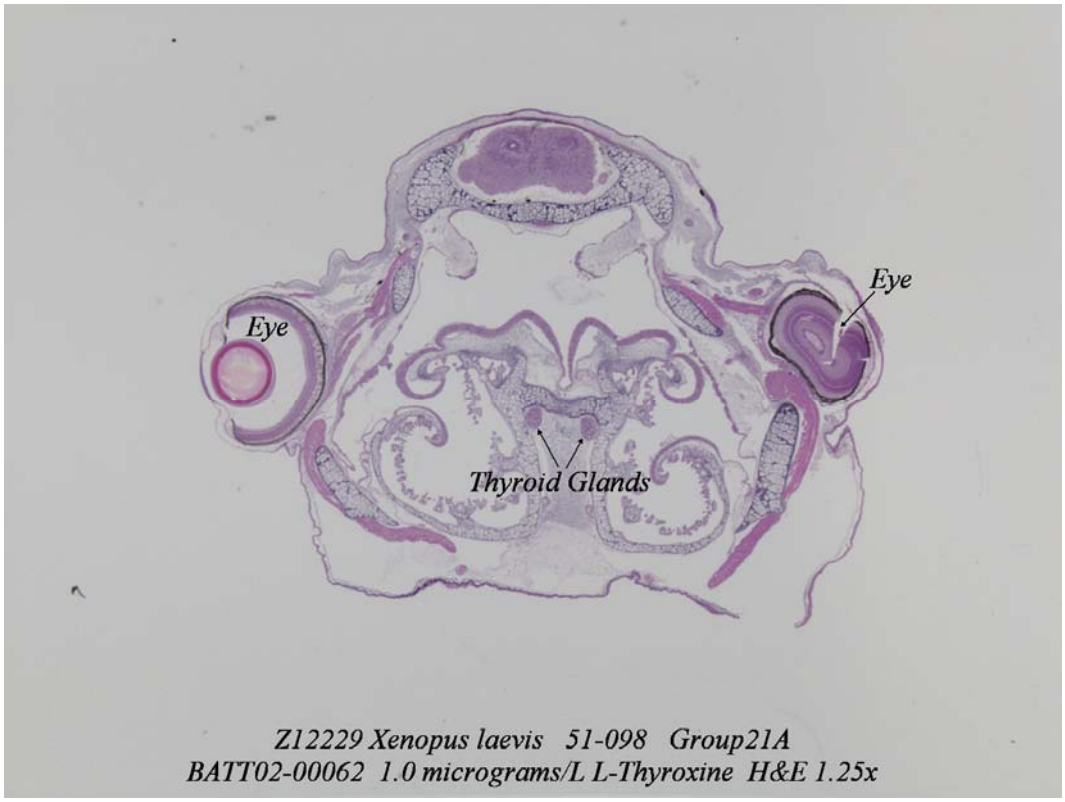


Z12223 (1.25x) and Z12224 (10x). Thyroid glands of frog 51-053 exposed to 0.5 µg/L L-thyroxine. There is a moderately severe decreased quantity of colloid and a moderate decrease in the size of the thyroid follicles as compared to control frogs. There is no interfollicular pigment.



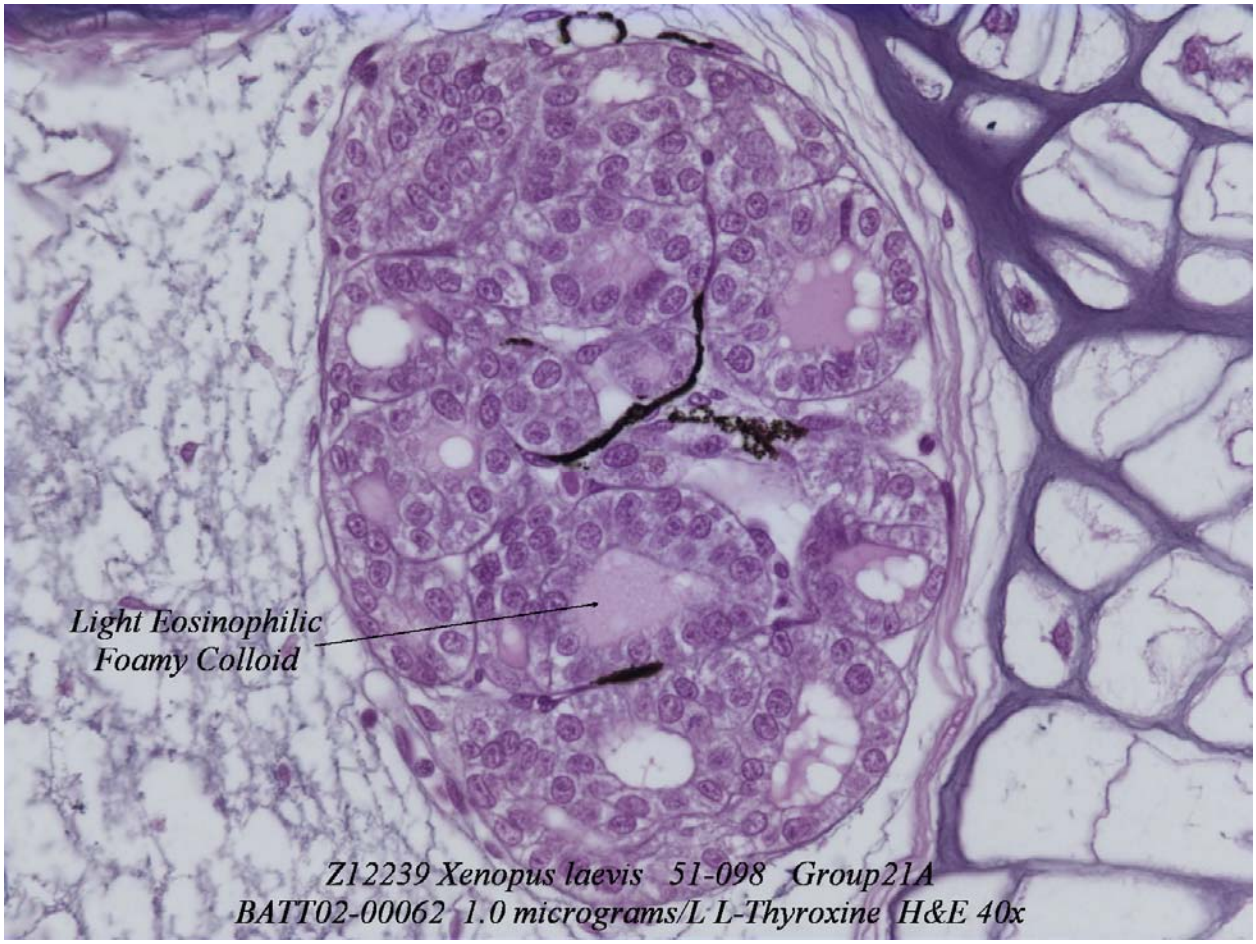


Z12231 (1.25x) and Z12232 (10x). Thyroid glands of frog 51-111 exposed to 1.0 µg/L L-thyroxine. The thyroid follicles are normal and colloid-filled. There is minimal interfollicular pigment.

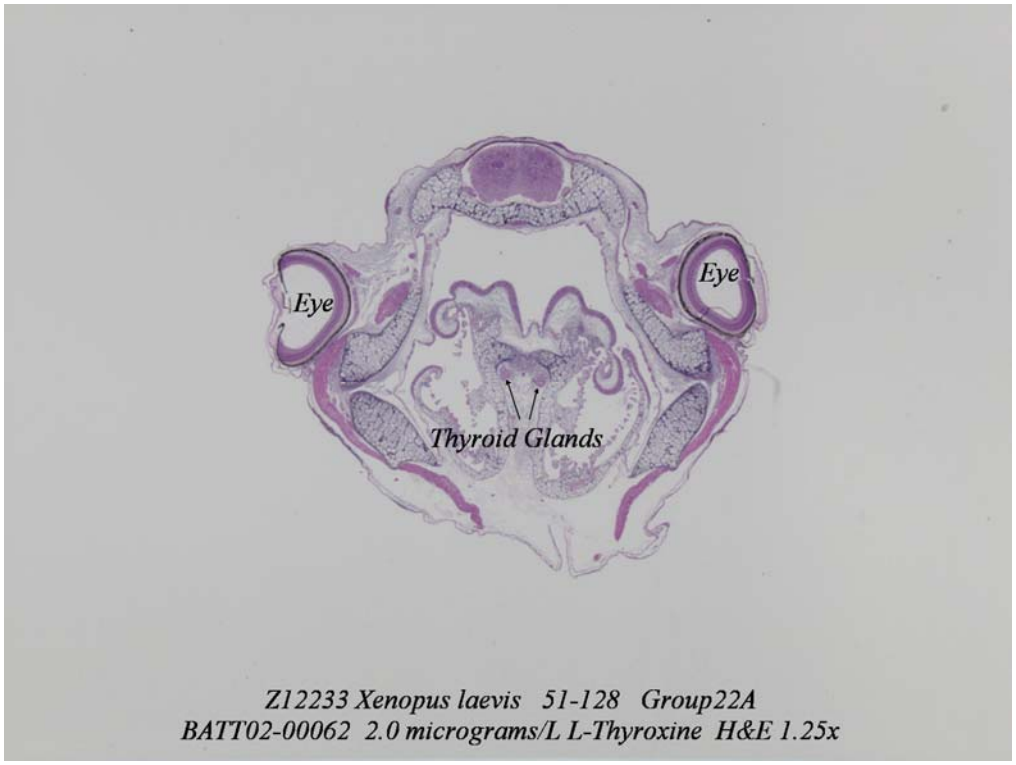


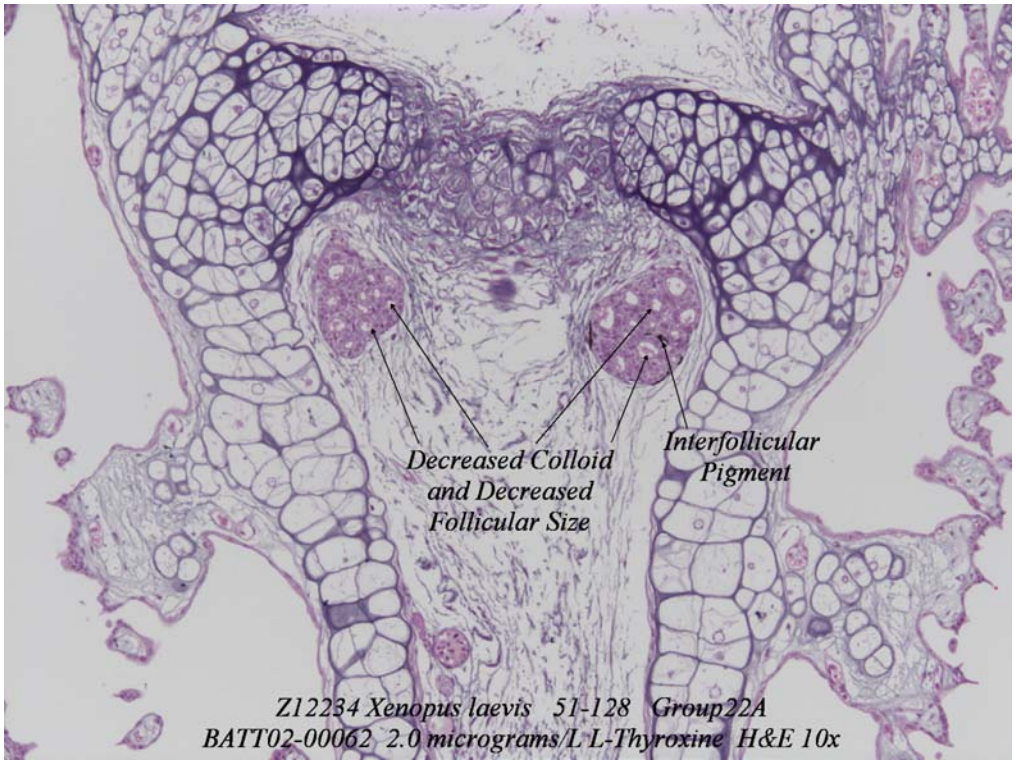


Z12229 (1.25x) and Z12230 (10x). Thyroid glands of frog 51-098 exposed to 1.0 µg/L L-thyroxine. There is a moderate decreased quantity of colloid and decreased size of thyroid follicles as compared to control frogs. There is minimal interfollicular pigment.

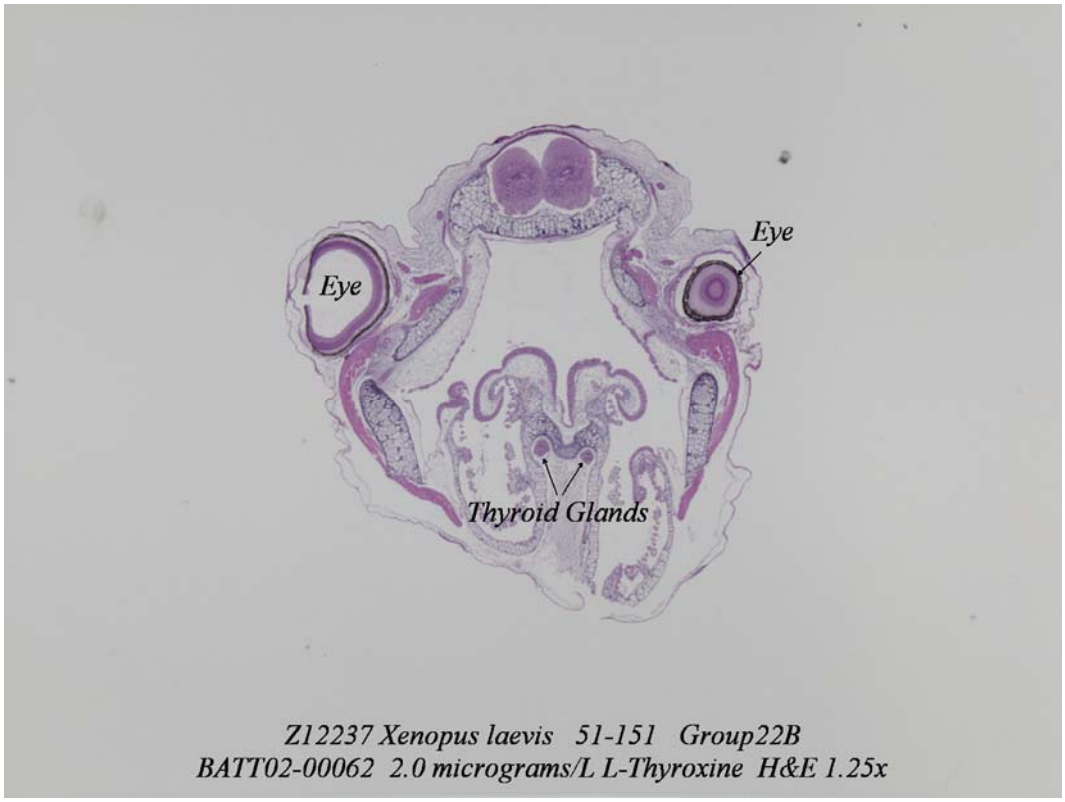


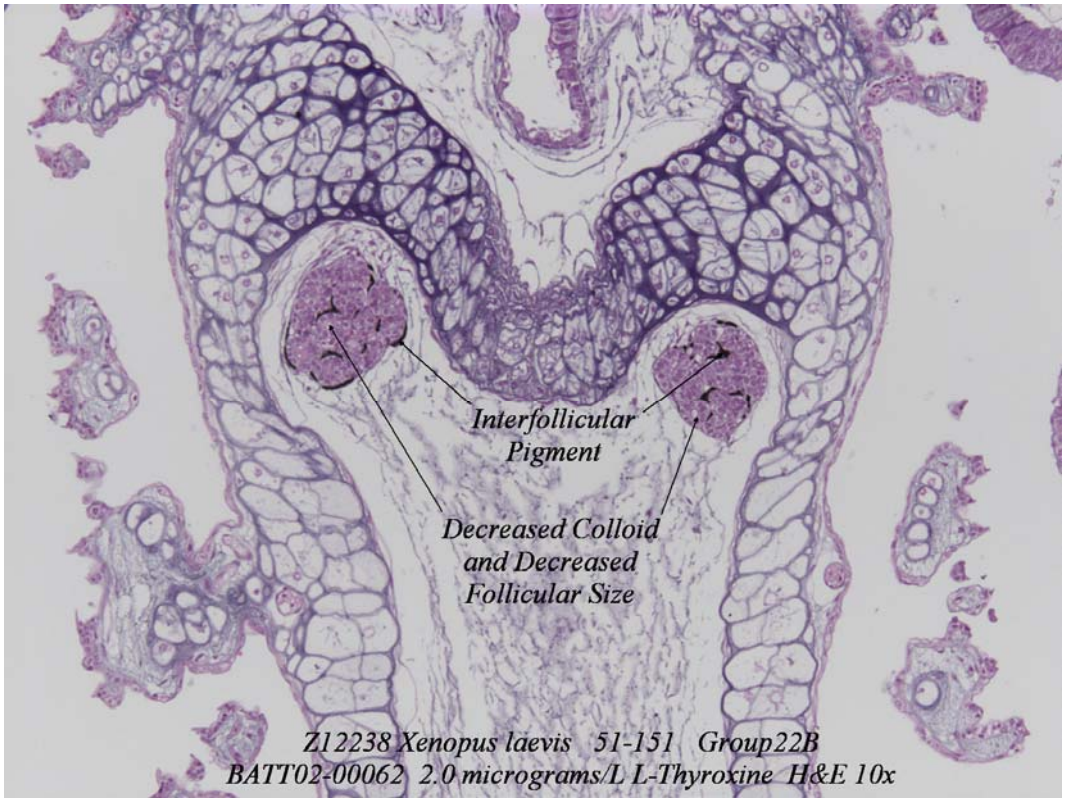
Z12239 (40x). Thyroid gland of frog 51-098 exposed to 1.0 µg/L L-thyroxine. There is minimal light eosinophilic foamy colloid in the thyroid follicle as compared to the normal eosinophilic homogeneous colloid.





Z12233 (1.25x) and Z12234 (10x). Thryoid glands of frog 51-128 exposed to 2.0 µg/L L-thyroxine. There is a moderately severe decreased quantity of colloid and a moderate decreased size of the thyroid follicles as compared to control frogs. There is minimal interfollicular pigment.

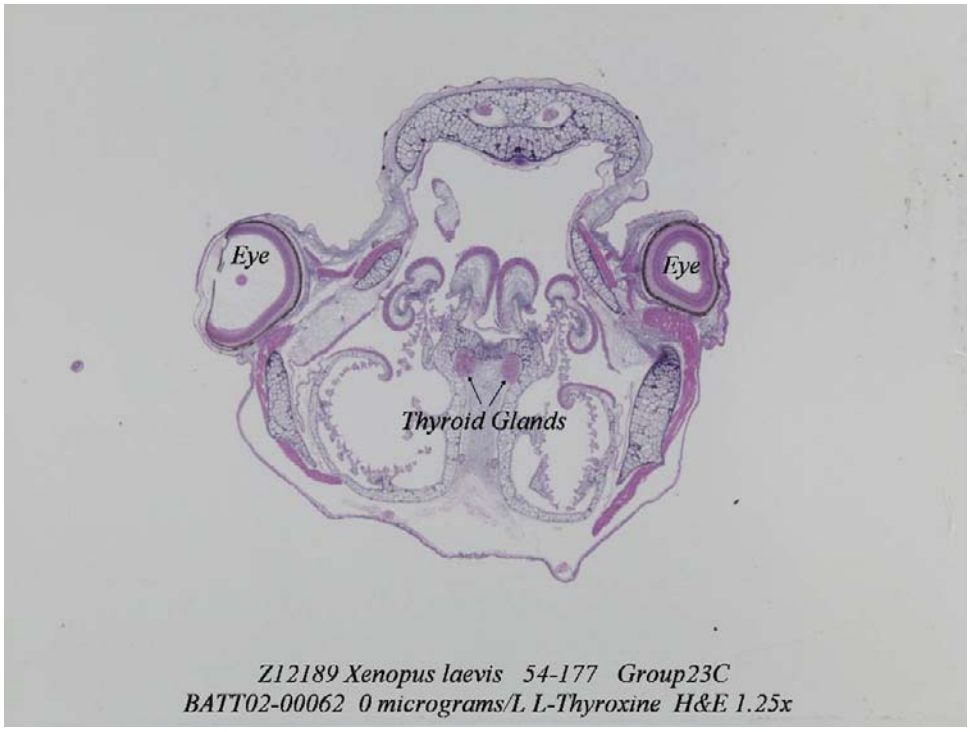


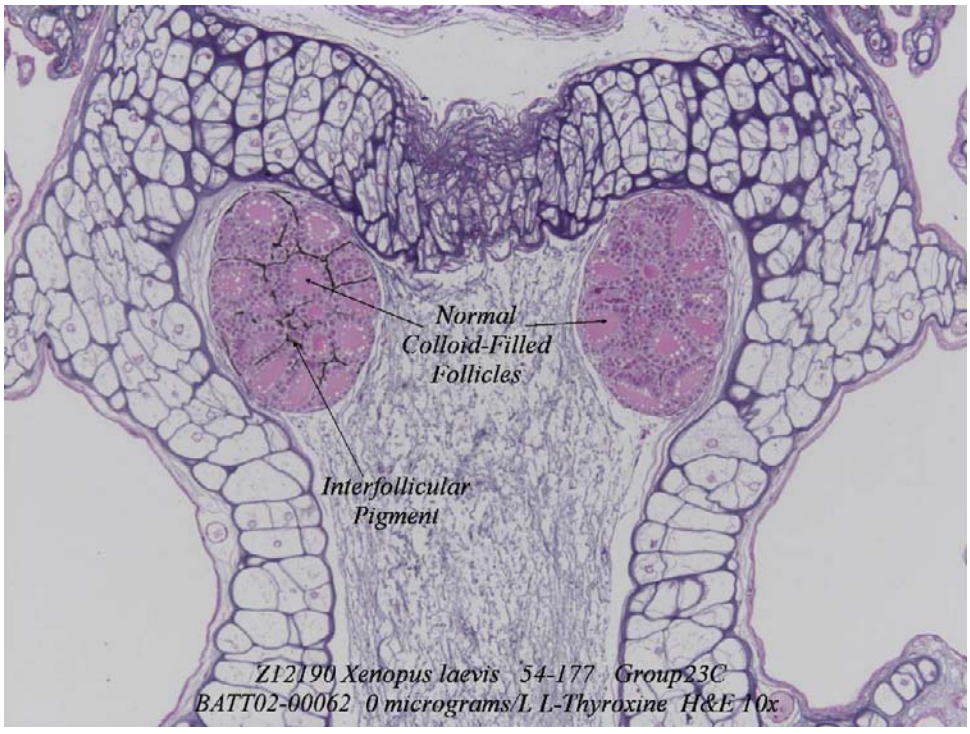


Z12237 (1.25x) and Z12238 (10x). Thyroid glands of frog 51-151 exposed to 2.0 µg/L L-thyroxine. There is a moderately severe decreased quantity of colloid and decreased size of thyroid follicles as compared to control frogs. There is mild interfollicular pigment.

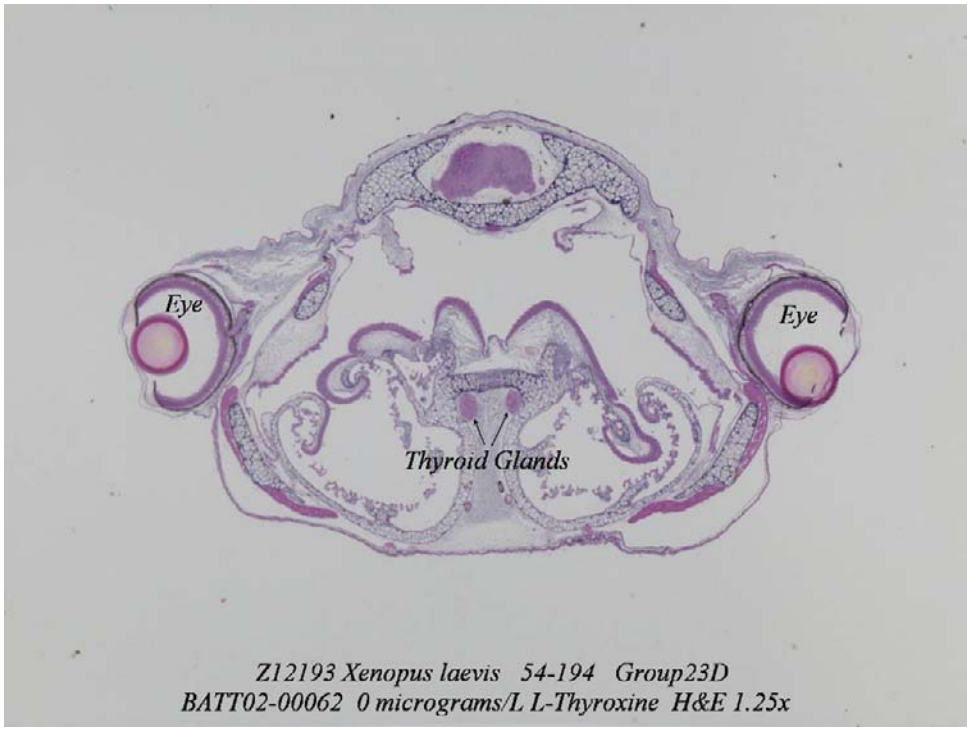
Appendix B:

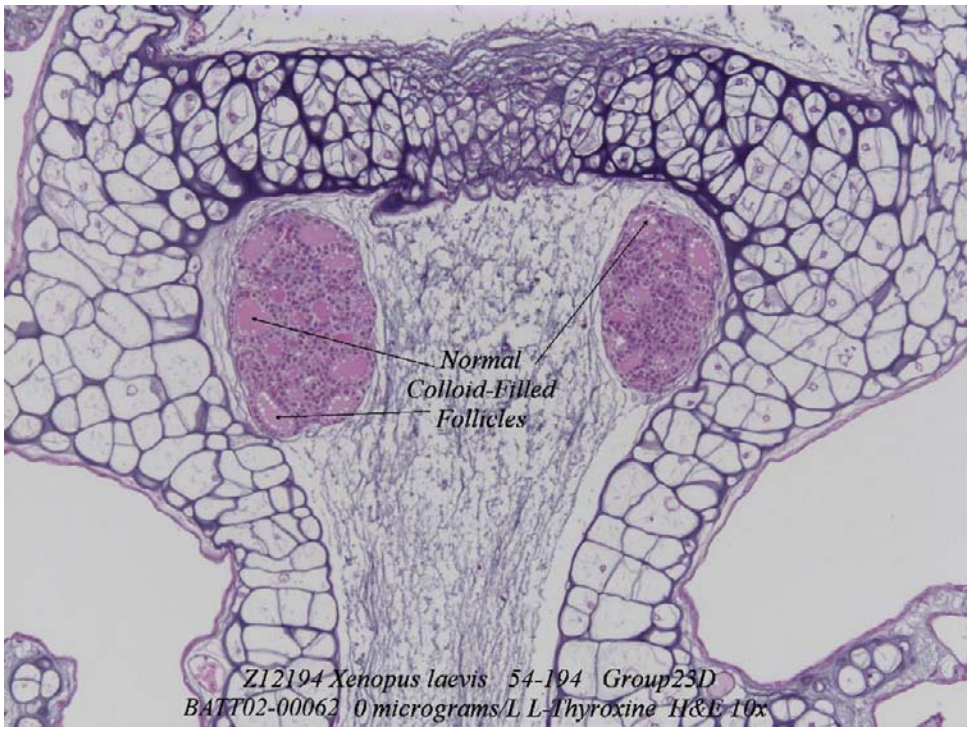
Stage 54 Photo Legends





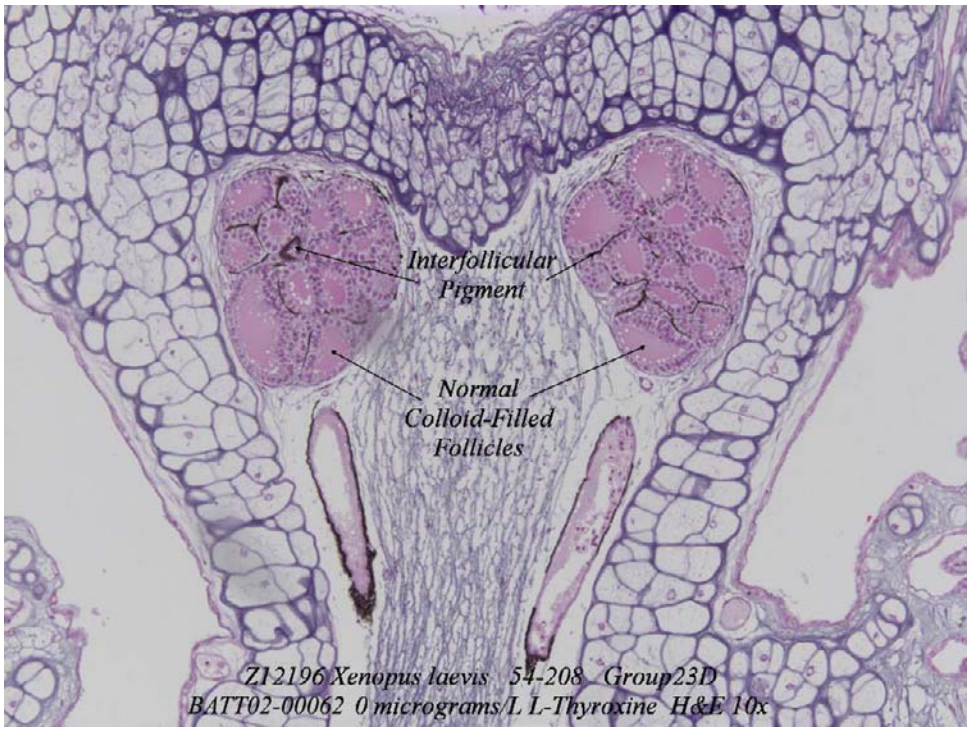
Z12189 (1.25) and Z12190 (10x). Thyroid glands of control frog 54-177. There is a variation in size of the normal colloid-filled thyroid follicles. There is a mild quantity of interfollicular pigment.



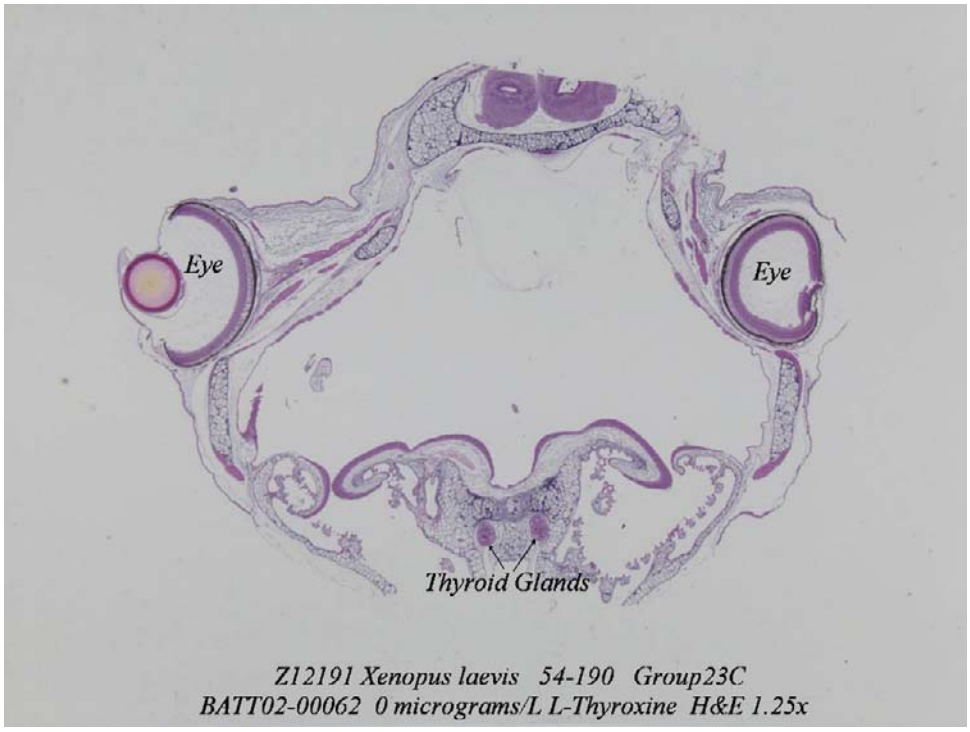


Z12193 (1.25x) and Z12194 (10x). Thyroid glands of control frog 54-194. There is a variation in size of the normal colloid-filled thyroid follicles. There is no interfollicular pigment.



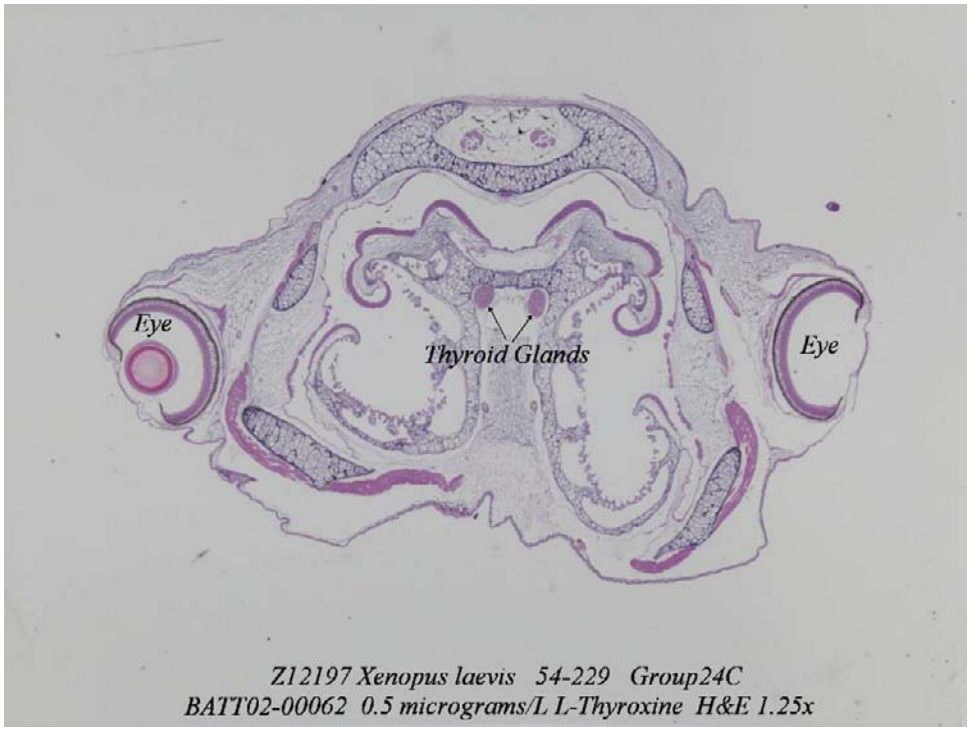


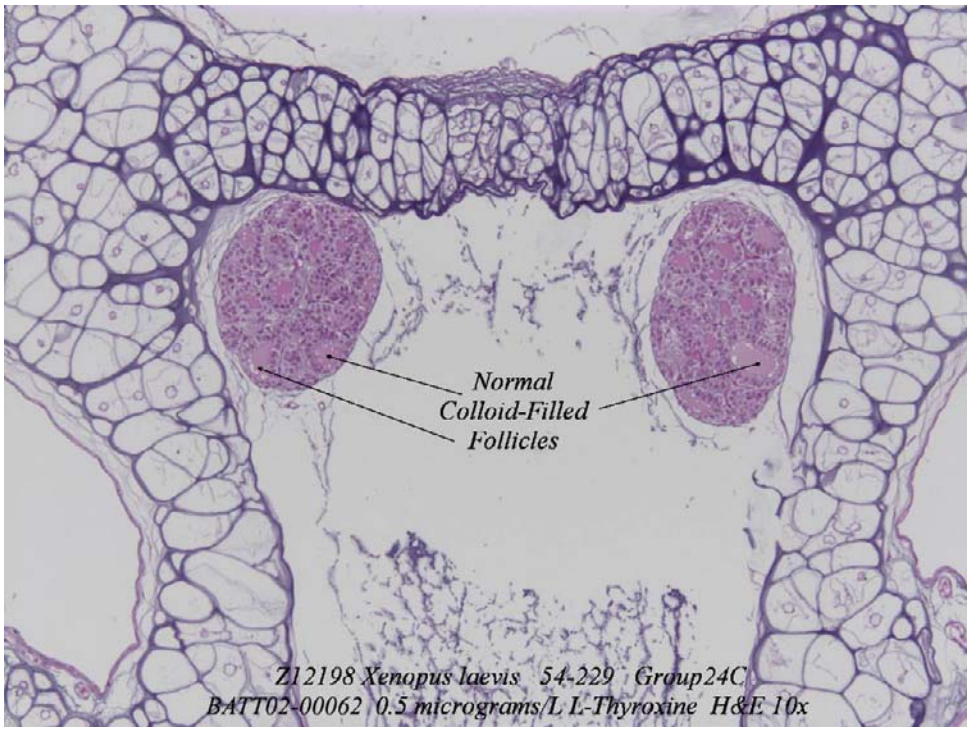
Z12195 (1.25x) and Z12196 (10x). Thyroid glands of control frog 54-208. There is a variation in size of the normal colloid-filled thyroid follicles. There is a mild quantity of interfollicular pigment.



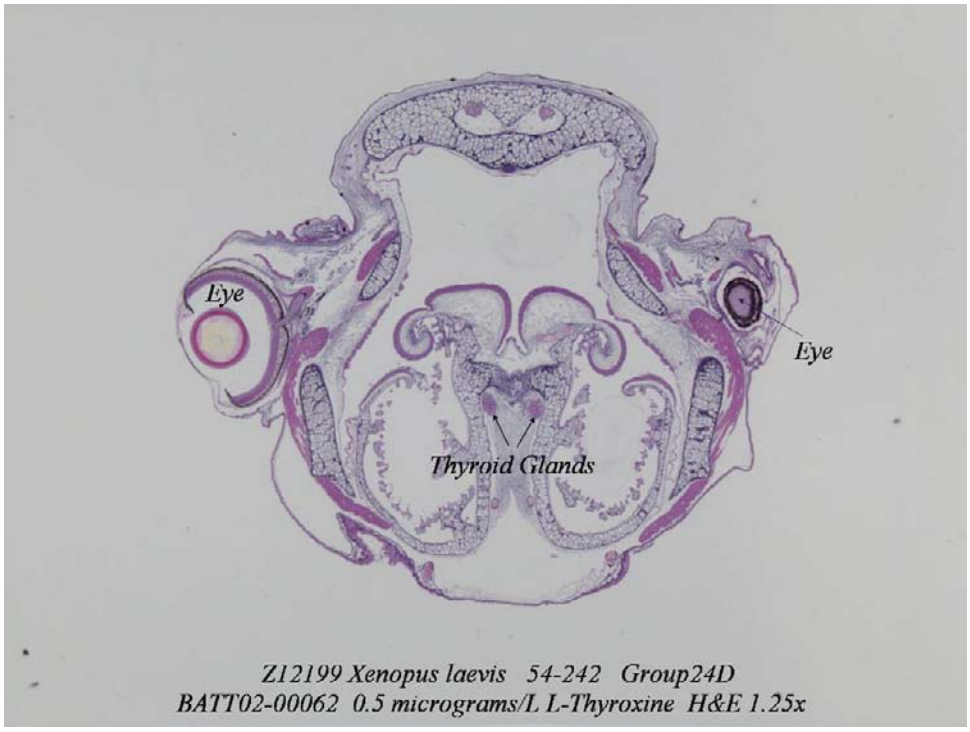


Z12191 (1.25x) and Z12192 (10x). Thyroid glands of control frog 54-190. There is minimal decreased quantity of colloid and a mild quantity of interfollicular pigment.





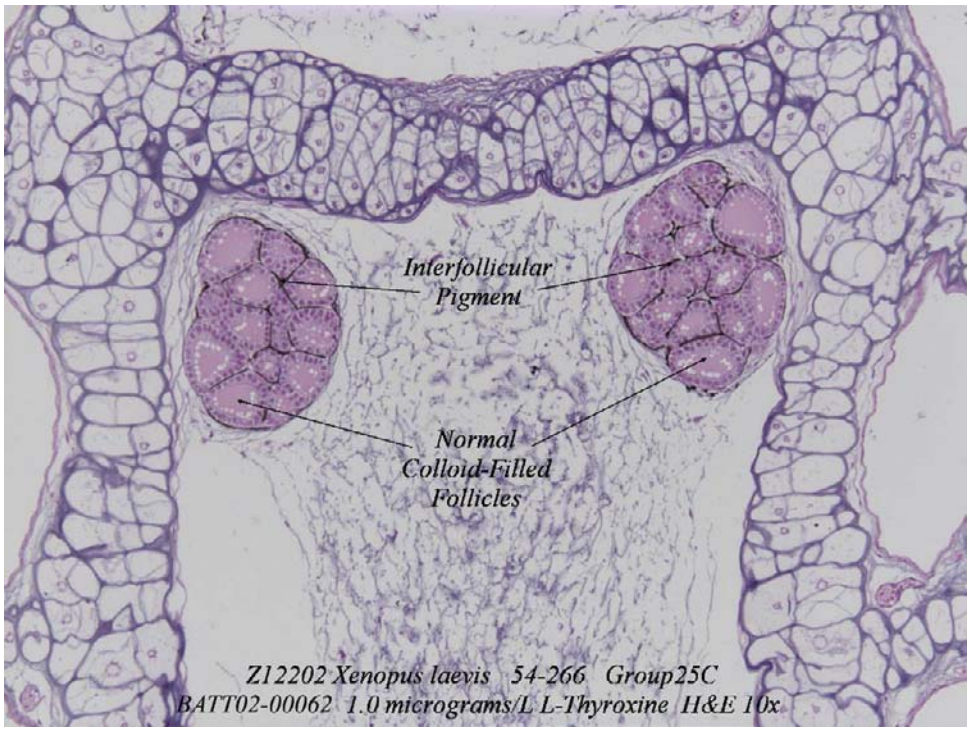
Z12197 (1.25x) and Z12198 (10x). Thyroid glands of frog 54-229 exposed to 0.5 µg/L L-thyroxine. The thyroid follicles are normal and colloid-filled.



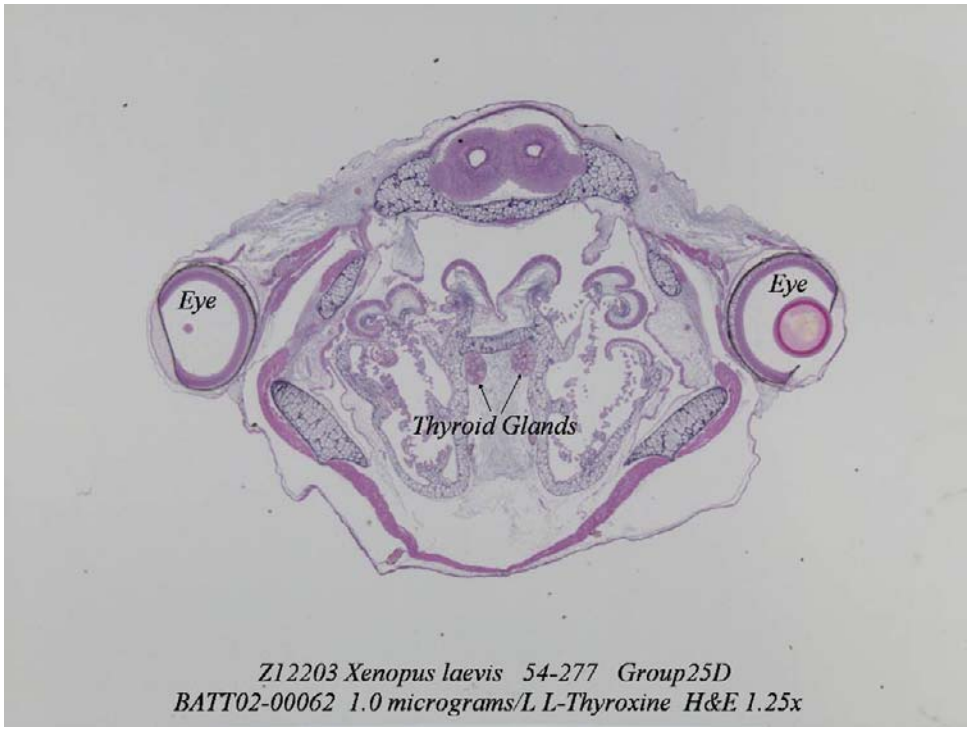


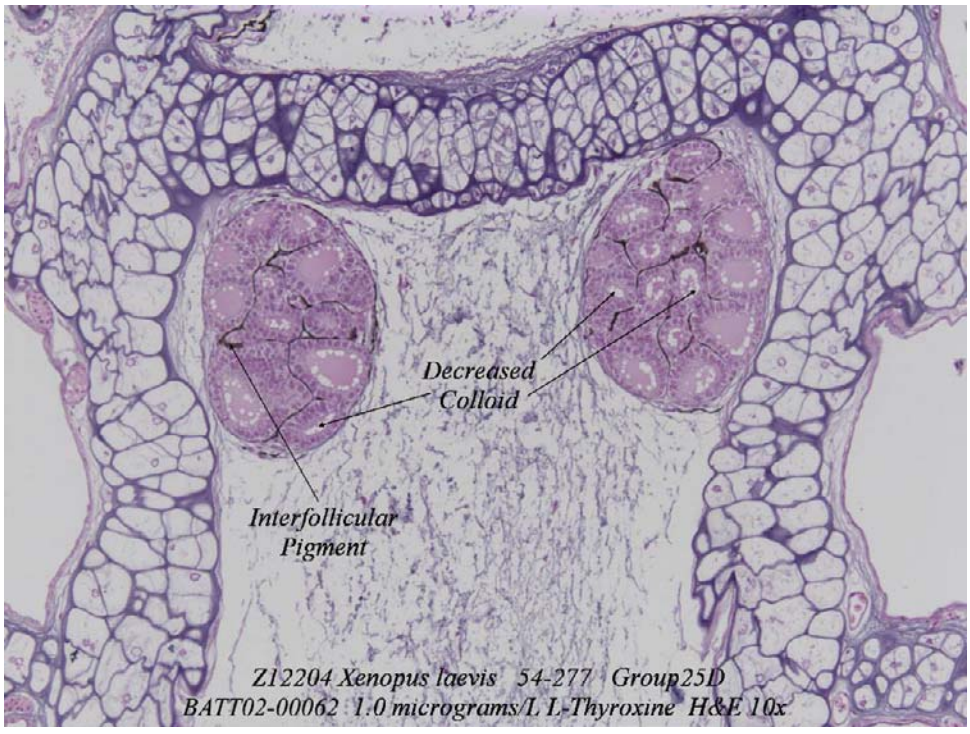
Z12199 (1.25x) and Z12200 (10x). Thyroid glands of frog 54-242 exposed to 0.5 µg/L L-thyroxine. There is a mild decreased quantity of colloid and a mild quantity of interfollicular pigment.



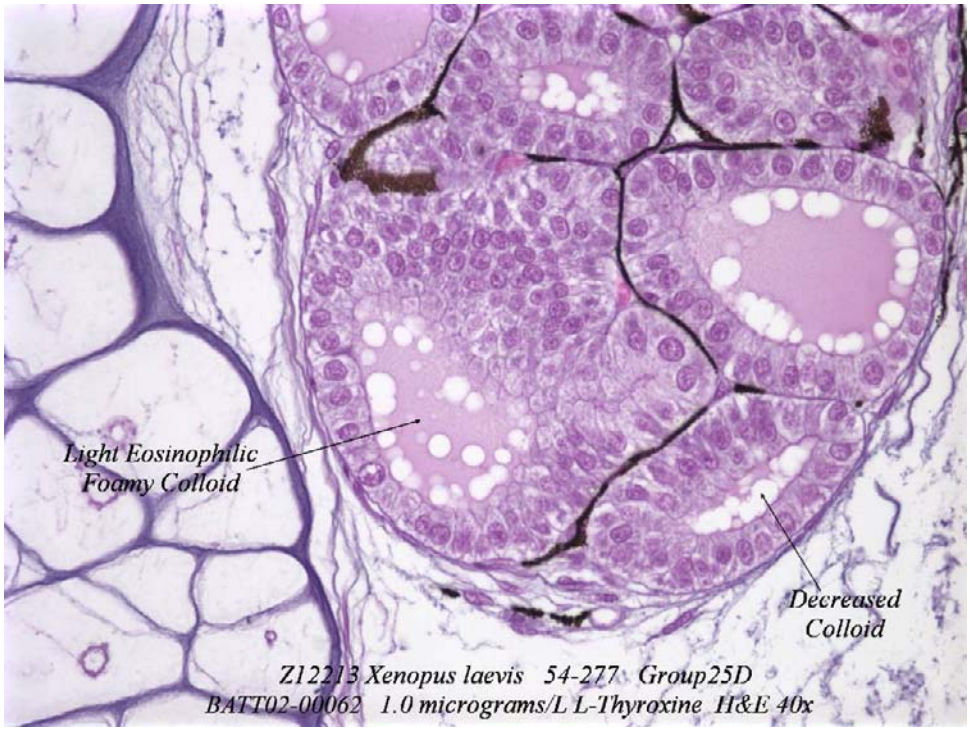


Z12201 (1.25x) and Z12202 (10x). Thyroid glands of frog 54-266 exposed to 1.0 µg/L L-thyroxine. The thyroid follicles are normal and colloid-filled. There is a moderate quantity of interfollicular pigment.



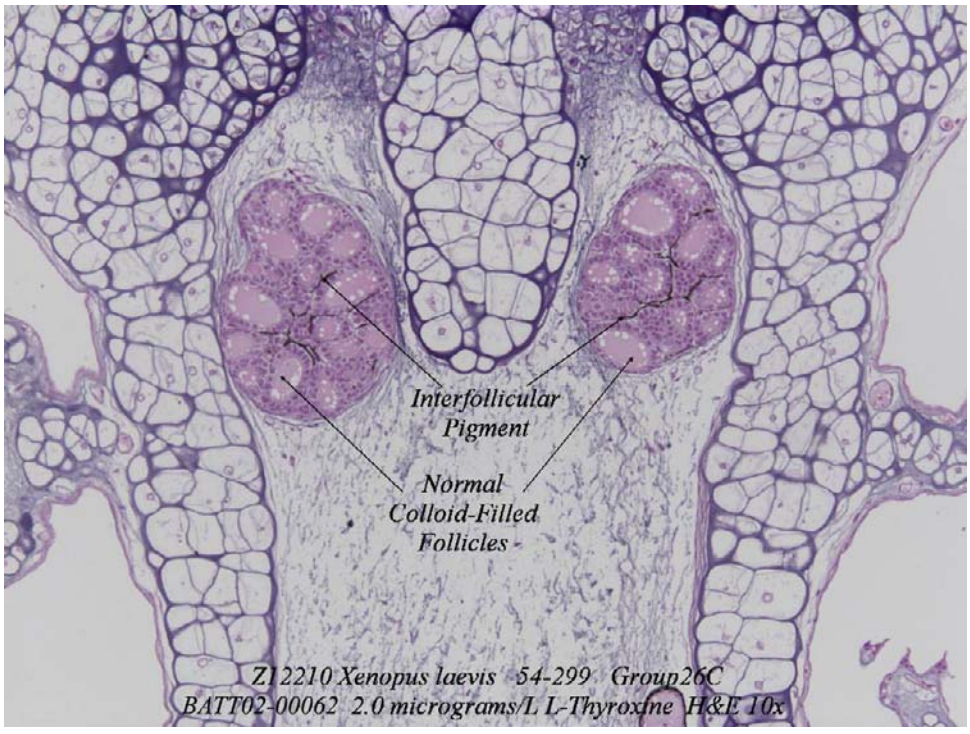


Z12203 (1.25x) and Z12204 (10x). Thyroid glands of frog 54-277 exposed to 1.0 µg/L L-thyroxine. There is minimal decreased quantity of colloid and a mild quantity of interfollicular pigment.

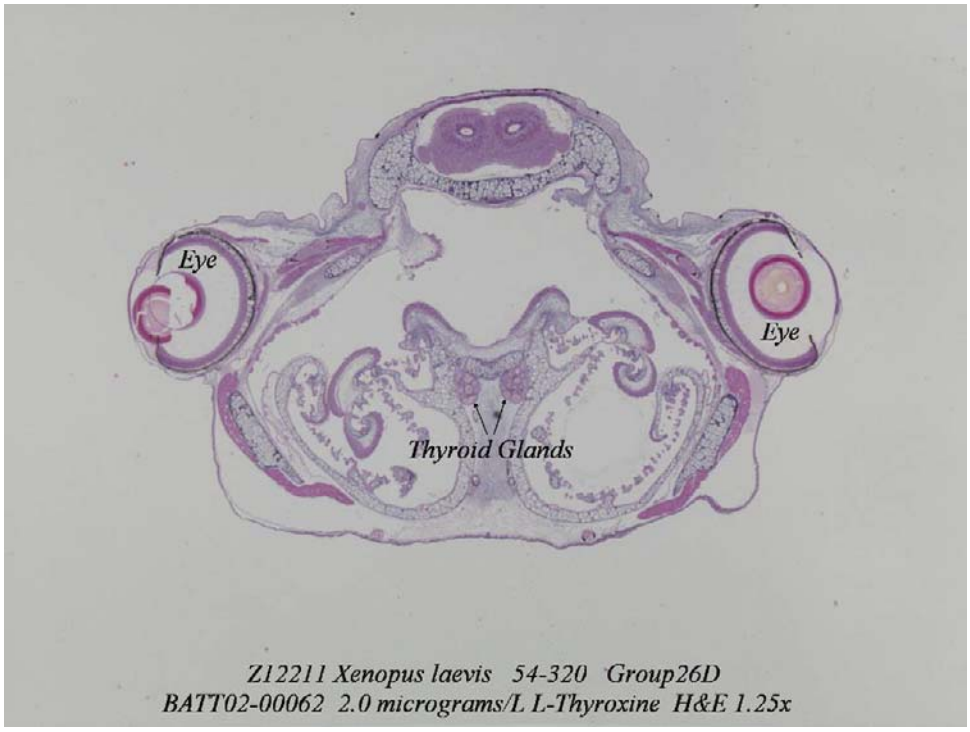


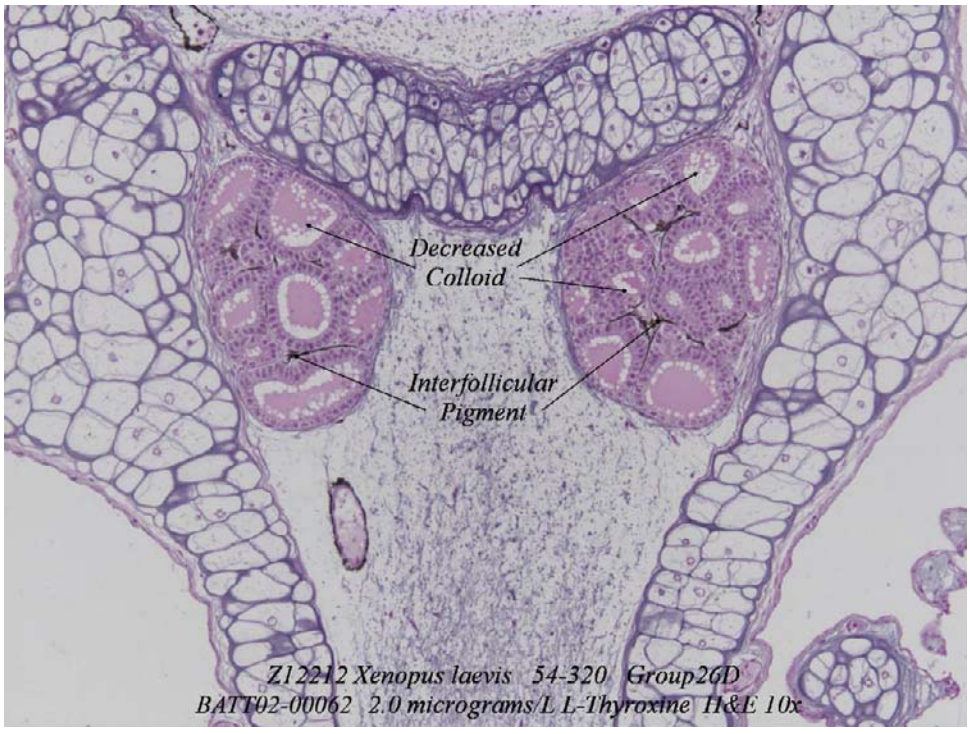
Z12213 (40x). Thyroid gland of frog 54-277 exposed to 1.0 µg/L L-thyroxine. There is minimal light eosinophilic foamy colloid in the thyroid follicle as compared to the normal eosinophilic homogeneous colloid.



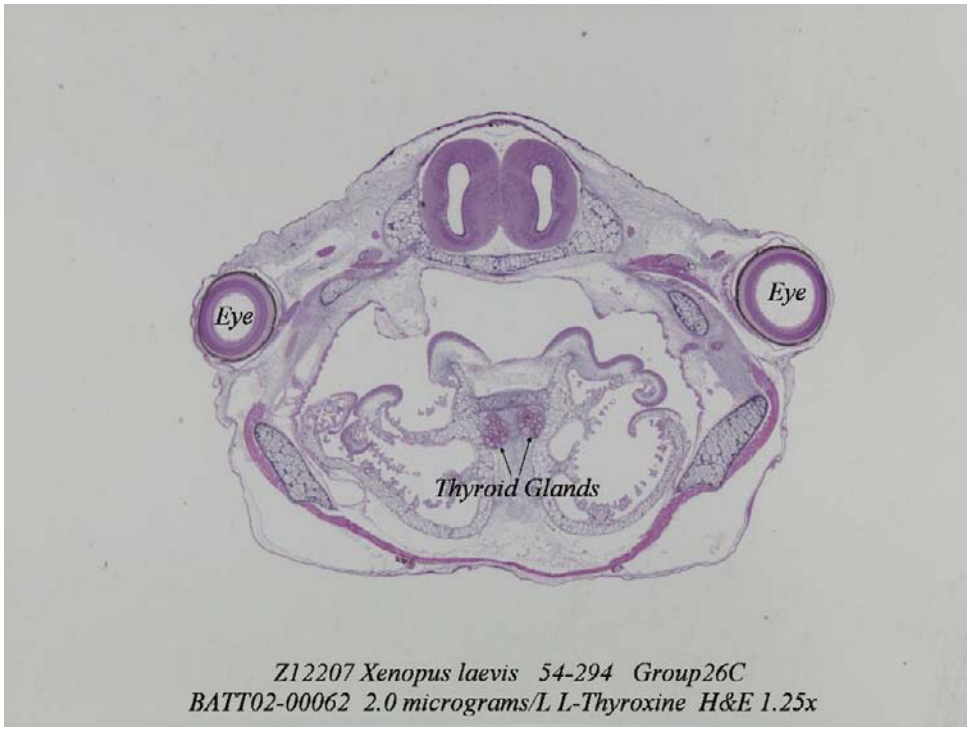


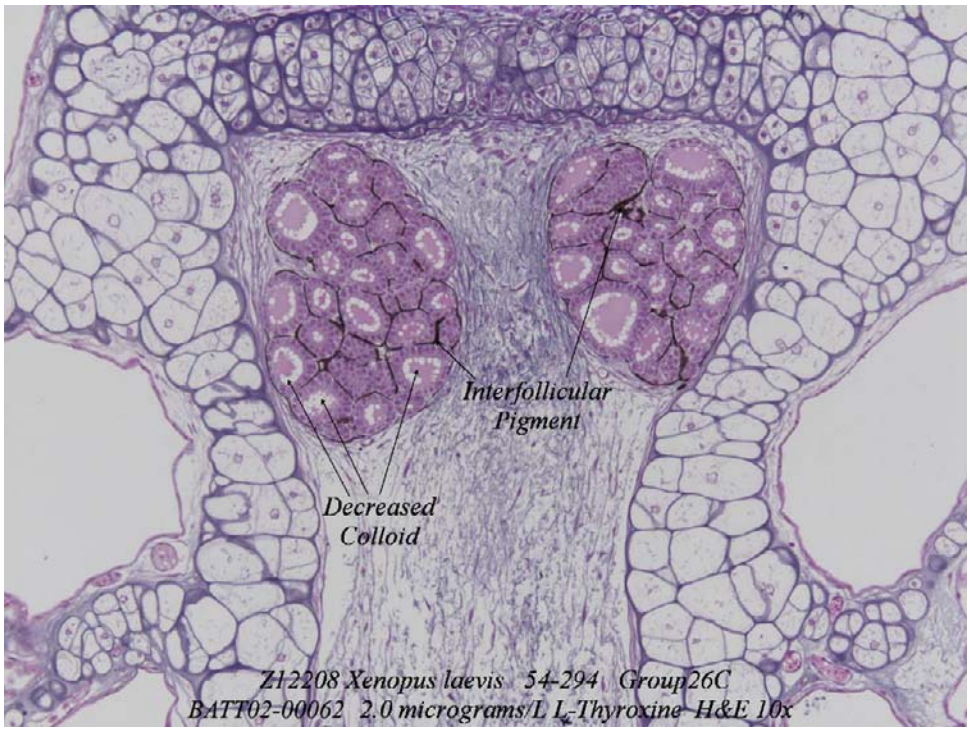
Z12209 (1.25x) and Z12210 (10x). Thyroid glands of frog 54-299 exposed to 2.0 µg/L L-thyroxine. The thyroid follicles are normal and colloid-filled. There is a mild quantity of interfollicular pigment.



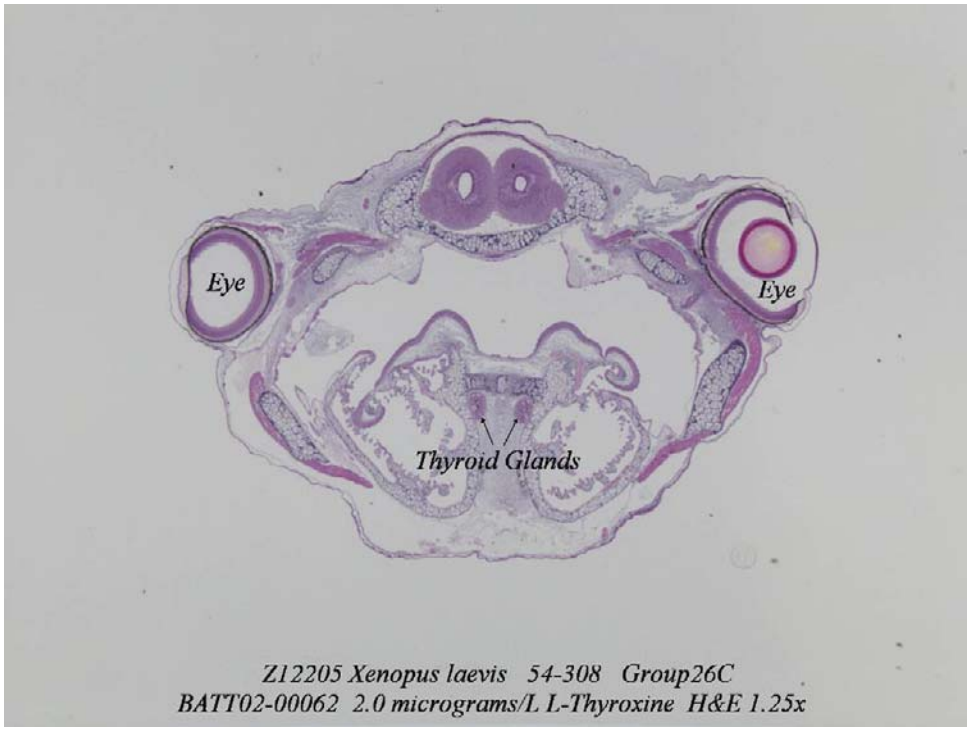


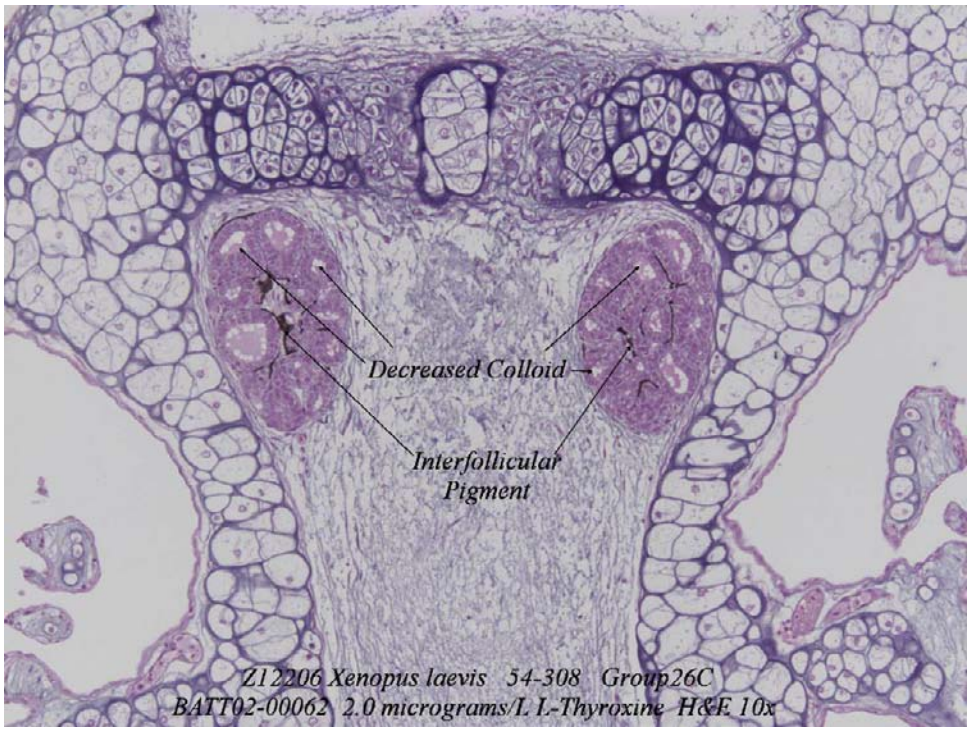
Z12211 (1.25x) and Z12212 (10x). Thyroid glands of frog 54-320 exposed to 2.0 µg/L L-thyroxine. There is minimal decreased quantity of colloid and a mild quantity of interfollicular pigment.





Z12207 (1.25x) and Z12208 (10x). Thyroid glands of frog 54-294 exposed to 2.0 µg/L L-thyroxine. There is a mild decreased quantity of colloid and a moderate quantity of interfollicular pigment.





Z12205 (1.25x) and Z12206 (10x). Thyroid glands of frog 54-308 exposed to 2.0 µg/L L-thyroxine. There is a moderate decreased quantity of colloid and a mild quantity of interfollicular pigment.



Z12214 (40x). Thyroid gland of frog 54-308 exposed to 2.0 µg/L L-thyroxine. There is minimal light eosinophilic foamy colloid in the thyroid follicle as compared to the normal eosinophilic homogeneous colloid.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	
P.O. #:	

BATT#2 - 00062

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
019	Specimen #'s: 008, 012, 015,	11/29/04 1300	10	G	10% Formalin	Histological examination of thyroid gland as per protocol # BATT#2 - 1 ①
↓	017, 019, 022, 024, 027,	↓ ↓	↓	↓	↓	↓
↓	029, 031					
020	Specimen #'s: 041, 046, 050,		10	G		
↓	053, 058, 064, 067, 070,		↓	↓	↓	↓
↓	073, 077					
021	Specimen #'s: 082, 086, 089,		10	G		
↓	092, 098, 102, 105, 111,		↓	↓	↓	↓
↓	114, 117					
022	Specimen #'s: 124, 128, 133,		10	G		
↓	136, 139, 145, 149, 151,		↓	↓	↓	↓
↓	154, 157					
023	Specimen #'s: 175, 177, 183,		10	G		
↓	188, 190, 194, 195, 206,		↓	↓	↓	↓
↓	208, 209	↓ ↓				

SPECIAL INSTRUCTIONS: Organisms exposed to L-thyroxine (T4). No samples from T4 40 ng/L Rep. A & B sent due to high mortality.

DATE:
INITIALS:

Andria Walker	12/2/04 1000	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

① N/A - JT - 12/2/04

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

Client:	BatTelle
Submit Report To:	Doug Fort
Sampler's Signature:	
P.O. #:	

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

BATT02 - 00062

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
024	Specimen #'s: 213, 218, 225,	11/29/04 1300	10	G	10% formalin	Histological examination of thyroid gland as per protocol # BATT02-1 (1)
↓	227, 229, 237, 239, 242,	↓ ↓			↓	↓
↓	244, 245					
025	Specimen #'s: 253, 255, 258,		10	G		
↓	264, 266, 271, 274, 277,					
↓	283, 287					
026	Specimen #'s: 292, 294, 299,		10	G		
↓	308, 310, 311, 315, 318,					
↓	320, 322					
027	Specimen #'s: 337, 341, 344,		10	G		
↓	346, 348, 350, 354, 356					
↓	364, 368	↓ ↓			↓	↓

SPECIAL INSTRUCTIONS: Organisms exposed to L-thyroxine (T4).

DATE:
INITIALS:

Andrea Walker	12/2/04 1000	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

(1) N/A - ST - 12/2/04

Appendix F
Dexamethasone

Metamorphosis Assay – Raw Data:

Daily Operational Data
Test Termination Data – Stage and Growth
Statistical Data – Stage and Growth

Test Chemical Analytical Data from Battelle:

Purity / Stability Report
In-Life Test Chemical Analysis Report

Thyroid Histology Data from EPL:

Sample Identification Key
Thyroid Pathology Report

Dexamethasone Metamorphosis Assay: Daily Operational Data

DAILY DATA

Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 0.0 ug/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc[1] (µg/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/15/04			100.0	22.6	7.4	7.2	1245	26			100.0	22.6	7.3	7.1	1345	26	100.0	22.6	7.4	7.2	1295	26	0.68
1	07/16/04		20	100.0	20.3	7.1	7.0	1379	27		20	100.0	20.4	7.2	7.2	1665	27	100.0	20.4	7.2	7.1	1522	27	
2	07/17/04		20	100.0	21.6	7.0	6.9	1391	26		20	100.0	21.7	7.2	7.0	1576	26	100.0	21.7	7.1	7.0	1484	26	
3	07/18/04		20	100.0	21.7	6.9	6.7	1409	27		20	100.0	21.9	7.1	7.2	1515	27	100.0	21.8	7.0	7.0	1462	27	
4	07/19/04		20	100.0	21.9	7.1	6.5	1405	27		20	100.0	21.8	7.2	6.6	1515	27	100.0	21.9	7.2	6.6	1460	27	
5	07/20/04		20	100.0	21.7	6.9	6.1	1394	26		20	100.0	21.8	7.0	6.5	1525	26	100.0	21.8	7.0	6.3	1460	26	
6	07/21/04		20	100.0	22.0	7.1	6.1	1380	26		20	100.0	22.1	7.1	6.0	1515	26	100.0	22.1	7.1	6.1	1448	26	0.68
7	07/22/04		20	100.0	22.0	7.0	6.3	1370	25		20	100.0	22.0	7.0	6.4	1620	25	100.0	22.0	7.0	6.4	1495	25	
8	07/23/04		20	100.0	20.9	7.0	6.3	1297	25		20	100.0	21.1	7.1	6.3	1444	25	100.0	21.0	7.1	6.3	1371	25	
9	07/24/04		20	100.0	20.7	7.1	6.2	1305	25		20	100.0	20.7	7.1	6.1	1400	25	100.0	20.7	7.1	6.2	1353	25	
10	07/25/04		20	100.0	21.0	6.9	5.9	1385	25		20	100.0	20.9	7.0	5.8	1485	25	100.0	21.0	7.0	5.9	1435	25	
11	07/26/04		20	100.0	20.2	7.0	5.7	1390	25		20	100.0	20.4	7.0	5.7	1445	25	100.0	20.3	7.0	5.7	1418	25	
12	07/27/04		20	100.0	20.4	6.9	6.1	1326	25		20	100.0	20.5	7.0	6.3	1369	25	100.0	20.5	7.0	6.2	1348	25	
13	07/28/04		20	100.0	20.4	7.0	6.1	1275	25		20	100.0	20.3	7.0	6.1	1405	25	100.0	20.4	7.0	6.1	1340	25	
14	07/29/04		20	100.0	20.4	6.9	6.1	1399	25		20	100.0	20.3	6.9	6.0	1530	25	100.0	20.4	6.9	6.1	1465	25	0.68
15	07/30/04		20	100.0	20.1	6.7	5.8	1357	24		20	100.0	20.2	6.8	5.4	1509	24	100.0	20.2	6.8	5.6	1433	24	
16	07/31/04		20	100.0	20.0	6.7	6.4	1250	25		20	100.0	20.1	6.9	6.2	1408	25	100.0	20.1	6.8	6.3	1329	25	
17	08/01/04		20	100.0	20.2	6.7	5.8	1494	23		20	100.0	20.1	6.8	5.4	1588	23	100.0	20.2	6.8	5.6	1541	23	
18	08/02/04		20	100.0	21.0	6.8	5.5	1364	23		20	100.0	20.8	6.8	4.9	1418	23	100.0	20.9	6.8	5.2	1391	23	
19	08/03/04		20	100.0	20.1	6.9	5.7	1320	23		20	100.0	20.2	6.9	5.1	1539	23	100.0	20.2	6.9	5.4	1430	23	
20	08/04/04		20	100.0	20.2	6.8	5.9	1280	23		20	100.0	20.3	6.9	6.0	1470	23	100.0	20.3	6.9	6.0	1375	23	0.68
21	08/05/04		20	100.0							20	100.0						100.0						
	Median:				**20.7**	**6.9**	**6.1**	**1370**	**25**				**20.7**	**7.0**	**6.1**	**1509**	**25**		**20.7**	**7.0**	**6.2**	**1433**	**25**	
	Mode:				**20.6**	**6.9**	**6.1**	**#N/A**	**25**				**20.3**	**7.0**	**6.0**	**1515**	**25**		**20.4**	**7.0**	**7.0**	**#N/A**	**25**	
	Mean:			**100.0**	**20.9**	**6.9**	**6.2**	**1353**	**25.0**			**100.0**	**21.0**	**7.0**	**6.2**	**1490**	**25.0**	**100.0**	**20.9**	**7.0**	**6.2**	**1421**	**25.0**	**0.680**
	SEM:			**0.00**	**0.17**	**0.04**	**0.10**	**13.46**	**0.28**			**0.00**	**0.17**	**0.03**	**0.14**	**18.22**	**0.28**	**0.00**	**0.17**	**0.03**	**0.12**	**14.49**	**0.28**	**0.00**
	% CV:			**0.00**	**17.49**	**6.31**	**18.11**	**167.73**	**25.65**			**0.00**	**17.48**	**5.38**	**26.20**	**216.34**	**25.65**	**0.00**	**17.44**	**5.66**	**21.77**	**176.18**	**25.65**	**0.00**

[1]All measured test concentrations reported by Battelle as less than method detection limit (MDL) [1.36 µg/L] or none detected (ND) are expressed as 0.68 µg/L (1/2 of MDL).

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 1.0 ug/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/15/04			100.0	22.7	7.3	7.1	880	26			100.0	22.8	7.5	7.0	1295	26	100.0	22.8	7.4	7.1	1088	26	
1	07/16/04		20	100.0	20.6	7.3	6.8	1123	26		20	100.0	20.5	7.3	6.8	1768	26	100.0	20.6	7.3	6.8	1446	26	
2	07/17/04		20	100.0	21.6	7.2	6.8	1205	27		20	100.0	21.8	7.3	6.7	1761	27	100.0	21.7	7.3	6.8	1483	27	
3	07/18/04		20	100.0	21.7	7.2	6.6	1053	27		20	100.0	21.9	7.2	6.3	1628	27	100.0	21.8	7.2	6.5	1341	27	
4	07/19/04		20	100.0	21.8	7.2	6.5	1090	27		20	100.0	21.9	7.2	6.2	1660	27	100.0	21.9	7.2	6.4	1375	27	
5	07/20/04		20	100.0	21.8	7.0	5.9	969	26		20	100.0	21.9	7.1	5.8	1504	26	100.0	21.9	7.1	5.9	1237	26	
6	07/21/04		20	100.0	22.1	7.1	6.0	1000	26		20	100.0	22.2	7.2	6.0	1512	26	100.0	22.2	7.2	6.0	1256	26	
7	07/22/04		20	100.0	22.0	7.0	6.1	1081	26		20	100.0	22.0	7.0	6.0	1299	26	100.0	22.0	7.0	6.1	1190	26	
8	07/23/04		20	100.0	21.1	7.1	6.1	922	25		20	100.0	20.9	7.0	6.1	1387	25	100.0	21.0	7.1	6.1	1155	25	
9	07/24/04		20	100.0	20.6	7.0	6.0	1105	25		20	100.0	20.7	7.0	6.1	1350	25	100.0	20.7	7.0	6.1	1228	25	
10	07/25/04		20	100.0	20.8	7.1	5.8	1190	26		20	100.0	20.9	7.2	5.9	1335	26	100.0	20.9	7.2	5.9	1263	26	
11	07/26/04		20	100.0	20.5	7.1	5.6	1195	25		20	100.0	20.5	7.0	5.6	1380	25	100.0	20.5	7.1	5.6	1288	25	
12	07/27/04		20	100.0	20.6	7.0	6.0	1045	25		20	100.0	20.3	7.0	6.1	1478	25	100.0	20.5	7.0	6.1	1262	25	
13	07/28/04		20	100.0	20.4	7.0	5.9	1115	25		20	100.0	20.4	7.1	6.0	1535	25	100.0	20.4	7.1	6.0	1325	25	
14	07/29/04		20	100.0	20.3	7.0	5.8	949	25		20	100.0	20.4	7.0	5.9	1475	25	100.0	20.4	7.0	5.9	1212	25	
15	07/30/04		20	100.0	20.2	6.9	5.6	960	25		20	100.0	20.1	6.9	5.3	1560	25	100.0	20.2	6.9	5.5	1260	25	
16	07/31/04		20	100.0	20.0	6.9	6.1	1014	25		20	100.0	20.1	7.0	6.1	1510	25	100.0	20.1	7.0	6.1	1262	25	
17	08/01/04		20	100.0	20.3	7.0	5.6	1039	25		20	100.0	20.0	6.9	5.5	1672	25	100.0	20.2	7.0	5.6	1356	25	
18	08/02/04		20	100.0	20.9	6.9	5.0	993	24		20	100.0	20.7	6.9	4.8	1518	24	100.0	20.8	6.9	4.9	1256	24	
19	08/03/04		20	100.0	20.3	7.0	5.5	1050	24		20	100.0	20.2	7.0	4.9	1599	24	100.0	20.3	7.0	5.2	1325	24	
20	08/04/04		20	100.0	20.3	6.9	5.6	1105	24		20	100.0	20.4	7.0	4.7	1610	24	100.0	20.4	7.0	5.2	1358	24	
21	08/05/04		20	100.0							20	100.0						100.0						
	Median:				**20.6**	**7.0**	**6.0**	**1050**	**25**				**20.7**	**7.0**	**6.0**	**1512**	**25**		**20.7**	**7.1**	**6.0**	**1262**	**25**	
	Mode:				**20.6**	**7.0**	**5.6**	**1105**	**25**				**21.9**	**7.0**	**6.1**	**#N/A**	**25**		**21.9**	**7.0**	**5.9**	**#N/A**	**25**	
	Mean:			**100.0**	**21.0**	**7.1**	**6.0**	**1052**	**25.4**			**100.0**	**21.0**	**7.1**	**5.9**	**1516**	**25.4**	**100.0**	**21.0**	**7.1**	**6.0**	**1284**	**25.4**	**#DIV/0!**
	SEM:			**0.00**	**0.17**	**0.03**	**0.11**	**19.52**	**0.20**			**0.00**	**0.18**	**0.03**	**0.13**	**30.62**	**0.20**	**0.00**	**0.18**	**0.03**	**0.12**	**20.09**	**0.20**	**#DIV/0!**
	% CV:			**0.00**	**16.89**	**4.70**	**20.61**	**275.91**	**18.36**			**0.00**	**18.44**	**5.85**	**25.00**	**360.43**	**18.36**	**0.00**	**17.61**	**5.10**	**22.30**	**256.92**	**18.36**	**#DIV/0!**

DAILY DATA

Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 3.9 ug/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/15/04			100.0	22.8	7.4	7.3	1420	25			100.0	22.8	7.4	7.1	1360	25	100.0	22.8	7.4	7.2	1390	25	
1	07/16/04		20	100.0	20.2	7.3	6.8	1522	25		20	100.0	20.3	7.3	7.0	1347	25	100.0	20.3	7.3	6.9	1435	25	
2	07/17/04		20	100.0	21.7	7.3	6.6	1657	24		20	100.0	21.5	7.3	6.8	1261	24	100.0	21.6	7.3	6.7	1459	24	
3	07/18/04		20	100.0	21.8	7.2	6.2	1676	24		20	100.0	21.7	7.2	6.2	1222	24	100.0	21.8	7.2	6.2	1449	24	
4	07/19/04		20	100.0	22.0	7.3	6.1	1621	24		20	100.0	21.9	7.2	6.2	1290	24	100.0	22.0	7.3	6.2	1456	24	
5	07/20/04		20	100.0	21.7	7.1	5.6	1472	25		20	100.0	21.4	7.1	6.0	1211	25	100.0	21.6	7.1	5.8	1342	25	
6	07/21/04		20	100.0	22.2	7.1	5.7	1490	25		20	100.0	22.2	7.1	5.8	1270	25	100.0	22.2	7.1	5.8	1380	25	
7	07/22/04		20	100.0	22.0	7.0	5.8	1433	24		20	100.0	21.9	7.0	6.1	1224	24	100.0	22.0	7.0	6.0	1329	24	
8	07/23/04		20	100.0	20.9	7.1	6.0	1361	24		20	100.0	20.9	7.1	6.4	1129	24	100.0	20.9	7.1	6.2	1245	24	
9	07/24/04		20	100.0	20.6	7.0	6.3	1295	24		20	100.0	20.6	7.0	5.9	1215	24	100.0	20.6	7.0	6.1	1255	24	
10	07/25/04		20	100.0	21.0	7.1	5.7	1275	25		20	100.0	21.1	7.1	5.6	1250	25	100.0	21.1	7.1	5.7	1263	25	
11	07/26/04		20	100.0	20.4	7.0	5.6	1245	25		20	100.0	20.3	7.2	5.7	1230	25	100.0	20.4	7.1	5.7	1238	25	
12	07/27/04		20	100.0	20.3	7.1	6.2	1418	23		20	100.0	20.4	7.1	6.3	1027	23	100.0	20.4	7.1	6.3	1223	23	
13	07/28/04		20	100.0	20.4	7.0	5.9	1470	23		20	100.0	20.3	7.1	5.8	1080	23	100.0	20.4	7.1	5.9	1275	23	
14	07/29/04		20	100.0	20.4	7.0	6.0	1575	23		20	100.0	20.2	7.0	6.0	1172	23	100.0	20.3	7.0	6.0	1374	23	
15	07/30/04		20	100.0	20.0	6.9	5.7	1547	23		20	100.0	20.1	7.0	5.9	1211	23	100.0	20.1	7.0	5.8	1379	23	
16	07/31/04		20	100.0	20.0	7.1	6.1	1571	23		20	100.0	20.0	7.1	6.4	1193	23	100.0	20.0	7.1	6.3	1382	23	
17	08/01/04		20	100.0	20.3	6.9	5.1	1750	23		20	100.0	20.0	7.0	5.6	1293	23	100.0	20.2	7.0	5.4	1522	23	
18	08/02/04		20	100.0	20.6	7.0	5.0	1504	23		20	100.0	20.5	7.0	5.4	1085	23	100.0	20.6	7.0	5.2	1295	23	
19	08/03/04		20	100.0	20.4	7.0	5.5	1570	23		20	100.0	20.4	7.0	5.8	1135	23	100.0	20.4	7.0	5.7	1353	23	
20	08/04/04		20	100.0	20.5	7.1	5.7	1475	23		20	100.0	20.4	7.0	5.8	1210	23	100.0	20.5	7.1	5.8	1343	23	
21	08/05/04		20	100.0							20	100.0						100.0						
	Median:				**20.6**	**7.1**	**5.9**	**1490**	**24**				**20.5**	**7.1**	**6.0**	**1215**	**24**		**20.6**	**7.1**	**6.0**	**1353**	**24**	
	Mode:				**20.6**	**7.1**	**5.7**	**#N/A**	**23**				**20.3**	**7.0**	**5.8**	**1211**	**23**		**20.4**	**7.1**	**5.7**	**#N/A**	**23**	
	Mean:			**100.0**	**21.0**	**7.1**	**5.9**	**1493**	**23.9**			**100.0**	**20.9**	**7.1**	**6.1**	**1210**	**23.9**	**100.0**	**20.9**	**7.1**	**6.0**	**1351**	**23.9**	**#DIV/0!**
	SEM:			**0.00**	**0.18**	**0.03**	**0.12**	**28.74**	**0.19**			**0.00**	**0.18**	**0.03**	**0.10**	**18.46**	**0.19**	**0.00**	**0.18**	**0.03**	**0.11**	**18.27**	**0.19**	**#DIV/0!**
	% CV:			**0.00**	**18.21**	**5.10**	**21.72**	**340.90**	**17.48**			**0.00**	**17.92**	**4.42**	**18.46**	**243.11**	**17.48**	**0.00**	**18.02**	**4.61**	**19.62**	**227.71**	**17.48**	**#DIV/0!**

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 15.6 ug/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/15/04			100.0	22.9	7.4	7.1	1405	25			100.0	22.9	7.4	7.1	1080	25	100.0	22.9	7.4	7.1	1243	25	
1	07/16/04		20	100.0	20.0	7.3	6.8	1791	25		20	100.0	20.3	7.3	6.9	1872	25	100.0	20.2	7.3	6.9	1832	25	
2	07/17/04		20	100.0	21.7	7.3	6.6	1670	25		20	100.0	21.9	7.3	6.6	1879	25	100.0	21.8	7.3	6.6	1775	25	
3	07/18/04		20	100.0	21.9	7.2	6.4	1659	25		20	100.0	21.8	7.2	6.4	1782	25	100.0	21.9	7.2	6.4	1721	25	
4	07/19/04		20	100.0	21.9	7.3	6.1	1640	25		20	100.0	22.0	7.3	6.1	1710	25	100.0	22.0	7.3	6.1	1675	25	
5	07/20/04		20	100.0	21.5	7.1	5.7	1794	24		20	100.0	21.6	7.1	5.6	1689	24	100.0	21.6	7.1	5.7	1742	24	
6	07/21/04		20	100.0	22.1	7.2	5.7	1760	25		20	100.0	22.2	7.2	5.7	1495	25	100.0	22.2	7.2	5.7	1628	25	
7	07/22/04		20	100.0	21.8	7.0	6.3	1555	24		20	100.0	21.8	7.1	6.9	1775	24	100.0	21.8	7.1	6.6	1665	24	
8	07/23/04		20	100.0	20.7	7.1	6.1	1671	24		20	100.0	21.0	7.1	6.0	1584	24	100.0	20.9	7.1	6.1	1628	24	
9	07/24/04		20	100.0	20.6	7.1	6.0	1650	24		20	100.0	20.5	7.1	6.1	1585	24	100.0	20.6	7.1	6.1	1618	24	
10	07/25/04		20	100.0	21.0	7.1	5.7	1615	24		20	100.0	21.0	7.0	5.8	1575	24	100.0	21.0	7.1	5.8	1595	24	
11	07/26/04		20	100.0	20.2	7.1	5.8	1610	24		20	100.0	20.3	7.2	5.6	1575	24	100.0	20.3	7.2	5.7	1593	24	
12	07/27/04		20	100.0	20.1	7.1	6.2	1622	22		20	100.0	20.2	7.1	6.2	1581	22	100.0	20.2	7.1	6.2	1602	22	
13	07/28/04		20	100.0	20.2	7.0	6.1	1605	23		20	100.0	20.2	7.1	6.0	1550	23	100.0	20.2	7.1	6.1	1578	23	
14	07/29/04		20	100.0	20.3	7.0	6.3	1685	23		20	100.0	20.1	7.0	6.0	1748	23	100.0	20.2	7.0	6.2	1717	23	
15	07/30/04		20	100.0	20.1	7.0	6.1	1701	23		20	100.0	20.0	7.0	5.9	1688	23	100.0	20.1	7.0	6.0	1695	23	
16	07/31/04		20	100.0	20.0	7.1	6.2	1603	23		20	100.0	20.0	7.1	6.7	1613	23	100.0	20.0	7.1	6.5	1608	23	
17	08/01/04		20	100.0	20.0	7.0	5.7	1750	23		20	100.0	20.0	7.0	6.2	1738	23	100.0	20.0	7.0	6.0	1744	23	
18	08/02/04		20	100.0	20.3	7.0	5.4	1570	23		20	100.0	20.5	7.0	5.8	1630	23	100.0	20.4	7.0	5.6	1600	23	
19	08/03/04		20	100.0	20.3	7.1	5.7	1646	23		20	100.0	20.3	7.1	6.0	1680	23	100.0	20.3	7.1	5.9	1663	23	
20	08/04/04		20	100.0	20.5	7.0	5.7	1555	23		20	100.0	20.4	7.0	5.7	1625	23	100.0	20.5	7.0	5.7	1590	23	
21	08/05/04		20	100.0							20	100.0						100.0						
	Median:				**20.5**	**7.1**	**6.1**	**1646**	**24**				**20.5**	**7.1**	**6.0**	**1630**	**24**		**20.5**	**7.1**	**6.1**	**1628**	**24**	
	Mode:				**20.4**	**7.1**	**5.7**	**1555**	**23**				**20.3**	**7.1**	**6.0**	**1575**	**23**		**20.2**	**7.1**	**6.1**	**1628**	**23**	
	Mean:			**100.0**	**20.9**	**7.1**	**6.1**	**1646**	**23.8**			**100.0**	**20.9**	**7.1**	**6.2**	**1641**	**23.8**	**100.0**	**20.9**	**7.1**	**6.1**	**1643**	**23.8**	**#DIV/0!**
	SEM:			**0.00**	**0.19**	**0.03**	**0.09**	**19.49**	**0.20**			**0.00**	**0.19**	**0.03**	**0.10**	**35.98**	**0.20**	**0.00**	**0.19**	**0.03**	**0.09**	**25.21**	**0.20**	**#DIV/0!**
	% CV:			**0.00**	**19.14**	**4.53**	**16.90**	**220.14**	**19.03**			**0.00**	**19.40**	**4.45**	**18.05**	**407.04**	**19.03**	**0.00**	**19.22**	**4.42**	**16.76**	**285.03**	**19.03**	**#DIV/0!**

DAILY DATA

Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 62.5 ug/L**

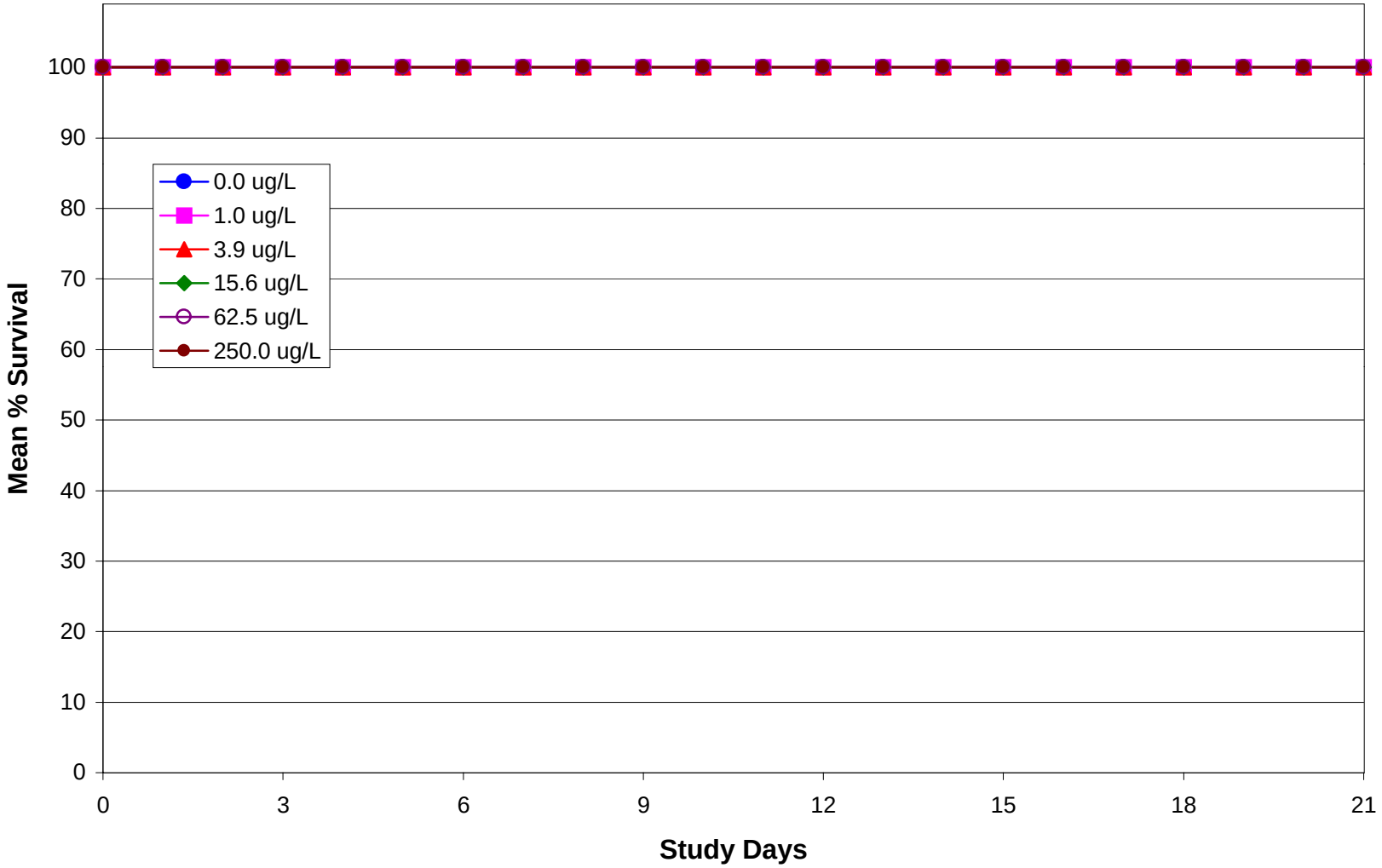
		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/15/04			100.0	22.8	7.5	7.1	1530	25			100.0	22.9	7.4	7.2	1190	25	100.0	22.9	7.5	7.2	1360	25	66.40
1	07/16/04		20	100.0	20.4	7.3	6.8	1551	25		20	100.0	20.5	7.3	7.0	1589	25	100.0	20.5	7.3	6.9	1570	25	
2	07/17/04		20	100.0	21.8	7.3	6.6	1601	25		20	100.0	21.4	7.4	7.0	1609	25	100.0	21.6	7.4	6.8	1605	25	
3	07/18/04		20	100.0	22.0	7.2	6.3	1440	26		20	100.0	21.9	7.3	6.6	1483	26	100.0	22.0	7.3	6.5	1462	26	
4	07/19/04		20	100.0	21.9	7.2	6.0	1480	26		20	100.0	22.0	7.3	6.4	1415	26	100.0	22.0	7.3	6.2	1448	26	
5	07/20/04		20	100.0	21.6	7.2	5.9	1552	26		20	100.0	21.8	7.2	6.0	1352	26	100.0	21.7	7.2	6.0	1452	26	
6	07/21/04		20	100.0	22.2	7.1	5.9	1390	26		20	100.0	22.2	7.1	5.8	1275	26	100.0	22.2	7.1	5.9	1333	26	68.80
7	07/22/04		20	100.0	21.7	7.1	6.4	1508	25		20	100.0	21.6	6.9	5.0	1477	25	100.0	21.7	7.0	5.7	1493	25	
8	07/23/04		20	100.0	21.0	7.1	6.2	1481	25		20	100.0	20.9	7.1	6.0	1231	25	100.0	21.0	7.1	6.1	1356	25	
9	07/24/04		20	100.0	20.5	7.0	5.8	1490	25		20	100.0	20.6	7.1	5.7	1210	25	100.0	20.6	7.1	5.8	1350	25	
10	07/25/04		20	100.0	21.0	7.0	5.8	1425	25		20	100.0	20.9	7.1	5.9	1275	25	100.0	21.0	7.1	5.9	1350	25	
11	07/26/04		20	100.0	20.2	7.0	5.5	1460	24		20	100.0	20.4	7.1	5.6	1275	24	100.0	20.3	7.1	5.6	1368	24	
12	07/27/04		20	100.0	20.4	7.1	6.5	1423	25		20	100.0	20.0	7.0	5.0	1311	25	100.0	20.2	7.1	5.8	1367	25	
13	07/28/04		20	100.0	20.3	7.0	6.0	1425	25		20	100.0	20.2	7.1	6.1	1395	25	100.0	20.3	7.1	6.1	1410	25	
14	07/29/04		20	100.0	20.3	7.0	6.0	1544	25		20	100.0	20.3	7.0	6.1	1345	25	100.0	20.3	7.0	6.1	1445	25	76.90
15	07/30/04		20	100.0	20.1	7.0	5.9	1467	25		20	100.0	20.0	7.0	5.9	1438	25	100.0	20.1	7.0	5.9	1453	25	
16	07/31/04		20	100.0	20.0	7.1	6.3	1417	24		20	100.0	20.0	7.1	6.5	1295	24	100.0	20.0	7.1	6.4	1356	24	
17	08/01/04		20	100.0	20.0	7.1	6.0	1665	25		20	100.0	20.2	7.1	5.7	1404	25	100.0	20.1	7.1	5.9	1535	25	
18	08/02/04		20	100.0	20.7	7.1	5.5	1529	25		20	100.0	20.7	7.1	5.6	1279	25	100.0	20.7	7.1	5.6	1404	25	
19	08/03/04		20	100.0	20.2	7.1	6.0	1394	25		20	100.0	20.2	7.1	5.9	1320	25	100.0	20.2	7.1	6.0	1357	25	
20	08/04/04		20	100.0	20.6	7.1	5.4	1330	25		20	100.0	20.5	7.1	5.9	1375	25	100.0	20.6	7.1	5.7	1353	25	79.40
21	08/05/04		20	100.0							20	100.0						100.0						
	Median:				**20.6**	**7.1**	**6.0**	**1480**	**25**				**20.6**	**7.1**	**5.9**	**1345**	**25**		**20.6**	**7.1**	**6.0**	**1404**	**25**	
	Mode:				**20.6**	**7.1**	**6.0**	**1425**	**25**				**20.0**	**7.1**	**5.9**	**1275**	**25**		**22.0**	**7.1**	**5.9**	**1356**	**25**	
	Mean:			**100.0**	**20.9**	**7.1**	**6.1**	**1481**	**25.1**			**100.0**	**20.9**	**7.1**	**6.0**	**1359**	**25.1**	**100.0**	**20.9**	**7.1**	**6.1**	**1420**	**25.1**	**72.875**
	SEM:			**0.00**	**0.18**	**0.03**	**0.09**	**17.07**	**0.12**			**0.00**	**0.19**	**0.03**	**0.13**	**24.91**	**0.12**	**0.00**	**0.18**	**0.03**	**0.10**	**17.18**	**0.12**	**3.13**
	% CV:			**0.00**	**18.41**	**4.73**	**17.28**	**203.26**	**10.76**			**0.00**	**18.56**	**4.95**	**23.67**	**309.58**	**10.76**	**0.00**	**18.40**	**4.60**	**17.98**	**208.90**	**10.76**	**73.25**

DAILY DATA

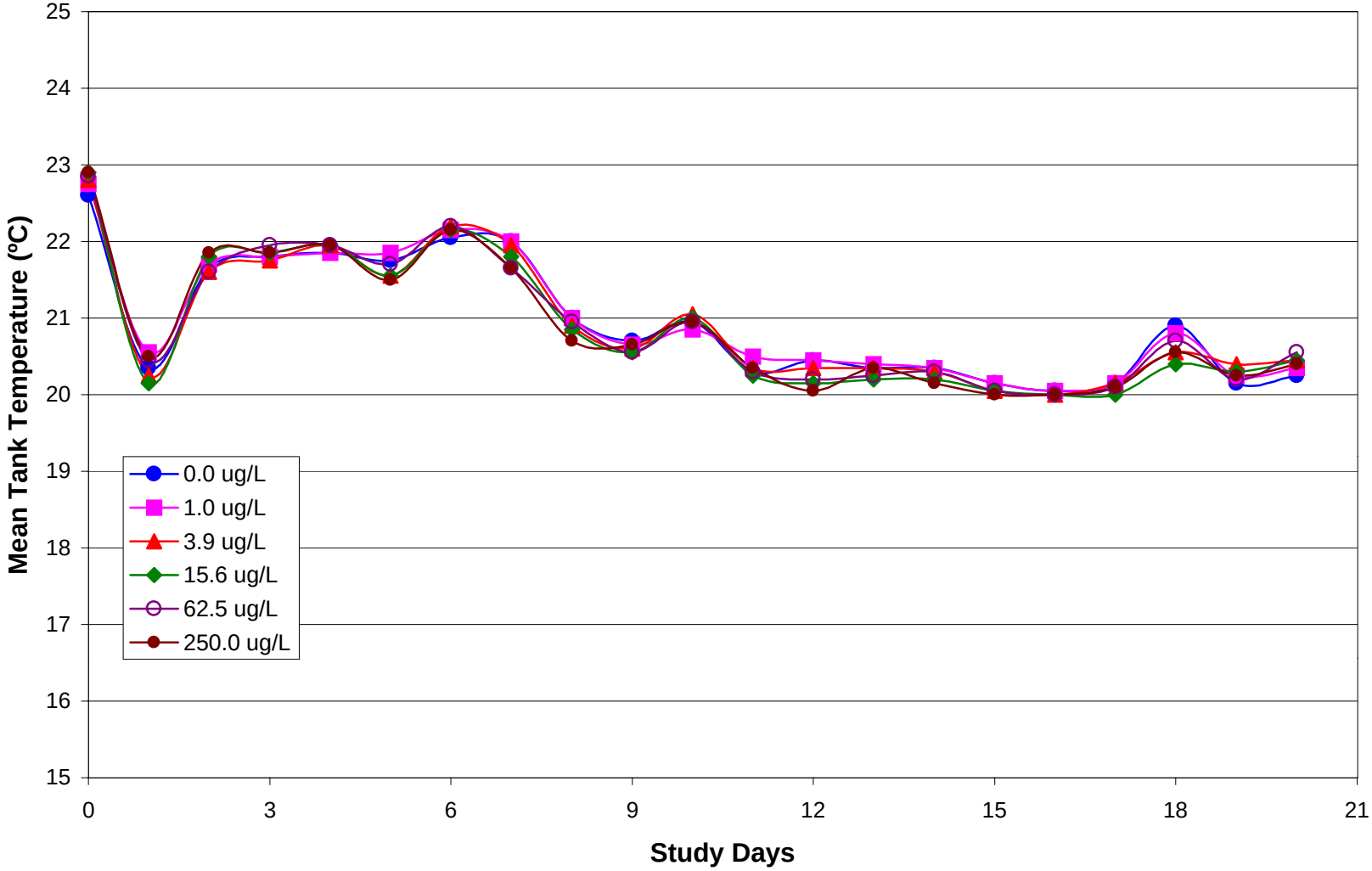
Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 250.0 ug/L**

		INITIAL STAGE 51 REPLICATE A								INITIAL STAGE 51 REPLICATE B								INITIAL STAGE 51 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/15/04			100.0	22.9	7.5	7.2	1560	25			100.0	22.9	7.4	7.3	1290	25	100.0	22.9	7.5	7.3	1425	25	226.90
1	07/16/04		20	100.0	20.5	7.3	6.8	1545	25		20	100.0	20.5	7.3	6.7	1796	25	100.0	20.5	7.3	6.8	1671	25	
2	07/17/04		20	100.0	21.7	7.3	6.5	1511	25		20	100.0	22.0	7.3	6.6	1691	25	100.0	21.9	7.3	6.6	1601	25	
3	07/18/04		20	100.0	21.9	7.2	6.2	1515	25		20	100.0	21.8	7.2	6.9	1607	25	100.0	21.9	7.2	6.6	1561	25	
4	07/19/04		20	100.0	22.0	7.2	6.1	1520	25		20	100.0	21.9	7.2	6.1	1465	25	100.0	22.0	7.2	6.1	1493	25	
5	07/20/04		20	100.0	21.5	7.2	5.8	1430	25		20	100.0	21.5	7.2	5.8	1634	25	100.0	21.5	7.2	5.8	1532	25	
6	07/21/04		20	100.0	22.1	7.1	5.9	1190	24		20	100.0	22.2	7.2	6.0	1725	24	100.0	22.2	7.2	6.0	1458	24	253.40
7	07/22/04		20	100.0	21.5	7.0	6.1	1223	24		20	100.0	21.8	7.0	6.1	1633	24	100.0	21.7	7.0	6.1	1428	24	
8	07/23/04		20	100.0	20.6	7.1	6.1	1487	24		20	100.0	20.8	7.1	6.0	1443	24	100.0	20.7	7.1	6.1	1465	24	
9	07/24/04		20	100.0	20.6	7.1	5.9	1405	25		20	100.0	20.7	7.0	6.0	1425	25	100.0	20.7	7.1	6.0	1415	25	
10	07/25/04		20	100.0	20.9	7.0	5.7	1440	25		20	100.0	21.0	6.9	5.9	1360	25	100.0	21.0	7.0	5.8	1400	25	
11	07/26/04		20	100.0	20.3	7.1	5.5	1380	25		20	100.0	20.4	7.0	5.5	1470	25	100.0	20.4	7.1	5.5	1425	25	
12	07/27/04		20	100.0	20.0	7.0	5.9	1428	23		20	100.0	20.1	7.1	6.0	1475	23	100.0	20.1	7.1	6.0	1452	23	
13	07/28/04		20	100.0	20.4	7.0	5.7	1405	23		20	100.0	20.3	7.0	5.8	1465	23	100.0	20.4	7.0	5.8	1435	23	
14	07/29/04		20	100.0	20.1	7.0	5.7	1531	23		20	100.0	20.2	7.0	5.7	1601	23	100.0	20.2	7.0	5.7	1566	23	269.40
15	07/30/04		20	100.0	20.0	7.0	5.6	1485	24		20	100.0	20.0	7.0	5.4	1662	24	100.0	20.0	7.0	5.5	1574	24	
16	07/31/04		20	100.0	20.0	7.1	6.1	1484	23		20	100.0	20.0	7.1	5.9	1515	23	100.0	20.0	7.1	6.0	1500	23	
17	08/01/04		20	100.0	20.1	7.1	5.4	1705	24		20	100.0	20.1	7.1	5.6	1590	24	100.0	20.1	7.1	5.5	1648	24	
18	08/02/04		20	100.0	20.6	7.0	5.2	1438	24		20	100.0	20.5	7.0	5.2	1502	24	100.0	20.6	7.0	5.2	1470	24	
19	08/03/04		20	100.0	20.2	7.1	5.4	1502	24		20	100.0	20.3	7.1	5.4	1623	24	100.0	20.3	7.1	5.4	1563	24	
20	08/04/04		20	100.0	20.4	7.0	5.5	1440	24		20	100.0	20.4	7.1	5.6	1505	24	100.0	20.4	7.1	5.6	1473	24	271.10
21	08/05/04		20	100.0							20	100.0						100.0						
	Median:				**20.6**	**7.1**	**5.9**	**1484**	**24**				**20.5**	**7.1**	**5.9**	**1515**	**24**		**20.6**	**7.1**	**6.0**	**1473**	**24**	
	Mode:				**20.6**	**7.0**	**6.1**	**1405**	**25**				**20.5**	**7.0**	**6.0**	**1465**	**25**		**21.9**	**7.0**	**6.0**	**1425**	**25**	
	Mean:			**100.0**	**20.9**	**7.1**	**5.9**	**1458**	**24.2**			**100.0**	**20.9**	**7.1**	**6.0**	**1547**	**24.2**	**100.0**	**20.9**	**7.1**	**5.9**	**1502**	**24.2**	**255.200**
	SEM:			**0.00**	**0.19**	**0.03**	**0.11**	**23.90**	**0.17**			**0.00**	**0.19**	**0.03**	**0.11**	**27.29**	**0.17**	**0.00**	**0.19**	**0.03**	**0.11**	**17.20**	**0.17**	**10.24**
	% CV:			**0.00**	**18.63**	**4.93**	**19.78**	**286.79**	**15.61**			**0.00**	**18.84**	**4.73**	**21.41**	**318.03**	**15.61**	**0.00**	**18.69**	**4.70**	**20.27**	**203.39**	**15.61**	**128.22**

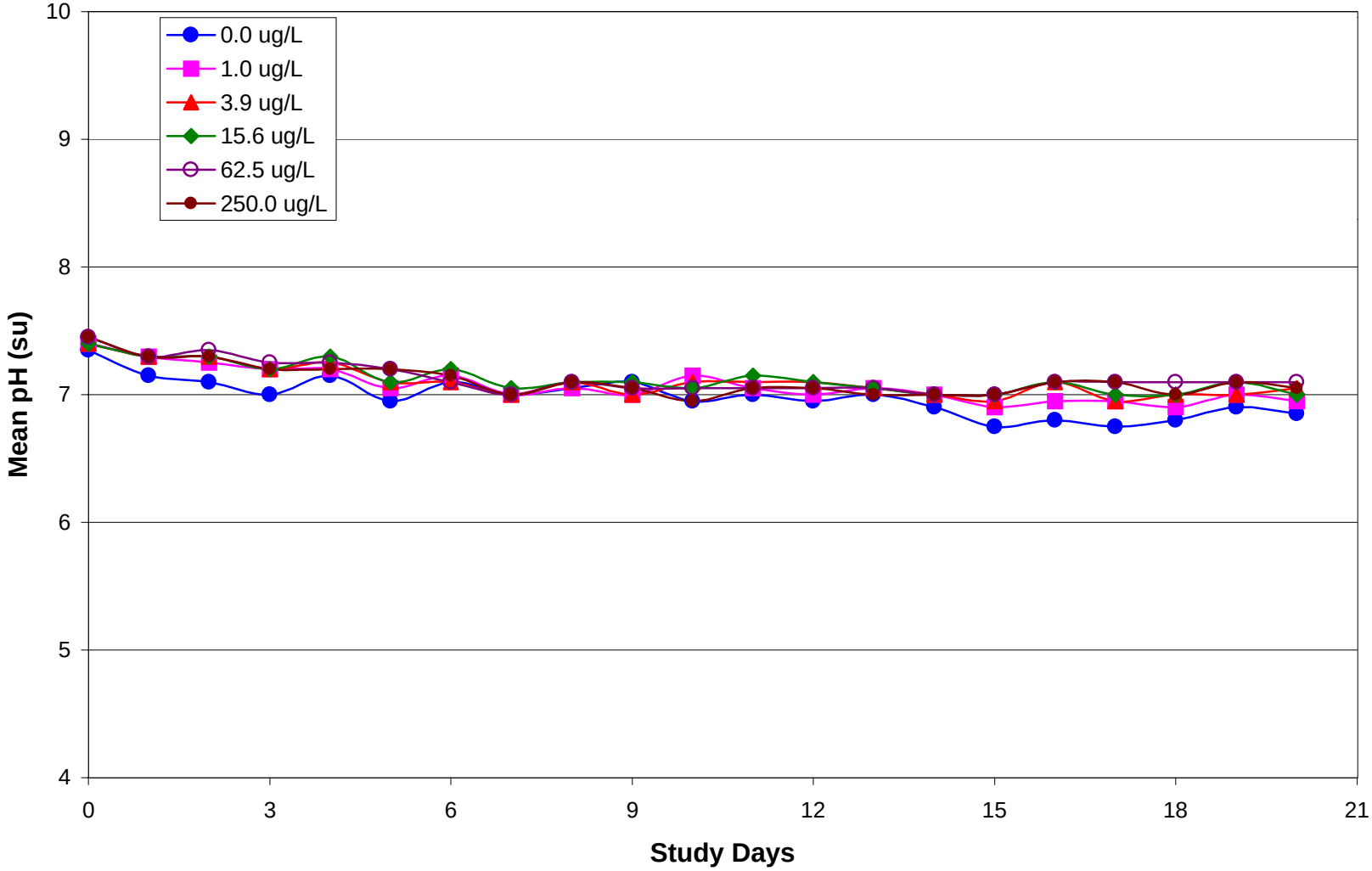
BATT02-00063
Dexamethasone
Daily Average of Larval Survival for Initial Stage 51 Test



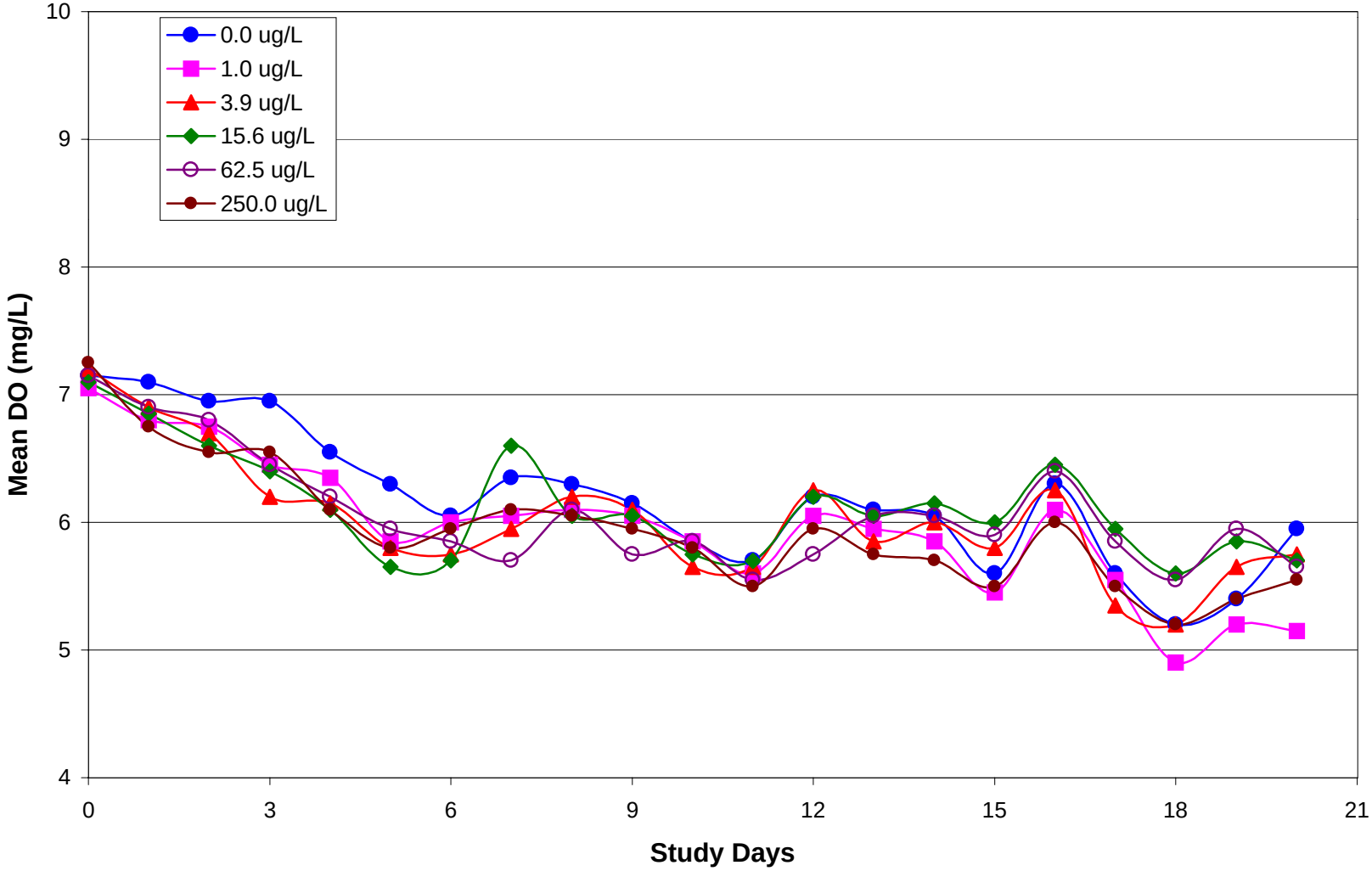
BATT02-00063
Dexamethasone
Daily Average Tank Temperatures for Initial Stage 51 Test



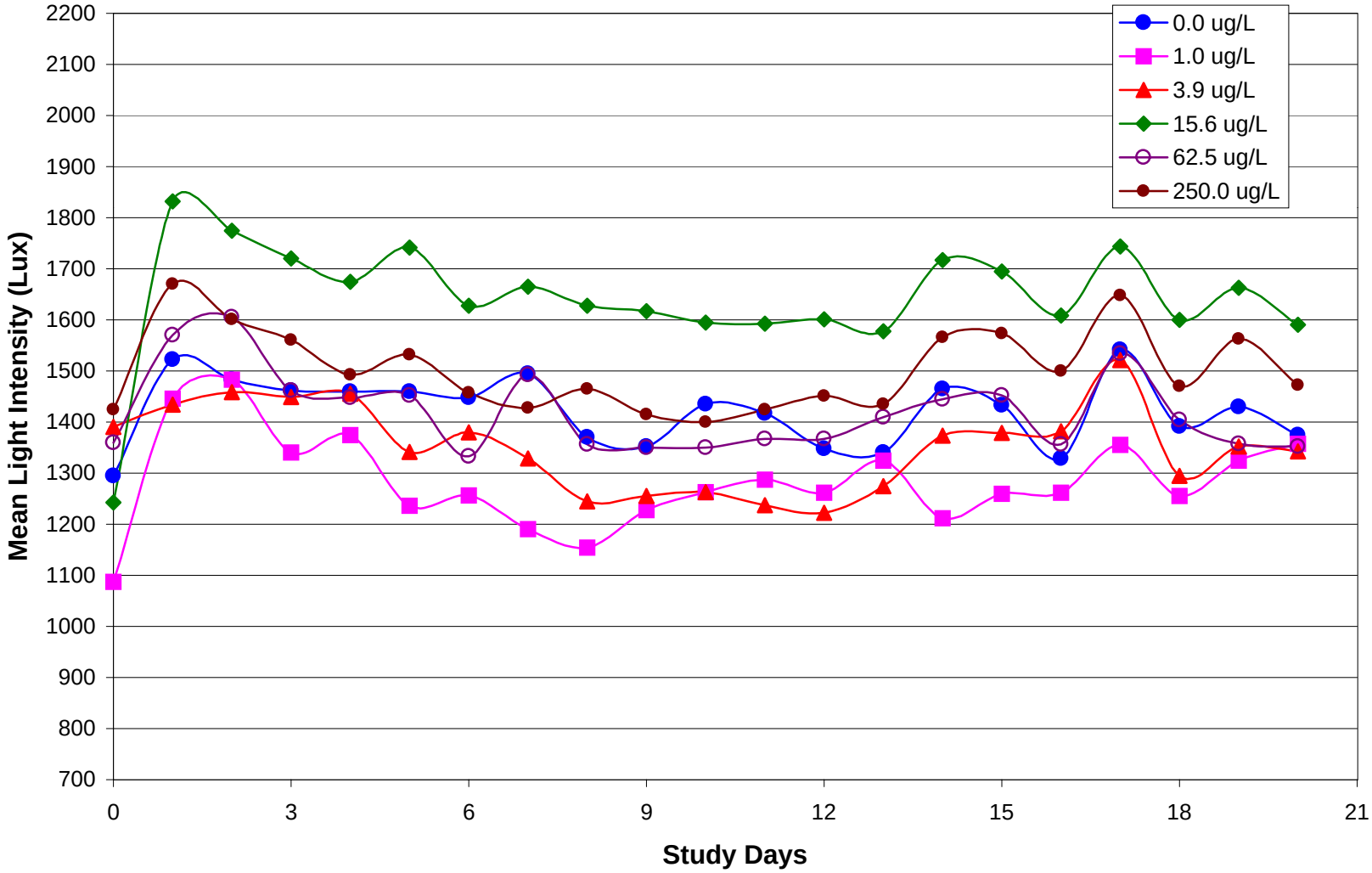
BATT02-00063
Dexamethasone
Daily Average pH for Initial Stage 51 Test



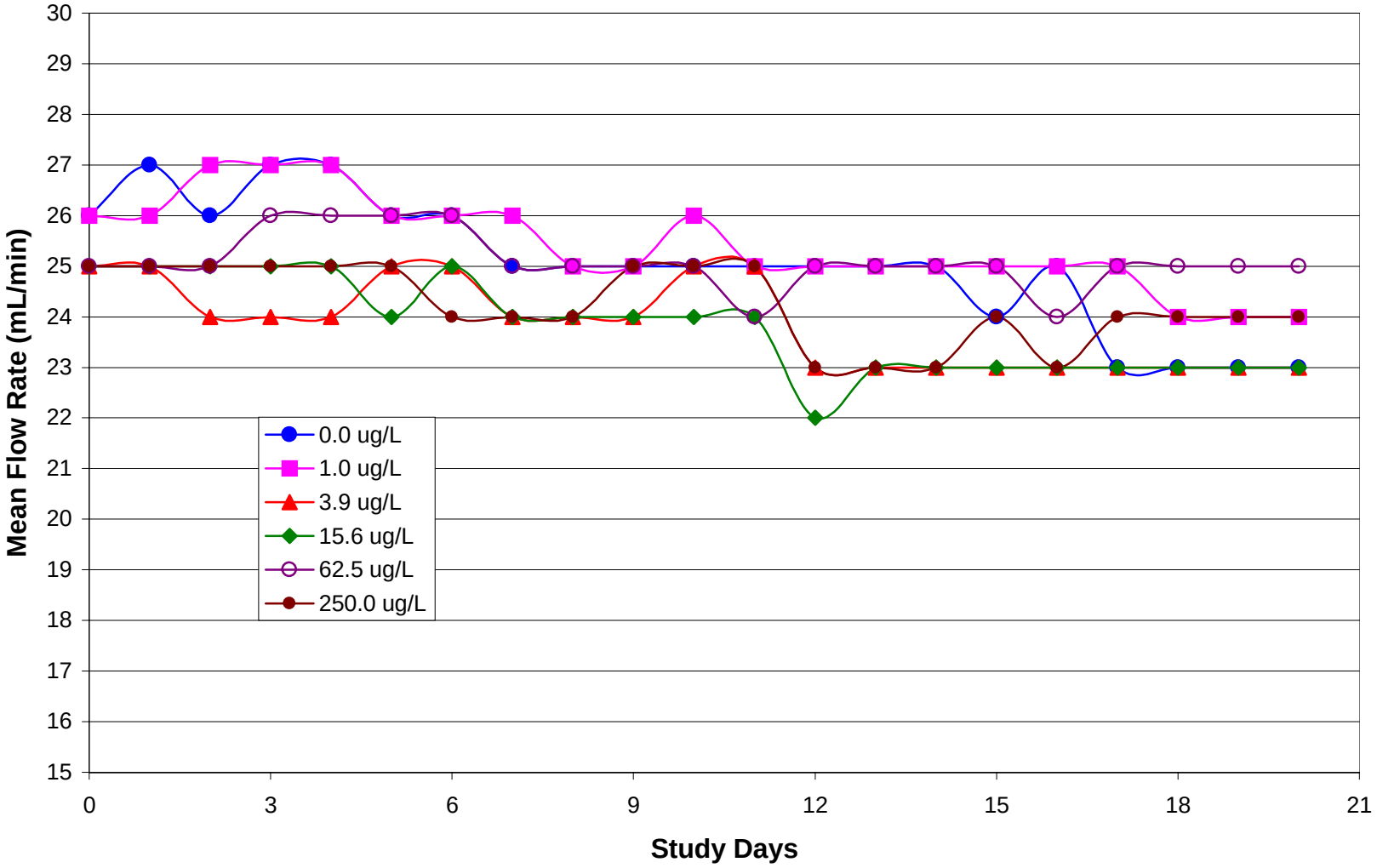
BATT02-00063
Dexamethasone
Daily Average Dissolved Oxygen (DO) for Initial Stage 51 Test



BATT02-00063
Dexamethasone
Daily Average Light Intensity for Initial Stage 51 Test



BATT02-00063
Dexamethasone
Daily Average Flow Rates for Initial Stage 51 Test



WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA

Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 0.0 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc[1] (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/21/04			100.0	22.0	7.2	7.1	1315	26			100.0	22.2	7.2	7.2	1020	26	100.0	22.1	7.2	7.2	1168	26	0.68
1	07/22/04		20	100.0	21.9	6.9	6.0	1269	25		20	100.0	22.0	7.0	6.1	1245	25	100.0	22.0	7.0	6.1	1257	25	
2	07/23/04		20	100.0	21.0	7.0	5.7	1437	25		20	100.0	20.9	7.0	6.1	1241	25	100.0	21.0	7.0	5.9	1339	25	
3	07/24/04		20	100.0	20.7	7.1	6.1	1390	25		20	100.0	20.7	7.0	6.2	1320	25	100.0	20.7	7.1	6.2	1355	25	
4	07/25/04		20	100.0	21.0	7.0	5.7	1405	25		20	100.0	20.9	7.1	5.7	1505	25	100.0	21.0	7.1	5.7	1455	25	
5	07/26/04		20	100.0	20.2	7.0	5.8	1330	25		20	100.0	20.4	7.1	5.7	1440	25	100.0	20.3	7.1	5.8	1385	25	
6	07/27/04		20	100.0	20.3	6.9	6.2	1380	25		20	100.0	20.4	6.9	6.0	1273	25	100.0	20.4	6.9	6.1	1327	25	
7	07/28/04		20	100.0	20.3	7.0	5.7	1295	25		20	100.0	20.4	7.1	5.8	1270	25	100.0	20.4	7.1	5.8	1283	25	
8	07/29/04		20	100.0	20.3	6.7	5.9	1540	25		20	100.0	20.4	6.8	5.9	1372	25	100.0	20.4	6.8	5.9	1456	25	0.68
9	07/30/04		20	100.0	20.2	6.8	5.7	1444	24		20	100.0	20.1	6.7	5.7	1132	24	100.0	20.2	6.8	5.7	1288	24	
10	07/31/04		20	100.0	20.0	6.7	6.1	1374	25		20	100.0	20.0	6.6	6.0	1277	25	100.0	20.0	6.7	6.1	1326	25	
11	08/01/04		20	100.0	20.1	6.8	5.7	1485	23		20	100.0	20.0	6.7	6.0	1426	23	100.0	20.1	6.8	5.9	1456	23	
12	08/02/04		20	100.0	20.0	6.9	5.1	1374	23		20	100.0	20.2	6.9	5.5	1261	23	100.0	20.1	6.9	5.3	1318	23	
13	08/03/04		20	100.0	20.1	6.9	5.0	1440	23		20	100.0	20.0	6.9	5.5	1266	23	100.0	20.1	6.9	5.3	1353	23	
14	08/04/04		20	100.0							20	100.0						100.0						0.68
	Median:				**20.3**	**6.9**	**5.8**	**1385**	**25**				**20.4**	**7.0**	**6.0**	**1272**	**25**		**20.4**	**6.9**	**5.9**	**1333**	**25**	
	Mode:				**20.3**	**6.9**	**5.7**	**1374**	**25**				**20.4**	**7.0**	**5.7**	**#N/A**	**25**		**20.4**	**7.1**	**6.1**	**#N/A**	**25**	
	Mean:			**100.0**	**20.6**	**6.9**	**5.8**	**1391**	**24.6**			**100.0**	**20.6**	**6.9**	**6.0**	**1289**	**24.6**	**100.0**	**20.6**	**6.9**	**5.9**	**1340**	**24.6**	**0.680**
	SEM:			**0.00**	**0.18**	**0.04**	**0.13**	**20.01**	**0.25**			**0.00**	**0.19**	**0.05**	**0.11**	**33.10**	**0.25**	**0.00**	**0.18**	**0.04**	**0.12**	**21.74**	**0.25**	**0.00**
	% CV:			**0.00**	**14.70**	**5.41**	**20.64**	**200.70**	**18.92**			**0.00**	**15.40**	**6.74**	**17.21**	**344.94**	**18.92**	**0.00**	**15.00**	**5.90**	**18.48**	**222.17**	**18.92**	**0.00**

[1]All measured test concentrations reported by Battelle as less than method detection limit (MDL) [1.36 µg/L] or none detected (ND) are expressed as 0.68 µg/L (1/2 of MDL).

DAILY DATA

Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 1.0 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/21/04			100.0	22.1	7.3	7.2	1480	26			100.0	22.2	7.2	7.4	1245	26	100.0	22.2	7.3	7.3	1363	26	
1	07/22/04		20	100.0	21.7	7.0	5.9	1438	26		20	100.0	21.7	7.0	5.9	1494	26	100.0	21.7	7.0	5.9	1466	26	
2	07/23/04		20	100.0	20.8	7.1	5.9	1458	25		20	100.0	21.0	7.0	5.8	1701	25	100.0	20.9	7.1	5.9	1580	25	
3	07/24/04		20	100.0	20.8	7.1	6.1	1470	25		20	100.0	20.7	7.1	6.3	1715	25	100.0	20.8	7.1	6.2	1593	25	
4	07/25/04		20	100.0	20.8	7.0	5.6	1705	26		20	100.0	20.9	7.1	5.8	1275	26	100.0	20.9	7.1	5.7	1490	26	
5	07/26/04		20	100.0	20.1	7.0	5.8	1735	25		20	100.0	20.2	7.2	5.9	1555	25	100.0	20.2	7.1	5.9	1645	25	
6	07/27/04		20	100.0	20.2	7.0	6.1	1311	25		20	100.0	20.2	7.0	6.4	1502	25	100.0	20.2	7.0	6.3	1407	25	
7	07/28/04		20	100.0	20.5	7.0	5.8	1285	25		20	100.0	20.4	6.9	5.7	1340	25	100.0	20.5	7.0	5.8	1313	25	
8	07/29/04		20	100.0	20.1	6.9	5.7	1643	25		20	100.0	20.3	6.9	6.1	1701	25	100.0	20.2	6.9	5.9	1672	25	
9	07/30/04		20	100.0	20.1	6.9	5.6	1474	25		20	100.0	20.2	6.9	5.9	1621	25	100.0	20.2	6.9	5.8	1548	25	
10	07/31/04		20	100.0	20.0	6.8	6.2	1295	25		20	100.0	20.0	6.9	6.4	1635	25	100.0	20.0	6.9	6.3	1465	25	
11	08/01/04		20	100.0	20.1	6.9	5.9	1563	25		20	100.0	20.1	6.9	6.1	1730	25	100.0	20.1	6.9	6.0	1647	25	
12	08/02/04		20	100.0	20.4	6.9	5.4	1366	24		20	100.0	20.4	6.9	5.4	1613	24	100.0	20.4	6.9	5.4	1490	24	
13	08/03/04		20	100.0	20.1	7.0	5.6	1367	24		20	100.0	20.0	7.1	5.7	1609	24	100.0	20.1	7.1	5.7	1488	24	
14	08/04/04		20	100.0							20	100.0						100.0						
	Median:				**20.3**	**7.0**	**5.9**	**1464**	**25**				**20.4**	**7.0**	**5.9**	**1611**	**25**		**20.3**	**7.0**	**5.9**	**1490**	**25**	
	Mode:				**20.1**	**7.0**	**5.9**	**#N/A**	**25**				**20.2**	**6.9**	**5.9**	**1701**	**25**		**20.2**	**6.9**	**5.9**	**#N/A**	**25**	
	Mean:			**100.0**	**20.6**	**7.0**	**5.9**	**1471**	**25.1**			**100.0**	**20.6**	**7.0**	**6.1**	**1553**	**25.1**	**100.0**	**20.6**	**7.0**	**6.0**	**1512**	**25.1**	**#DIV/0!**
	SEM:			**0.00**	**0.17**	**0.03**	**0.12**	**39.00**	**0.16**			**0.00**	**0.18**	**0.03**	**0.13**	**43.42**	**0.16**	**0.00**	**0.17**	**0.03**	**0.12**	**28.98**	**0.16**	**#DIV/0!**
	% CV:			**0.00**	**14.18**	**4.56**	**17.80**	**380.54**	**12.30**			**0.00**	**14.53**	**4.31**	**19.50**	**412.28**	**12.30**	**0.00**	**14.31**	**4.13**	**18.43**	**278.89**	**12.30**	**#DIV/0!**

DAILY DATA

Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 3.9 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/21/04			100.0	22.3	7.3	7.1	1390	25			100.0	22.3	7.3	7.1	1270	25	100.0	22.3	7.3	7.1	1330	25	
1	07/22/04		20	100.0	21.7	7.0	5.9	1294	24		20	100.0	22.0	7.0	6.0	1656	24	100.0	21.9	7.0	6.0	1475	24	
2	07/23/04		20	100.0	20.9	7.0	5.5	1292	24		20	100.0	20.9	7.1	6.0	1515	24	100.0	20.9	7.1	5.8	1404	24	
3	07/24/04		20	100.0	20.7	7.1	6.1	1280	24		20	100.0	20.7	7.0	5.9	1550	24	100.0	20.7	7.1	6.0	1415	24	
4	07/25/04		20	100.0	21.0	7.1	5.9	1520	25		20	100.0	21.0	7.1	5.7	1505	25	100.0	21.0	7.1	5.8	1513	25	
5	07/26/04		20	100.0	20.1	7.2	5.7	1535	25		20	100.0	20.2	7.1	5.8	1570	25	100.0	20.2	7.2	5.8	1553	25	
6	07/27/04		20	100.0	20.3	7.0	5.8	1298	23		20	100.0	20.2	7.0	6.1	1446	23	100.0	20.3	7.0	6.0	1372	23	
7	07/28/04		20	100.0	20.4	7.0	5.6	1275	23		20	100.0	20.5	7.1	5.7	1390	23	100.0	20.5	7.1	5.7	1333	23	
8	07/29/04		20	100.0	20.4	6.9	5.7	1423	23		20	100.0	20.4	7.0	5.8	1670	23	100.0	20.4	7.0	5.8	1547	23	
9	07/30/04		20	100.0	20.0	7.0	5.6	1271	23		20	100.0	20.1	7.0	5.7	1555	23	100.0	20.1	7.0	5.7	1413	23	
10	07/31/04		20	100.0	20.0	7.0	5.9	1268	23		20	100.0	20.0	7.0	6.2	1504	23	100.0	20.0	7.0	6.1	1386	23	
11	08/01/04		20	100.0	20.1	7.0	5.5	1382	23		20	100.0	20.1	7.0	5.7	1573	23	100.0	20.1	7.0	5.6	1478	23	
12	08/02/04		20	100.0	20.4	6.9	5.0	1279	23		20	100.0	20.4	6.9	5.2	1539	23	100.0	20.4	6.9	5.1	1409	23	
13	08/03/04		20	100.0	20.1	7.1	5.2	1265	23		20	100.0	20.1	7.1	5.5	1479	23	100.0	20.1	7.1	5.4	1372	23	
14	08/04/04		20	100.0							20	100.0						100.0						
	Median:				**20.4**	**7.0**	**5.7**	**1293**	**23**				**20.4**	**7.0**	**5.8**	**1527**	**23**		**20.4**	**7.0**	**5.8**	**1411**	**23**	
	Mode:				**20.1**	**7.0**	**5.9**	**#N/A**	**23**				**20.1**	**7.0**	**5.7**	**#N/A**	**23**		**20.4**	**7.0**	**5.8**	**1372**	**23**	
	Mean:			**100.0**	**20.6**	**7.0**	**5.8**	**1341**	**23.6**			**100.0**	**20.6**	**7.1**	**5.9**	**1516**	**23.6**	**100.0**	**20.6**	**7.0**	**5.8**	**1428**	**23.6**	**#DIV/0!**
	SEM:			**0.00**	**0.18**	**0.03**	**0.13**	**25.12**	**0.23**			**0.00**	**0.19**	**0.03**	**0.12**	**27.30**	**0.23**	**0.00**	**0.19**	**0.03**	**0.12**	**19.54**	**0.23**	**#DIV/0!**
	% CV:			**0.00**	**15.02**	**4.11**	**20.22**	**256.71**	**17.31**			**0.00**	**15.70**	**3.54**	**17.77**	**262.33**	**17.31**	**0.00**	**15.33**	**3.65**	**18.61**	**193.40**	**17.31**	**#DIV/0!**

DAILY DATA

Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 15.6 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/21/04			100.0	22.2	7.3	7.2	1005	25			100.0	22.3	7.2	7.3	1235	25	100.0	22.3	7.3	7.3	1120	25	
1	07/22/04		20	100.0	21.8	7.0	6.2	1110	24		20	100.0	21.6	7.0	6.0	1004	24	100.0	21.7	7.0	6.1	1057	24	
2	07/23/04		20	100.0	20.8	7.0	5.8	928	24		20	100.0	20.8	7.1	6.0	1003	24	100.0	20.8	7.1	5.9	966	24	
3	07/24/04		20	100.0	20.6	7.1	6.1	1000	24		20	100.0	20.7	7.0	6.0	1125	24	100.0	20.7	7.1	6.1	1063	24	
4	07/25/04		20	100.0	21.0	7.0	5.6	1175	24		20	100.0	20.9	7.1	5.7	1265	24	100.0	21.0	7.1	5.7	1220	24	
5	07/26/04		20	100.0	20.4	7.2	5.9	1160	24		20	100.0	20.3	7.1	5.8	1295	24	100.0	20.4	7.2	5.9	1228	24	
6	07/27/04		20	100.0	20.2	7.0	5.5	1008	22		20	100.0	20.3	7.0	5.9	1027	22	100.0	20.3	7.0	5.7	1018	22	
7	07/28/04		20	100.0	20.5	7.0	5.9	1060	23		20	100.0	20.5	7.0	5.7	1265	23	100.0	20.5	7.0	5.8	1163	23	
8	07/29/04		20	100.0	20.3	6.9	5.3	1288	23		20	100.0	20.2	6.9	6.0	1000	23	100.0	20.3	6.9	5.7	1144	23	
9	07/30/04		20	100.0	20.0	7.0	5.3	1166	23		20	100.0	20.1	6.9	6.0	1051	23	100.0	20.1	7.0	5.7	1109	23	
10	07/31/04		20	100.0	20.0	7.0	5.9	1152	23		20	100.0	20.0	7.0	6.4	1125	23	100.0	20.0	7.0	6.2	1139	23	
11	08/01/04		20	100.0	20.0	7.0	5.5	1080	23		20	100.0	20.0	7.0	5.9	1060	23	100.0	20.0	7.0	5.7	1070	23	
12	08/02/04		20	100.0	20.4	6.9	5.0	972	23		20	100.0	20.4	7.0	5.3	1109	23	100.0	20.4	7.0	5.2	1041	23	
13	08/03/04		20	100.0	20.1	7.1	5.2	1106	23		20	100.0	20.0	7.1	5.6	1086	23	100.0	20.1	7.1	5.4	1096	23	
14	08/04/04		20	100.0							20	100.0						100.0						
	Median:				**20.4**	**7.0**	**5.7**	**1093**	**23**				**20.4**	**7.0**	**6.0**	**1098**	**23**		**20.4**	**7.0**	**5.8**	**1102**	**23**	
	Mode:				**20.0**	**7.0**	**5.9**	**#N/A**	**23**				**20.0**	**7.0**	**6.0**	**1125**	**23**		**20.3**	**7.0**	**5.7**	**#N/A**	**23**	
	Mean:			**100.0**	**20.6**	**7.0**	**5.7**	**1086**	**23.4**			**100.0**	**20.6**	**7.0**	**6.0**	**1118**	**23.4**	**100.0**	**20.6**	**7.0**	**5.9**	**1102**	**23.4**	**#DIV/0!**
	SEM:			**0.00**	**0.18**	**0.03**	**0.15**	**26.17**	**0.20**			**0.00**	**0.18**	**0.02**	**0.12**	**28.30**	**0.20**	**0.00**	**0.18**	**0.02**	**0.13**	**19.75**	**0.20**	**#DIV/0!**
	% CV:			**0.00**	**14.81**	**4.08**	**22.90**	**297.07**	**15.62**			**0.00**	**14.65**	**3.11**	**18.75**	**316.66**	**15.62**	**0.00**	**14.69**	**3.36**	**19.90**	**222.60**	**15.62**	**#DIV/0!**

DAILY DATA

Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 62.5 ug/L**

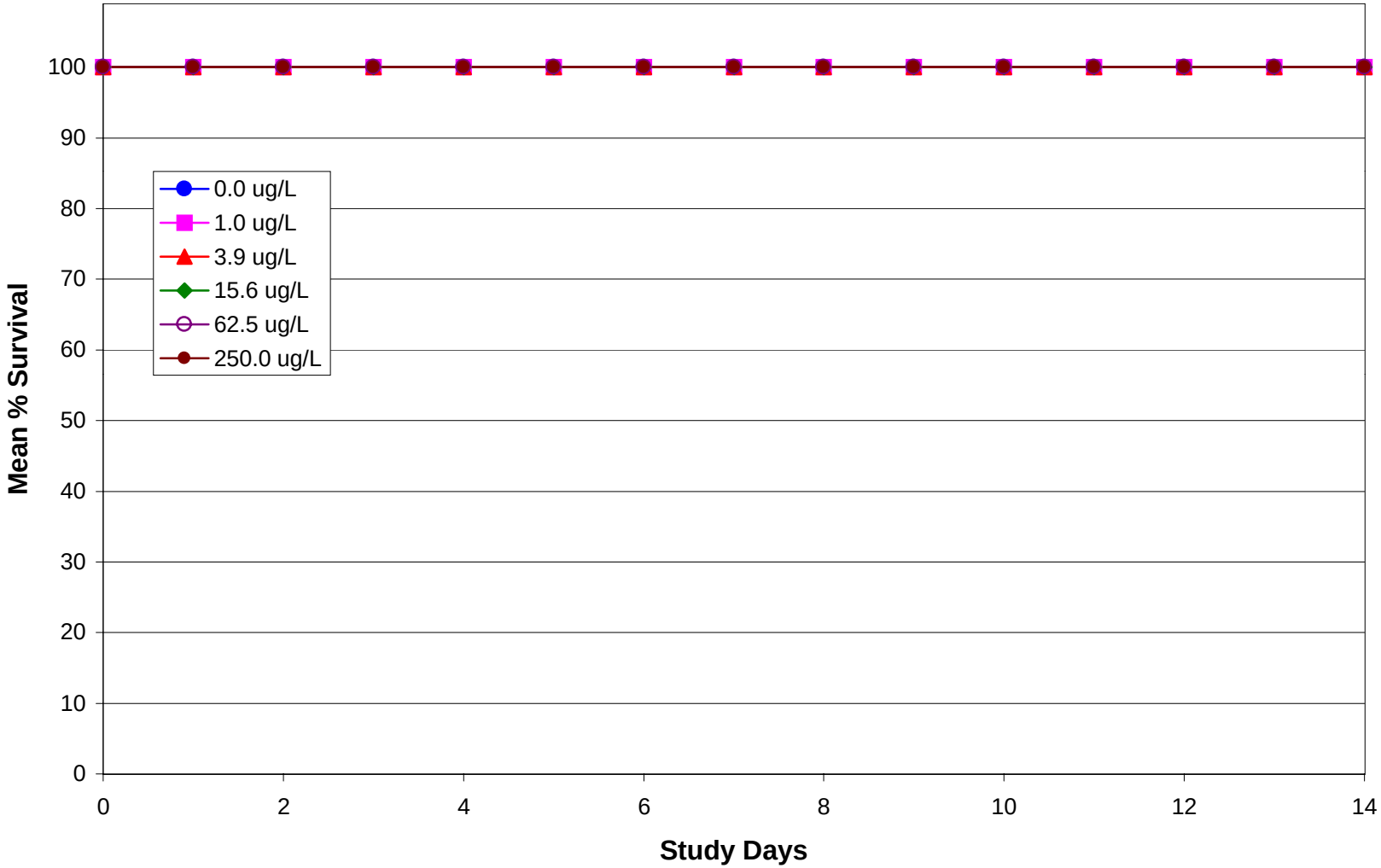
		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/21/04			100.0	22.3	7.2	7.2	1665	26			100.0	22.4	7.3	7.2	1090	26	100.0	22.4	7.3	7.2	1378	26	68.80
1	07/22/04		20	100.0	21.6	6.9	5.0	1414	25		20	100.0	22.0	7.0	6.5	1335	25	100.0	21.8	7.0	5.8	1375	25	
2	07/23/04		20	100.0	20.8	7.0	5.8	1533	25		20	100.0	21.0	7.0	6.0	1441	25	100.0	20.9	7.0	5.9	1487	25	
3	07/24/04		20	100.0	20.6	7.1	6.2	1480	25		20	100.0	20.6	7.1	6.1	1415	25	100.0	20.6	7.1	6.2	1448	25	
4	07/25/04		20	100.0	21.1	7.1	5.8	1485	25		20	100.0	21.1	7.1	5.8	1425	25	100.0	21.1	7.1	5.8	1455	25	
5	07/26/04		20	100.0	20.2	7.2	5.7	1505	24		20	100.0	20.3	7.2	5.6	1445	24	100.0	20.3	7.2	5.7	1475	24	
6	07/27/04		20	100.0	20.0	6.9	5.2	1546	25		20	100.0	21.0	7.1	6.4	1383	25	100.0	20.5	7.0	5.8	1465	25	
7	07/28/04		20	100.0	20.5	7.1	5.9	1440	25		20	100.0	20.4	7.1	5.8	1180	25	100.0	20.5	7.1	5.9	1310	25	
8	07/29/04		20	100.0	20.4	6.9	5.8	1736	25		20	100.0	20.4	7.0	5.9	1497	25	100.0	20.4	7.0	5.9	1617	25	76.90
9	07/30/04		20	100.0	20.1	7.0	5.7	1657	25		20	100.0	20.3	7.0	5.8	1429	25	100.0	20.2	7.0	5.8	1543	25	
10	07/31/04		20	100.0	20.0	7.1	6.2	1541	24		20	100.0	20.0	7.1	6.3	1602	24	100.0	20.0	7.1	6.3	1572	24	
11	08/01/04		20	100.0	20.1	7.0	5.9	1765	25		20	100.0	20.1	7.0	6.0	1639	25	100.0	20.1	7.0	6.0	1702	25	
12	08/02/04		20	100.0	20.3	7.0	5.1	1452	25		20	100.0	20.4	7.0	5.4	1483	25	100.0	20.4	7.0	5.3	1468	25	
13	08/03/04		20	100.0	20.0	7.1	5.7	1550	25		20	100.0	20.0	7.1	5.9	1509	25	100.0	20.0	7.1	5.8	1530	25	
14	08/04/04		20	100.0							20	100.0						100.0						79.40
	Median:				**20.4**	**7.1**	**5.8**	**1537**	**25**				**20.4**	**7.1**	**6.0**	**1435**	**25**		**20.4**	**7.1**	**5.8**	**1471**	**25**	
	Mode:				**20.0**	**7.1**	**5.8**	**#N/A**	**25**				**20.4**	**7.0**	**5.8**	**#N/A**	**25**		**20.0**	**7.0**	**5.8**	**#N/A**	**25**	
	Mean:			**100.0**	**20.6**	**7.0**	**5.8**	**1555**	**24.9**			**100.0**	**20.7**	**7.1**	**6.1**	**1420**	**24.9**	**100.0**	**20.6**	**7.1**	**5.9**	**1487**	**24.9**	**75.033**
	SEM:			**0.00**	**0.18**	**0.03**	**0.14**	**29.36**	**0.13**			**0.00**	**0.19**	**0.02**	**0.12**	**38.88**	**0.13**	**0.00**	**0.18**	**0.02**	**0.12**	**27.20**	**0.13**	**3.20**
	% CV:			**0.00**	**15.04**	**3.83**	**22.51**	**278.59**	**9.51**			**0.00**	**15.96**	**3.35**	**18.09**	**386.17**	**9.51**	**0.00**	**15.21**	**3.40**	**17.78**	**263.92**	**9.51**	**63.97**

DAILY DATA

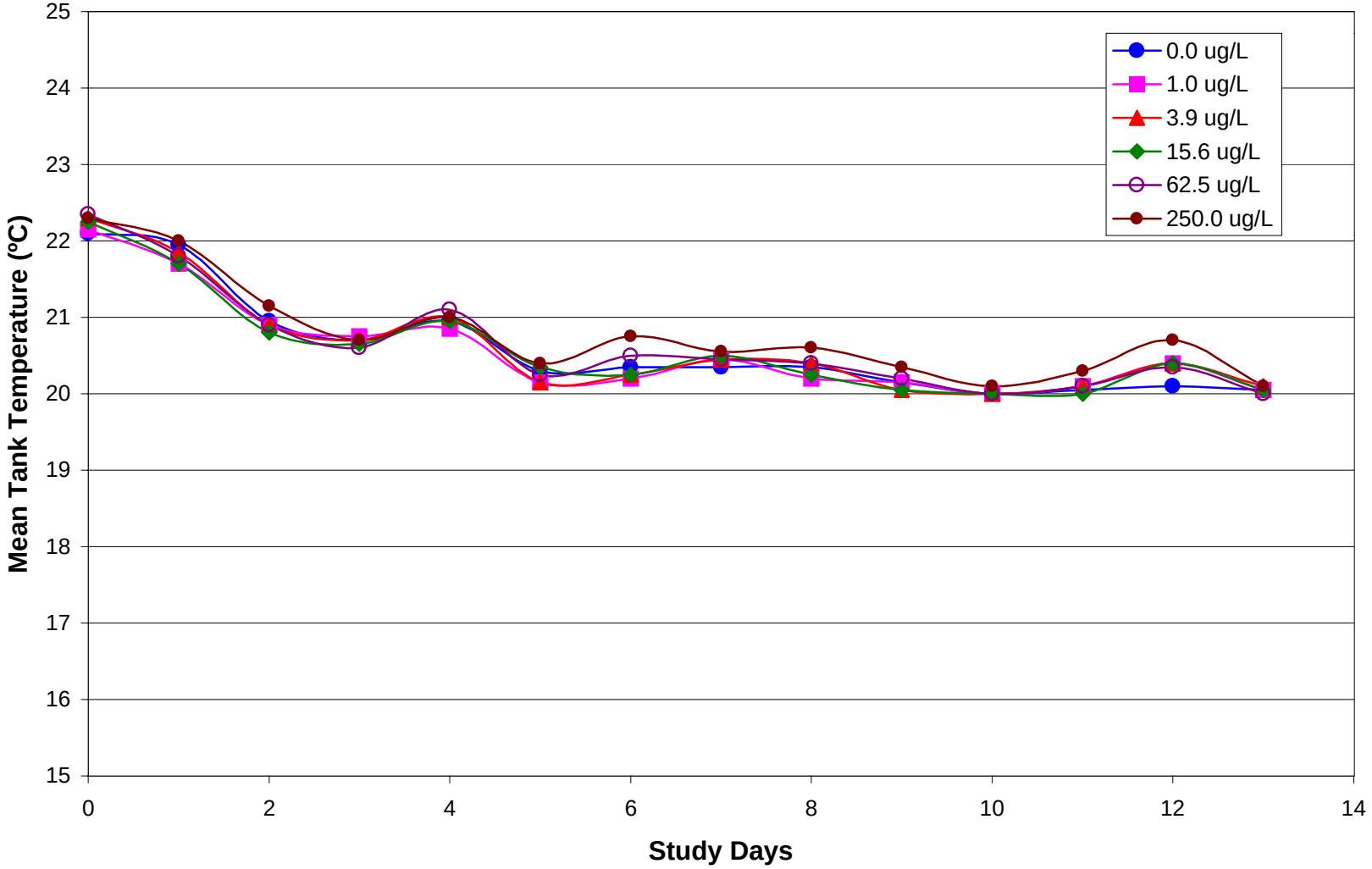
Workorder No: 00063 **Test No: 1**
Test Chemical: Dexamethasone (DXM) **Target Conc: 250.0 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES						Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Test Conc (ug/L)
		Initial No.= 20								**Initial No.= 20**														
0	07/21/04			100.0	22.2	7.3	7.2	1225	24			100.0	22.4	7.2	7.3	1620	24	100.0	22.3	7.3	7.3	1423	24	253.40
1	07/22/04		20	100.0	22.0	7.0	6.2	1424	24		20	100.0	22.0	7.1	6.4	1364	24	100.0	22.0	7.1	6.3	1394	24	
2	07/23/04		20	100.0	21.1	7.1	6.2	1395	24		20	100.0	21.2	7.1	6.4	1313	24	100.0	21.2	7.1	6.3	1354	24	
3	07/24/04		20	100.0	20.7	7.1	6.1	1405	25		20	100.0	20.7	7.0	6.0	1365	25	100.0	20.7	7.1	6.1	1385	25	
4	07/25/04		20	100.0	21.0	7.0	5.8	1360	25		20	100.0	21.0	7.1	5.7	1275	25	100.0	21.0	7.1	5.8	1318	25	
5	07/26/04		20	100.0	20.5	7.2	5.8	1360	25		20	100.0	20.3	7.1	5.9	1330	25	100.0	20.4	7.2	5.9	1345	25	
6	07/27/04		20	100.0	20.8	7.1	6.1	1380	23		20	100.0	20.7	7.1	6.2	1353	23	100.0	20.8	7.1	6.2	1367	23	
7	07/28/04		20	100.0	20.6	7.0	6.0	1335	23		20	100.0	20.5	7.1	5.8	1480	23	100.0	20.6	7.1	5.9	1408	23	
8	07/29/04		20	100.0	20.6	7.0	6.0	1570	23		20	100.0	20.6	7.0	6.2	1390	23	100.0	20.6	7.0	6.1	1480	23	269.40
9	07/30/04		20	100.0	20.4	7.0	6.2	1561	24		20	100.0	20.3	7.0	6.0	1435	24	100.0	20.4	7.0	6.1	1498	24	
10	07/31/04		20	100.0	20.1	7.1	6.6	1447	23		20	100.0	20.1	7.1	6.2	1483	23	100.0	20.1	7.1	6.4	1465	23	
11	08/01/04		20	100.0	20.3	7.1	5.9	1577	24		20	100.0	20.3	7.1	6.4	1585	24	100.0	20.3	7.1	6.2	1581	24	
12	08/02/04		20	100.0	20.7	7.0	5.4	1435	24		20	100.0	20.7	7.0	5.8	1444	24	100.0	20.7	7.0	5.6	1440	24	
13	08/03/04		20	100.0	20.1	7.1	5.8	1446	24		20	100.0	20.1	7.2	6.2	1501	24	100.0	20.1	7.2	6.0	1474	24	
14	08/04/04		20	100.0							20	100.0						100.0						271.10
	Median:				**20.7**	**7.1**	**6.1**	**1415**	**24**				**20.7**	**7.1**	**6.2**	**1413**	**24**		**20.7**	**7.1**	**6.1**	**1415**	**24**	
	Mode:				**20.7**	**7.0**	**6.2**	**1360**	**24**				**20.7**	**7.1**	**6.2**	**#N/A**	**24**		**20.7**	**7.1**	**6.3**	**#N/A**	**24**	
	Mean:			**100.0**	**20.8**	**7.1**	**6.1**	**1423**	**23.9**			**100.0**	**20.8**	**7.1**	**6.2**	**1424**	**23.9**	**100.0**	**20.8**	**7.1**	**6.1**	**1424**	**23.9**	**264.633**
	SEM:			**0.00**	**0.17**	**0.02**	**0.11**	**26.05**	**0.20**			**0.00**	**0.18**	**0.02**	**0.11**	**27.14**	**0.20**	**0.00**	**0.17**	**0.02**	**0.10**	**19.06**	**0.20**	**5.64**
	% CV:			**0.00**	**13.75**	**3.35**	**17.07**	**258.35**	**14.92**			**0.00**	**15.01**	**2.49**	**16.03**	**269.07**	**14.92**	**0.00**	**14.35**	**2.62**	**15.70**	**189.02**	**14.92**	**60.03**

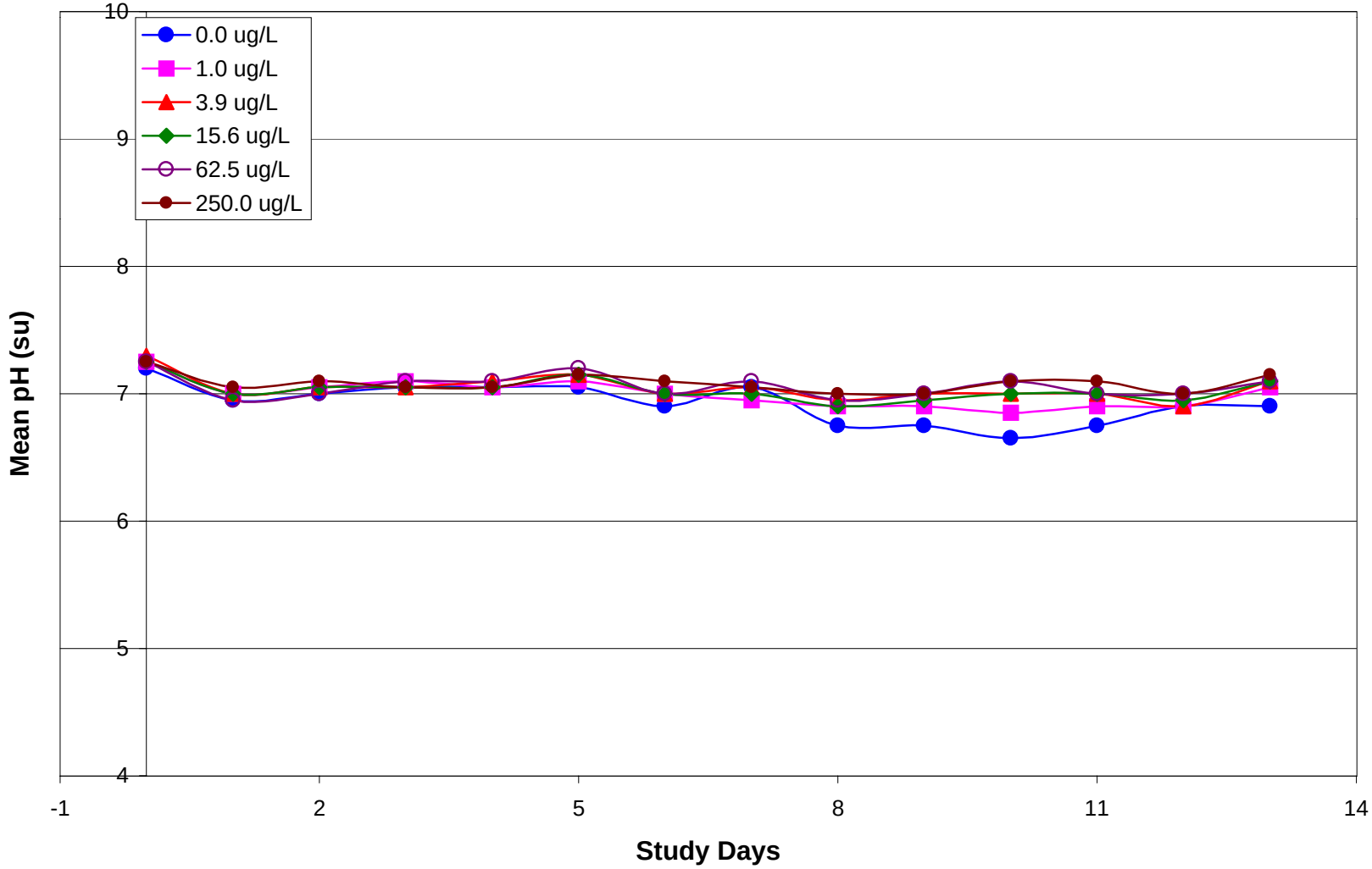
BATT02-00063
Dexamethasone
Daily Average of Larval Survival for Initial Stage 54 Test



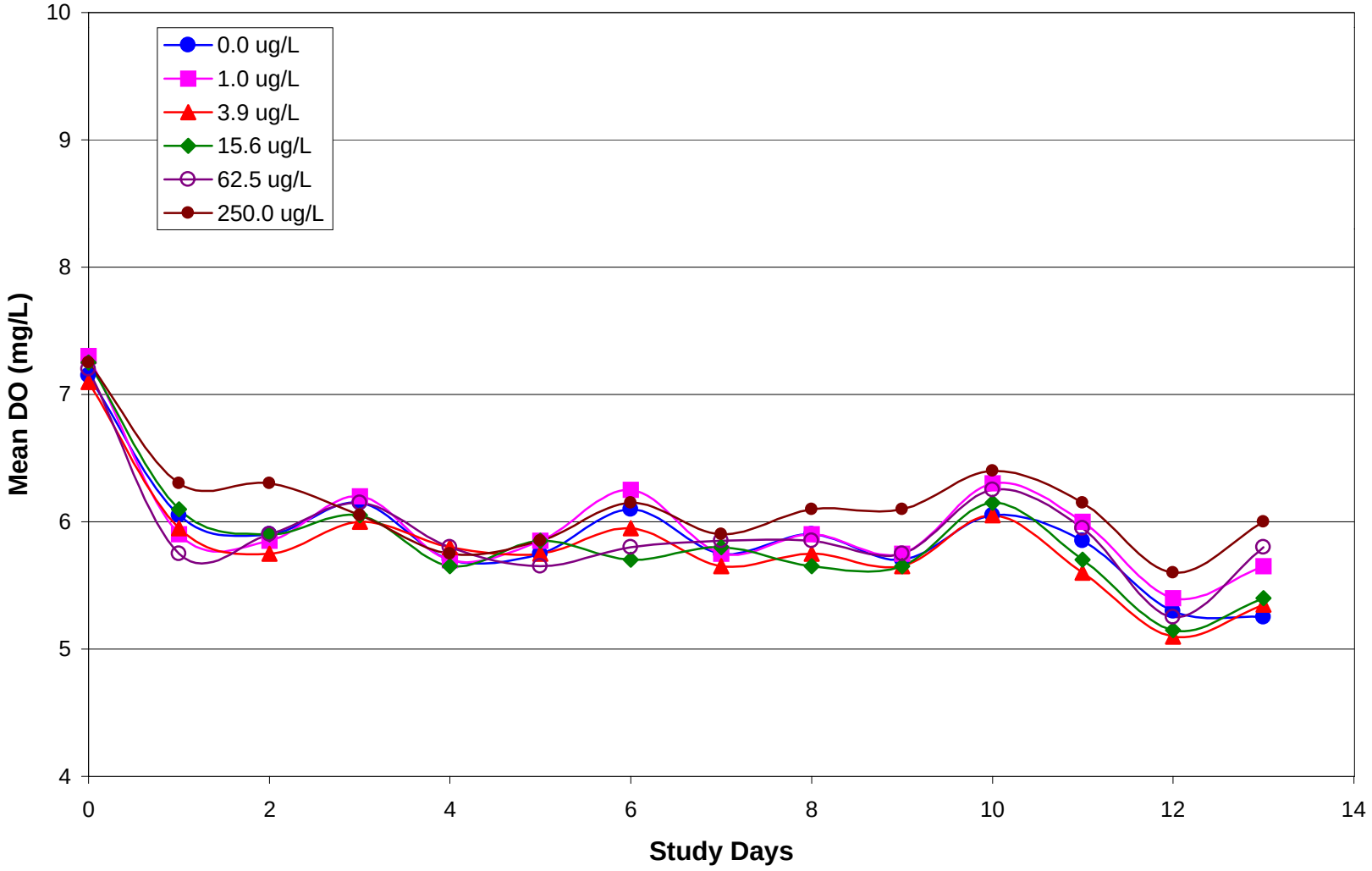
BATT02-00063
Dexamethasone
Daily Average Tank Temperatures for Initial Stage 54 Test



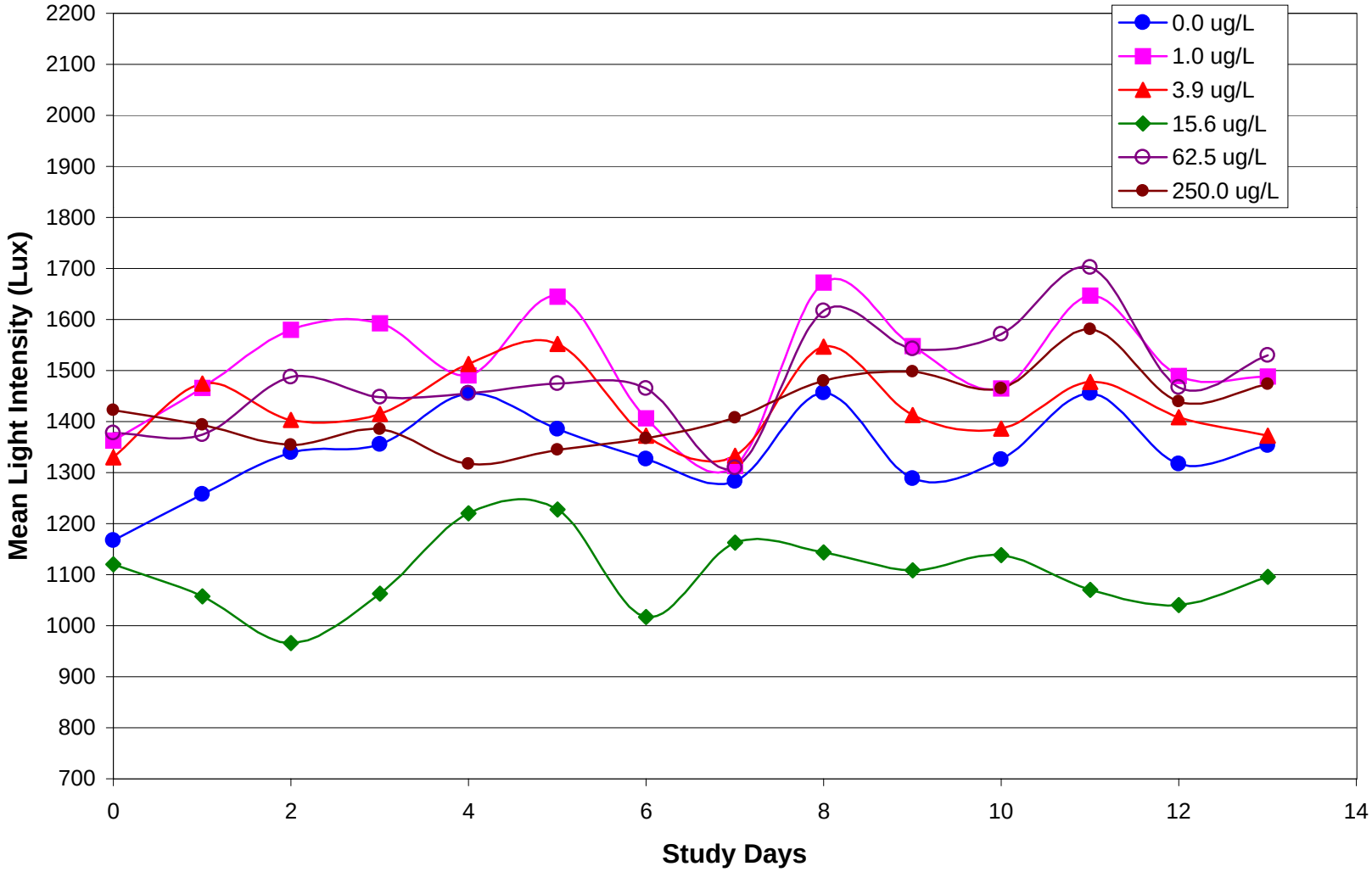
BATT02-00063
Dexamethasone
Daily Average pH for Initial Stage 54 Test



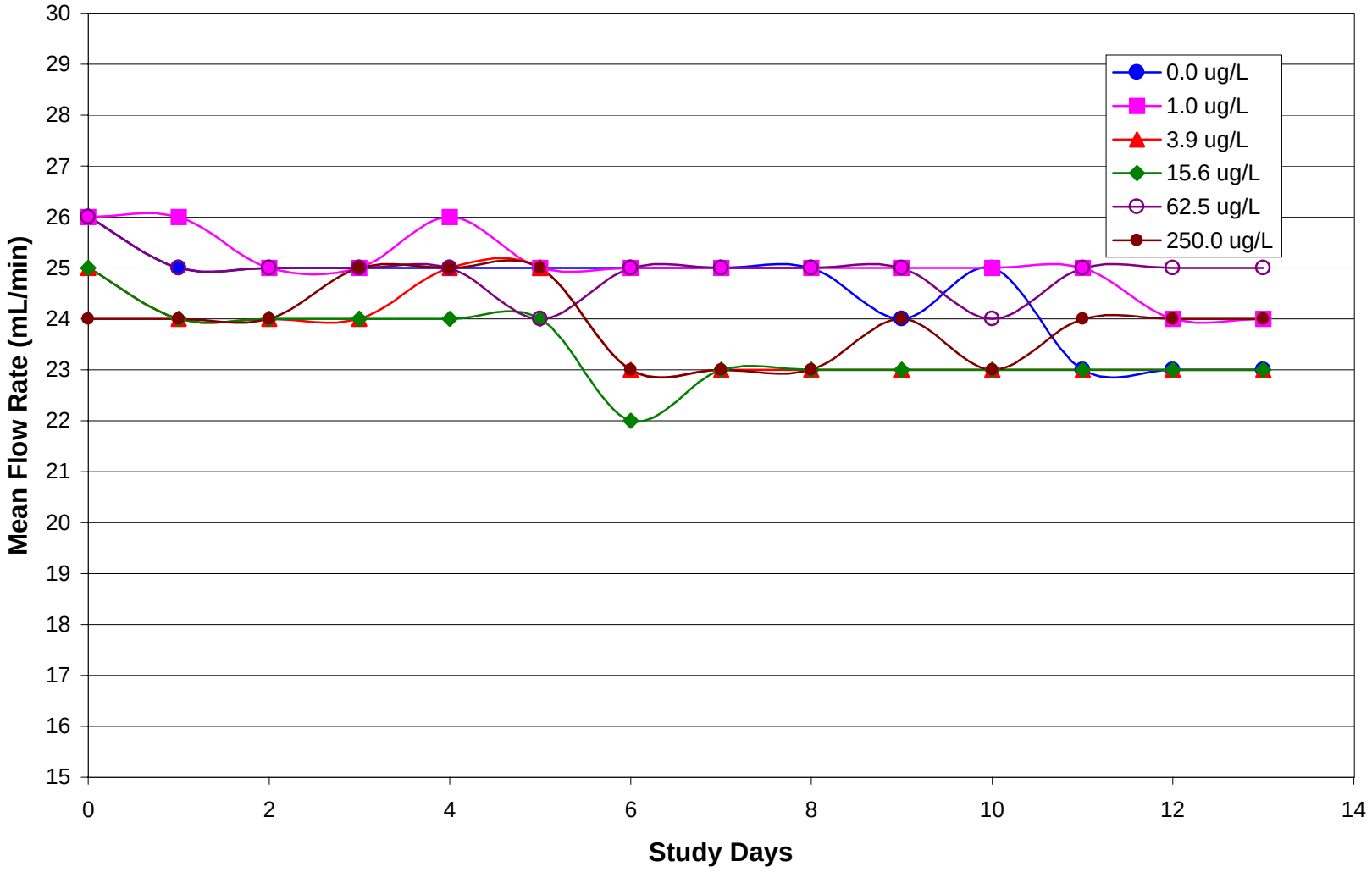
BATT02-00063
Dexamethasone
Daily Average Dissolved Oxygen (DO) for Initial Stage 54 Test



BATT02-00063
Dexamethasone
Daily Average Light Intensity for Initial Stage 54 Test



BATT02-00063
Dexamethasone
Daily Average Flow Rates for Initial Stage 54 Test



Dexamethasone
Metamorphosis Assay:
Test Termination Data – Stage and Growth

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 0.0 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	241	0.6345	55	
	242	0.4372	56	
	243	0.8246	57	
	244	1.1818	57	
	245	1.0320	59	
	246	0.8789	59	Kinked Tail
	247	0.3872	55	
	248	0.4949	55	
	249	0.4247	55	
	250	0.6683	56	Kinked Tail
	251	0.5913	57	Kinked Tail
	252	0.7340	57	
	253	0.4796	56	
	254	0.6842	56	Kinked Tail
	255	0.8464	56	Kinked Tail
	256	0.6240	56	
	257	0.4274	54	
	258	0.3508	54	
	259	0.5418	55	
	260	0.4623	55	
	Median:	**0.608**	**56**	
	Mode:		**55**	
	Mean:	**0.635**	**56.000**	
	SEM:	**0.050**	**0.308**	
	%CV:	**28.245**	**18.394**	
B	261	0.5348	55	
	262	0.4307	55	
	263	0.7651	56	Kinked Tail
	264	0.8937	57	Kinked Tail
	265	0.6471	57	
	266	1.1414	59	
	267	1.2312	59	
	268	0.2937	54	
	269	0.5053	55	
	270	0.6609	55	
	271	0.8102	56	
	272	1.1995	56	
	273	0.5877	56	
	274	1.3127	57	
	275	0.5183	55	
	276	0.6087	55	
	277	0.7946	56	
	278	0.7314	56	
	279	0.7710	56	
	280	0.6525	56	
	Median:	**0.696**	**56**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.755**	**56.050**	
	SEM:	**0.062**	**0.285**	
	%CV:	**32.073**	**17.048**	
	Median:	**0.650**	**56**	
	Mode:		**56**	
	Total Mean:	**0.695**	**56.025**	
	SEM:	**0.041**	**0.207**	
	%CV:	**30.853**	**17.508**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 1.0 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	281	0.7308	55	Kinked Tail
	282	0.8105	56	Kinked Tail
	283	0.8958	56	
	284	0.7970	57	
	285	0.8099	57	
	286	0.9883	59	
	287	0.6809	59	Kinked Tail
	288	0.5652	55	
	289	0.6081	56	Kinked Tail
	290	0.4530	56	
	291	0.6022	56	
	292	0.8423	57	
	293	1.0082	57	
	294	1.0591	57	Kinked Tail
	295	0.4002	55	
	296	0.5069	55	
	297	0.4376	56	
	298	0.5529	56	Kinked Tail
	299	0.8240	56	
	300	0.7868	57	
	Median:	**0.759**	**56**	
	Mode:		**56**	
	Mean:	**0.718**	**56.400**	
	SEM:	**0.044**	**0.255**	
	%CV:	**23.164**	**15.213**	
B	301	0.4360	54	
	302	0.7304	55	
	303	0.7379	55	Kinked Tail
	304	0.8683	56	
	305	0.9757	56	Kinked Tail
	306	1.0921	57	
	307	1.2475	57	
	308	0.4003	54	
	309	0.5457	55	
	310	0.4291	55	
	311	0.6727	55	
	312	0.6598	56	
	313	0.7718	56	
	314	0.8952	56	
	315	0.3563	54	
	316	0.5046	55	
	317	0.7495	56	
	318	0.7569	56	
	319	0.7233	56	
	320	0.9422	57	
	Median:	**0.734**	**56**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.725**	**55.550**	
	SEM:	**0.053**	**0.211**	
	%CV:	**27.894**	**12.673**	
	Median:	**0.734**	**56**	
	Mode:		**56**	
	Total Mean:	**0.721**	**55.975**	
	SEM:	**0.034**	**0.177**	
	%CV:	**25.322**	**14.978**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 3.9 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	321	0.3874	55	
	322	0.4879	55	Kinked Tail
	323	0.7177	56	Kinked Tail
	324	0.4852	56	
	325	0.5062	56	
	326	0.7000	57	
	327	0.8877	57	
	328	0.8592	57	Kinked Tail
	329	0.8489	59	
	330	0.5886	55	Kinked Tail
	331	0.4560	55	Kinked Tail
	332	0.7595	56	Kinked Tail
	333	0.7539	56	
	334	0.9014	57	Kinked Tail
	335	0.9967	59	
	336	0.5020	59	Kinked Tail
	337	0.4706	55	Kinked Tail
	338	0.4348	55	Kinked Tail
	339	0.6186	56	
	340	0.5116	56	
	Median:	**0.604**	**56**	
	Mode:		**56**	
	Mean:	**0.644**	**56.350**	
	SEM:	**0.042**	**0.302**	
	%CV:	**23.183**	**17.964**	
B	341	0.3409	55	
	342	0.3761	55	
	343	0.6255	56	
	344	0.7703	56	
	345	0.7482	56	
	346	0.5810	56	
	347	0.8061	57	
	348	0.6554	57	
	349	0.3230	55	
	350	0.4265	55	
	351	0.5242	55	
	352	0.6154	56	
	353	0.6809	56	Kinked Tail
	354	0.7819	56	
	355	0.4984	56	
	356	0.6133	56	
	357	0.2939	55	
	358	0.3792	55	
	359	0.7655	56	
	360	0.6680	56	
	Median:	**0.614**	**56**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.574**	**55.750**	
	SEM:	**0.038**	**0.143**	
	%CV:	**22.224**	**8.554**	
	Median:	**0.614**	**56**	
	Mode:		**56**	
	Total Mean:	**0.609**	**56.050**	
	SEM:	**0.028**	**0.172**	
	%CV:	**22.898**	**14.491**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 15.6 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	361	0.2900	54	
	362	0.2581	54	
	363	0.4006	55	
	364	0.4286	55	
	365	0.4213	55	
	366	0.6623	56	
	367	0.5209	56	Kinked Tail
	368	0.4416	56	Kinked Tail
	369	0.6848	56	
	370	0.4138	55	
	371	0.4072	55	Kinked Tail
	372	0.7443	55	
	373	0.4334	56	Kinked Tail
	374	0.4491	56	Kinked Tail
	375	0.5193	56	
	376	0.6311	56	Kinked Tail
	377	0.6289	56	Kinked Tail
	378	0.4900	56	
	379	0.5344	56	
	380	0.5720	57	Kinked Tail
	Median:	**0.470**	**56**	
	Mode:		**56**	
	Mean:	**0.497**	**55.550**	
	SEM:	**0.029**	**0.170**	
	%CV:	**18.164**	**10.186**	
B	381	0.1618	53	
	382	0.3274	54	
	383	0.4082	55	Kinked Tail
	384	0.3537	55	Kinked Tail
	385	0.4051	56	Kinked Tail
	386	0.3459	56	
	387	0.6041	56	Kinked Tail
	388	0.7521	57	
	389	0.5966	57	Kinked Tail
	390	0.6034	59	
	391	0.4187	55	Kinked Tail
	392	0.3728	55	
	393	0.3402	55	
	394	0.5629	56	Kinked Tail
	395	0.7919	56	
	396	0.4301	56	
	397	0.4116	56	
	398	0.3859	56	
	399	0.4835	56	
	400	0.8655	57	
	Median:	**0.415**	**56**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.481**	**55.800**	
	SEM:	**0.039**	**0.277**	
	%CV:	**25.430**	**16.596**	
	Median:	**0.438**	**56**	
	Mode:		**56**	
	Total Mean:	**0.489**	**55.675**	
	SEM:	**0.024**	**0.162**	
	%CV:	**21.785**	**13.704**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 62.5 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	401	0.1332	53	
	402	0.2104	54	
	403	0.4015	55	
	404	0.3631	55	
	405	0.4066	55	Kinked Tail
	406	0.2211	55	
	407	0.5295	56	
	408	0.3845	56	
	409	0.3978	56	
	410	0.5974	56	
	411	0.4968	57	
	412	0.2051	53	
	413	0.2714	54	
	414	0.3152	54	
	415	0.3144	55	
	416	0.2981	55	
	417	0.2985	55	
	418	0.3339	55	
	419	0.4291	55	
	420	0.3574	55	
	Median:	**0.346**	**55**	
	Mode:		**55**	
	Mean:	**0.348**	**54.950**	
	SEM:	**0.026**	**0.223**	
	%CV:	**19.403**	**13.472**	
B	421	0.2327	54	
	422	0.2882	55	
	423	0.3682	55	
	424	0.4179	55	
	425	0.3918	55	
	426	0.3308	55	
	427	0.5117	56	
	428	0.4280	56	
	429	0.5030	56	
	430	0.5843	56	
	431	0.4589	56	
	432	0.2679	54	
	433	0.3516	55	
	434	0.3418	55	
	435	0.4369	55	
	436	0.3433	55	
	437	0.4692	55	
	438	0.3708	56	
	439	0.5139	56	
	440	0.4430	56	Kinked Tail
	Median:	**0.405**	**55**	**Comments:**
	Mode:		**55**	
	Rep Mean:	**0.403**	**55.300**	
	SEM:	**0.020**	**0.147**	
	%CV:	**14.282**	**8.834**	
	Median:	**0.370**	**55**	
	Mode:		**55**	
	Total Mean:	**0.375**	**55.125**	
	SEM:	**0.017**	**0.135**	
	%CV:	**17.231**	**11.488**	

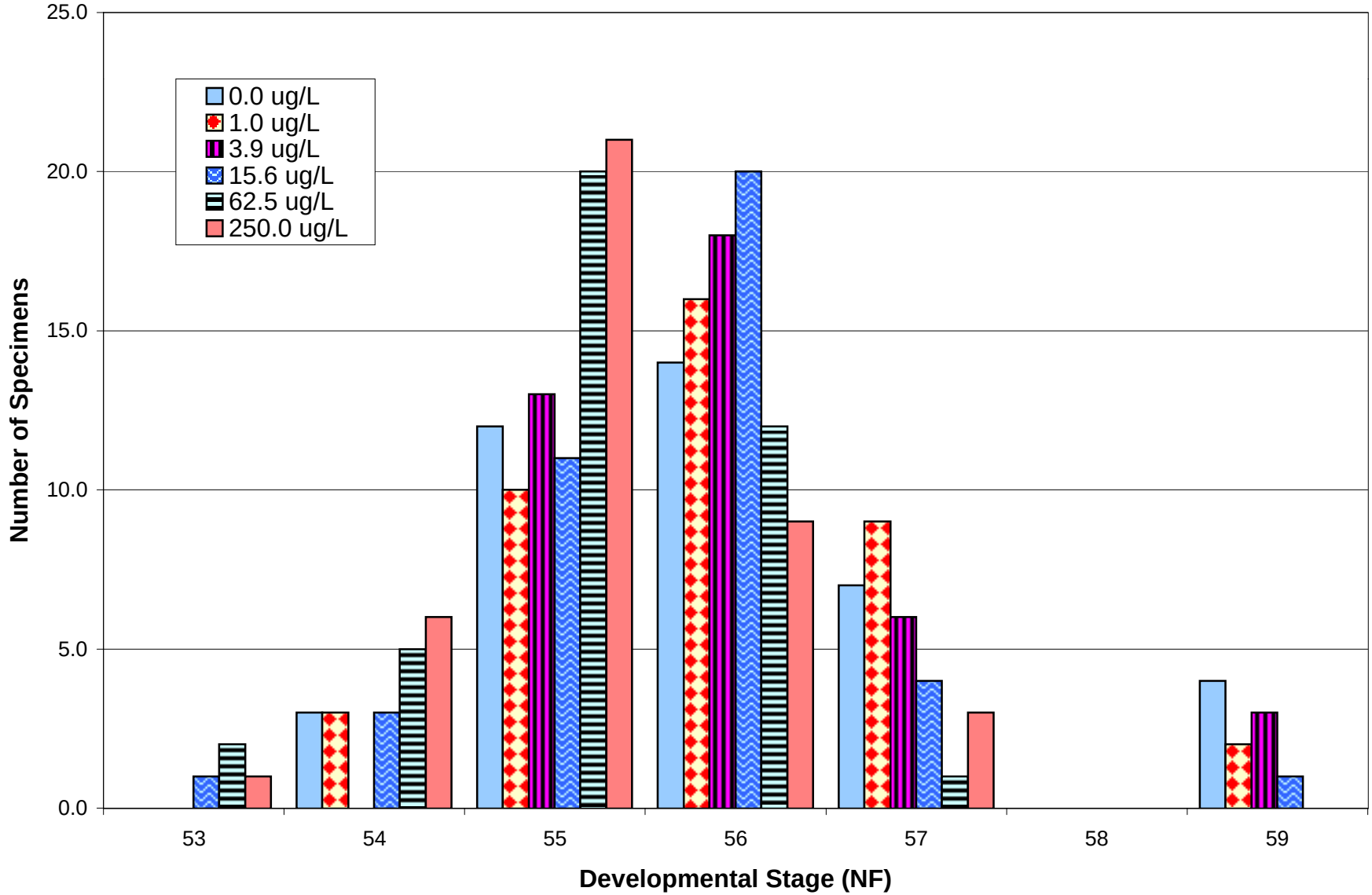
TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 250.0 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	461	0.1445	53	
	462	0.1132	54	
	463	0.1392	54	
	464	0.2596	54	
	465	0.3781	55	
	466	0.1954	55	
	467	0.2561	55	
	468	0.3095	55	
	469	0.5409	56	
	470	0.6794	57	
	471	0.2690	54	
	472	0.2648	54	
	473	0.2543	54	
	474	0.3196	55	
	475	0.3157	55	
	476	0.3957	55	
	477	0.3688	55	
	478	0.3672	55	
	479	0.3957	56	
	480	0.5220	56	
	Median:	**0.313**	**55**	
	Mode:		**55**	
	Mean:	**0.324**	**54.850**	
	SEM:	**0.032**	**0.209**	
	%CV:	**24.744**	**12.602**	
B	441	0.3068	55	
	442	0.3443	55	
	443	0.3078	55	
	444	0.3371	55	
	445	0.4531	55	
	446	0.4724	56	
	447	0.4877	56	
	448	0.3601	56	
	449	0.4229	56	
	450	0.4479	57	
	451	0.5491	57	
	452	0.3103	55	
	453	0.3804	55	
	454	0.3594	55	
	455	0.3253	55	
	456	0.3229	55	
	457	0.3873	55	
	458	0.2663	55	
	459	0.4988	56	
	460	0.3526	56	
	Median:	**0.360**	**55**	**Comments:**
	Mode:		**55**	
	Rep Mean:	**0.385**	**55.500**	
	SEM:	**0.017**	**0.154**	
	%CV:	**12.466**	**9.238**	
	Median:	**0.348**	**55**	
	Mode:		**55**	
	Total Mean:	**0.355**	**55.175**	
	SEM:	**0.018**	**0.138**	
	%CV:	**19.527**	**11.763**	

BATT02-00063
Dexamethasone
Stage Distribution for Initial Stage 51 Test



TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 0.0 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	001	0.9387	59	
	002	1.0160	57	
	003	0.6338	57	
	004	0.9256	57	
	005	0.8588	59	
	006	0.8817	58	
	007	0.8474	58	
	008	0.7115	56	
	009	0.7598	57	
	010	0.8288	57	
	011	1.1213	59	
	012	0.6569	56	
	013	0.9870	57	
	014	0.5401	56	
	015	0.6922	57	
	016	0.6321	56	
	017	0.5460	56	
	018	0.7752	57	
	019	0.7312	57	
	020	0.7067	57	
	Median:	**0.768**	**57**	
	Mode:		**57**	
	Mean:	**0.790**	**57.150**	
	SEM:	**0.035**	**0.221**	
	%CV:	**17.783**	**13.070**	
D	021	0.9922	57	
	022	0.8488	57	
	023	1.1364	57	
	024	0.9616	59	
	025	1.0241	59	
	026	0.7724	56	
	027	0.7901	58	
	028	0.9535	59	Kinked Tail
	029	0.6869	56	
	030	0.9997	57	
	031	0.6216	56	
	032	0.6998	56	
	033	0.6946	58	
	034	0.8543	58	
	035	0.4586	55	
	036	0.7390	57	
	037	0.7464	57	
	038	0.5662	56	
	039	0.6060	57	
	040	0.6727	56	
	Median:	**0.759**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.791**	**57.050**	
	SEM:	**0.039**	**0.256**	
	%CV:	**19.843**	**15.172**	
	Median:	**0.766**	**57**	
	Mode:		**57**	
	Total Mean:	**0.790**	**57.100**	
	SEM:	**0.026**	**0.167**	
	%CV:	**18.599**	**13.992**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 1.0 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	041	0.7227	57	
	042	0.8346	57	
	043	1.0395	59	
	044	0.9156	58	
	045	0.9043	57	Kinked Tail
	046	0.7349	56	Kinked Tail
	047	1.0993	57	
	048	0.7773	56	
	049	0.6506	57	
	050	0.8295	56	
	051	0.7932	57	
	052	0.5806	55	
	053	0.6636	56	
	054	0.6012	55	
	055	0.9762	57	Kinked Tail
	056	0.7314	56	
	057	0.6632	57	
	058	0.8247	56	
	059	0.8912	57	Kinked Tail
	060	0.8304	58	
	Median:	**0.809**	**57**	
	Mode:		**57**	
	Mean:	**0.803**	**56.700**	
	SEM:	**0.031**	**0.219**	
	%CV:	**15.712**	**12.998**	
D	061	0.8210	57	Kinked Tail
	062	0.8967	59	
	063	1.0359	57	Kinked Tail
	064	0.9770	58	Kinked Tail
	065	0.6759	57	Kinked Tail
	066	0.4560	56	
	067	0.7709	57	
	068	0.6527	56	Kinked Tail
	069	0.7276	57	
	070	0.9684	59	
	071	0.5920	55	Kinked Tail
	072	0.6511	57	
	073	0.7987	56	
	074	1.1201	58	Kinked Tail
	075	0.5895	60	
	076	0.5700	60	
	077	0.6782	56	
	078	0.8006	58	Kinked Tail
	079	0.7638	59	Kinked Tail
	080	0.6715	57	Kinked Tail
	Median:	**0.746**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.761**	**57.450**	
	SEM:	**0.038**	**0.312**	
	%CV:	**19.573**	**18.399**	
	Median:	**0.774**	**57**	
	Mode:		**57**	
	Total Mean:	**0.782**	**57.075**	
	SEM:	**0.025**	**0.197**	
	%CV:	**17.634**	**16.524**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 3.9 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	081	0.5767	58	
	082	0.5089	56	
	083	0.6938	59	
	084	0.6542	57	
	085	0.8750	57	Kinked Tail
	086	0.7930	57	
	087	0.6069	56	
	088	0.5573	57	Kinked Tail
	089	0.5536	60	
	090	0.5389	56	
	091	0.6708	59	
	092	0.7782	59	Kinked Tail
	093	0.7233	58	
	094	0.7100	59	
	095	0.4554	60	
	096	0.6824	59	
	097	0.6044	57	Kinked Tail
	098	0.6935	58	Kinked Tail
	099	0.6870	59	
	100	0.6404	59	
	Median:	**0.663**	**58**	
	Mode:		**59**	
	Mean:	**0.650**	**58.000**	
	SEM:	**0.023**	**0.290**	
	%CV:	**12.742**	**17.041**	
D	101	0.6146	56	Kinked Tail
	102	0.8548	57	
	103	0.6378	58	
	104	1.0636	57	
	105	0.8560	57	Kinked Tail
	106	0.7446	59	
	107	0.8027	57	
	108	0.5735	55	
	109	0.6577	56	
	110	0.6674	56	
	111	0.6374	57	
	112	0.7156	56	
	113	0.6029	55	
	114	0.7933	58	Kinked Tail
	115	0.8372	57	
	116	0.8516	56	
	117	0.6960	56	
	118	0.6705	58	
	119	0.9674	59	
	120	0.5834	60	
	Median:	**0.706**	**57**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.741**	**57.000**	
	SEM:	**0.030**	**0.299**	
	%CV:	**15.510**	**17.718**	
	Median:	**0.677**	**57**	
	Mode:		**57**	
	Total Mean:	**0.696**	**57.500**	
	SEM:	**0.020**	**0.221**	
	%CV:	**15.148**	**18.409**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 15.6 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	121	0.6325	57	Kinked Tail
	122	0.7188	57	
	123	0.6653	57	
	124	0.6255	56	
	125	0.4578	56	
	126	0.7216	57	
	127	0.6626	57	
	128	0.6683	57	
	129	0.5287	57	
	130	0.5890	57	
	131	0.7347	57	Kinked Tail
	132	0.5388	56	
	133	0.7469	57	
	134	0.7728	57	Kinked Tail
	135	0.6735	57	
	136	0.6274	57	
	137	0.6948	57	
	138	0.7168	57	
	139	0.7361	56	
	140	0.6789	57	
	Median:	**0.671**	**57**	
	Mode:		**57**	
	Mean:	**0.660**	**56.800**	
	SEM:	**0.018**	**0.092**	
	%CV:	**9.981**	**5.445**	
D	141	0.6184	57	
	142	0.6131	56	Kinked Tail
	143	0.8082	57	
	144	0.7194	56	
	145	0.7871	57	
	146	0.3523	55	
	147	0.3375	55	
	148	0.4587	56	
	149	0.5905	57	
	150	0.4173	55	
	151	0.5495	56	
	152	0.6665	57	
	153	0.4812	58	
	154	0.4931	56	
	155	0.7580	57	
	156	0.6091	59	
	157	0.6374	56	
	158	0.6930	56	
	159	0.5464	57	
	160	0.6119	57	
	Median:	**0.611**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.587**	**56.500**	
	SEM:	**0.030**	**0.224**	
	%CV:	**17.563**	**13.304**	
	Median:	**0.635**	**57**	
	Mode:		**57**	
	Total Mean:	**0.623**	**56.650**	
	SEM:	**0.018**	**0.122**	
	%CV:	**14.639**	**10.225**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 62.5 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	161	0.5605	55	
	162	0.6204	55	
	163	0.6202	56	
	164	0.6443	57	
	165	0.5438	56	
	166	0.6204	56	
	167	0.7136	57	
	168	0.5482	57	
	169	0.4989	56	
	170	0.4581	56	
	171	0.5099	56	
	172	0.6076	57	
	173	0.6027	56	
	174	0.4651	56	
	175	0.6032	56	
	176	0.5111	56	
	177	0.5472	56	
	178	0.5647	56	
	179	0.4299	56	
	180	0.6148	56	
	Median:	**0.563**	**56**	
	Mode:		**56**	
	Mean:	**0.564**	**56.100**	
	SEM:	**0.016**	**0.124**	
	%CV:	**9.451**	**7.377**	
D	181	0.4361	55	
	182	0.6600	56	
	183	0.4875	56	
	184	0.5088	57	
	185	0.5555	57	
	186	0.4962	55	
	187	0.6783	56	
	188	0.5742	57	
	189	0.6726	57	
	190	0.4614	55	
	191	0.4842	56	
	192	0.4988	56	
	193	0.5695	57	
	194	0.5736	57	
	195	0.4363	55	
	196	0.5863	56	
	197	0.5435	56	
	198	0.6121	56	
	199	0.6406	56	
	200	0.7964	57	
	Median:	**0.563**	**56**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.564**	**56.150**	
	SEM:	**0.021**	**0.167**	
	%CV:	**12.405**	**9.944**	
	Median:	**0.563**	**56**	
	Mode:		**56**	
	Total Mean:	**0.564**	**56.125**	
	SEM:	**0.013**	**0.102**	
	%CV:	**10.884**	**8.649**	

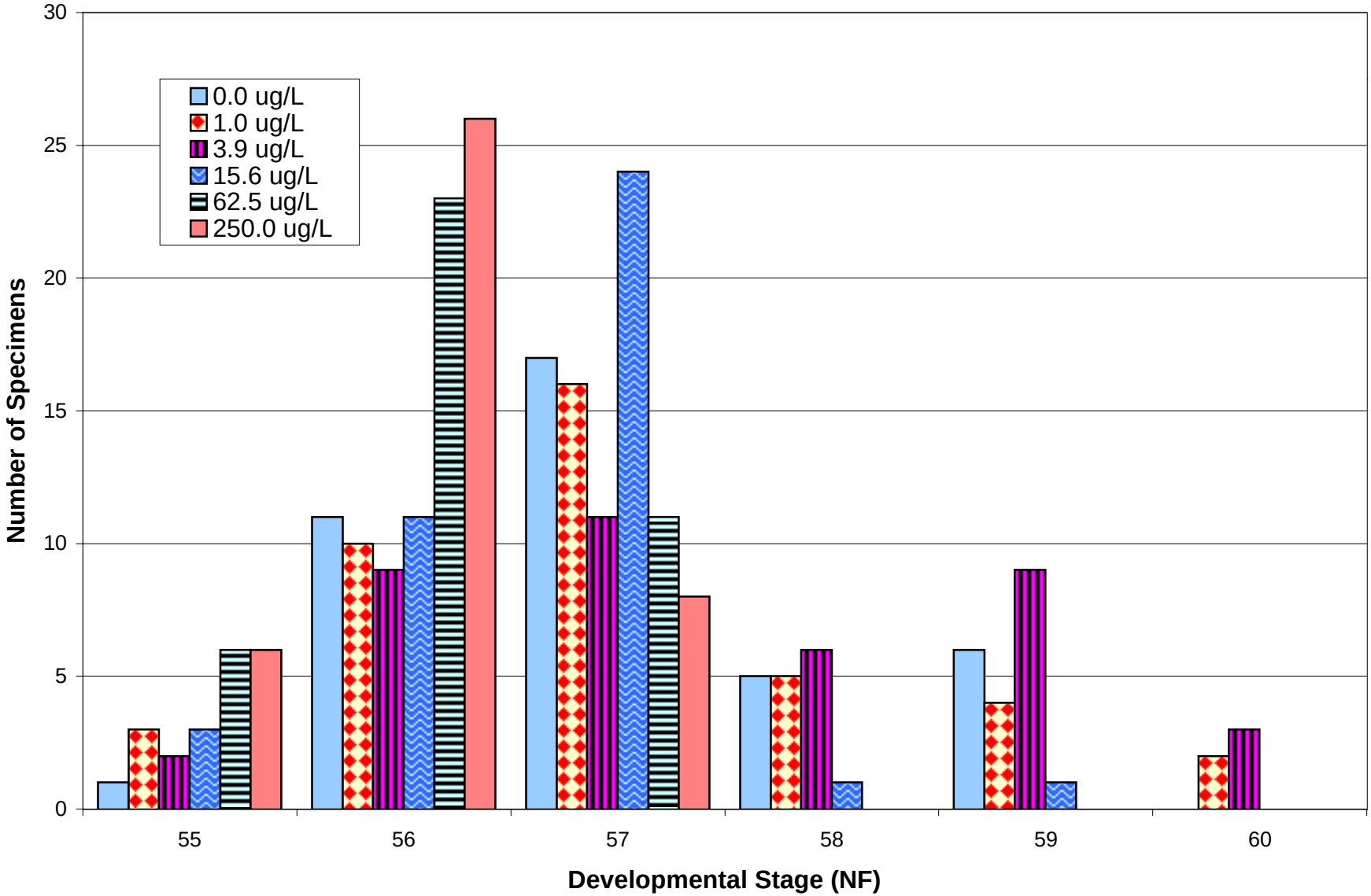
TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00063 **Test No: 1**

Test Chemical: Dexamethasone (DXM) **Target Conc: 250.0 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	201	0.3918	55	
	202	0.4774	56	
	203	0.5881	56	
	204	0.5378	57	
	205	0.7257	57	
	206	0.4067	55	
	207	0.7000	56	
	208	0.4751	56	
	209	0.5120	56	
	210	0.4591	56	
	211	0.5272	55	
	212	0.4373	56	
	213	0.4648	56	
	214	0.5084	56	
	215	0.5357	57	
	216	0.4480	56	
	217	0.5568	56	
	218	0.5377	56	
	219	0.6110	56	
	220	0.5657	57	
	Median:	**0.520**	**56**	
	Mode:		**56**	
	Mean:	**0.523**	**56.050**	
	SEM:	**0.019**	**0.135**	
	%CV:	**12.029**	**8.078**	
D	221	0.3932	55	
	222	0.3857	56	
	223	0.5285	56	
	224	0.4995	56	
	225	0.6194	57	
	226	0.5188	57	
	227	0.5270	57	
	228	0.4068	55	
	229	0.6360	56	
	230	0.5149	56	
	231	0.5843	56	
	232	0.5651	56	
	233	0.5347	56	
	234	0.4951	57	
	235	0.4154	55	
	236	0.6005	56	
	237	0.4085	56	
	238	0.4851	56	
	239	0.4490	56	
	240	0.4993	56	
	Median:	**0.507**	**56**	**Comments:**
	Mode:		**56**	
	Rep Mean:	**0.503**	**56.050**	
	SEM:	**0.017**	**0.135**	
	%CV:	**10.668**	**8.078**	
	Median:	**0.513**	**56**	
	Mode:		**56**	
	Total Mean:	**0.513**	**56.050**	
	SEM:	**0.013**	**0.094**	
	%CV:	**11.323**	**7.974**	

BATT02-00063
Dexamethasone
Stage Distribution for Initial Stage 54 Test



Dexamethasone Metamorphosis Assay: Statistical Data – Stage and Growth

One Way Analysis of Variance

Data source: BATT02-00063_DXM_Stage 51_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0ug/L	40	0	56	55	57
1.0ug/L	40	0	56	55	57
3.9ug/L	40	0	56	55	56
15.6ug/L	40	0	56	55	56
62.5ug/L	40	0	55	55	56
250.0ug/L	40	0	55	55	56

H = 29.726 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
62.5ug/L vs 0.0ug/L	49.225	3.171	Yes	**LOEC**
250.0ug/L vs 0.0ug/L	48.15	3.102	Yes	
15.6ug/L vs 0.0ug/L	12.063	0.777	No	**NOEC**
3.9ug/L vs 0.0ug/L	4.938	0.318	Do Not Test	
1.0ug/L vs 0.0ug/L	3.4	0.219	Do Not Test	

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00063_DXM_Stage 51_Growth

Normality Test: Passed (P = 0.113)

Equal Variance Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0ug/L	40	0	0.65	0.5	0.817
1.0ug/L	40	0	0.734	0.549	0.855
3.9ug/L	40	0	0.614	0.478	0.757
15.6ug/L	40	0	0.438	0.403	0.6
62.5ug/L	40	0	0.37	0.306	0.44
250.0ug/L	40	0	0.348	0.288	0.409

H = 106.452 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
250.0ug/L vs 0.0ug/L	106.825	6.881	Yes	
62.5ug/L vs 0.0ug/L	97.862	6.304	Yes	
15.6ug/L vs 0.0ug/L	54.15	3.488	Yes	**LOEC**
3.9ug/L vs 0.0ug/L	15.387	0.991	No	**NOEC**
1.0ug/L vs 0.0ug/L	11.875	0.765	Do Not Test	

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00063_DXM_Stage 54_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0ug/L	40	0	57	56	58
1.0ug/L	40	0	57	56	58
3.9ug/L	40	0	57	56	59
15.6ug/L	40	0	57	56	57
62.5ug/L	40	0	56	56	57
250.0ug/L	40	0	56	56	56

H = 53.228 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
250.0ug/L vs 0.0ug/L	68.262	4.397	Yes	
62.5ug/L vs 0.0ug/L	61.625	3.97	Yes	**LOEC**
15.6ug/L vs 0.0ug/L	21.212	1.366	No	**NOEC**
3.9ug/L vs 0.0ug/L	13.638	0.878	Do Not Test	
1.0ug/L vs 0.0ug/L	4.362	0.281	Do Not Test	

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00063_DXM_Stage 54_Growth

Normality Test: Passed (P = 0.087)

Equal Variance Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0ug/L	40	0	0.766	0.68	0.932
1.0ug/L	40	0	0.774	0.663	0.894
3.9ug/L	40	0	0.677	0.606	0.786
15.6ug/L	40	0	0.635	0.548	0.718
62.5ug/L	40	0	0.563	0.499	0.617
250.0ug/L	40	0	0.513	0.454	0.561

H = 106.963 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
250.0ug/L vs 0.0ug/L	123.125	7.931	Yes	
62.5ug/L vs 0.0ug/L	97.7	6.293	Yes	
15.6ug/L vs 0.0ug/L	60.888	3.922	Yes	**LOEC**
3.9ug/L vs 0.0ug/L	32.063	2.065	No	**NOEC**
1.0ug/L vs 0.0ug/L	1.375	0.0886	Do Not Test	

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Dexamethasone
Test Chemical Analytical Data from Battelle:
Purity / Stability Report

Battelle
Putting Technology To Work

Chemical Repository Services for the EDSP
EPA Contract No. 68-W-01-023

1.0 TITLE AND APPROVAL

Chemistry Report for WA 3-11 Dexamethasone in well water

October 25, 2004

Prepared By:

Tim Fortman — 10-25-04 (Date)
Chemical Repository Analyst

Approved By:

Richard M. Ecker — 10/25/04 (Date)
Director, Marine Sciences Laboratory

Michael E Cobb — 10-25-04 (Date)
Chemical Repository Study Director

Battelle Marine Sciences Laboratory
1529 West Sequim Bay Road
Sequim, WA 98382

Submitted to:

Doug Fort
Fort Environmental Laboratories
Stillwater, Oklahoma 74074

2.0 QA REVIEWER AND CONTRIBUTOR PAGE

Chemistry Report for WA 3-11
Dexamethasone in Well Water

Reviewed by: Mary E Lynn
Mary Lynn, EDSP Quality Assurance Representative
Windward Company, Gig Harbor

Date: 10/25/04

Personnel participating in this study:

Analyst: Tim Fortman, Rebecca Wood

Chemical Repository Study Director: Michael Cobb

3.0 EXECUTIVE SUMMARY

Chemistry Report for WA 3-11
Dexamethasone in Well Water

Parameter	Chemical
Compound Name	Dexamethasone
CAS #	50-02-2
Central File No.	2207-1b
Initial Receipt Date	05/11/2004
Expiration Date	05/11/2008
Manufacturer	Biomol
Lot Number	P4311
Battelle Study #	WA 3-11
Method	EDSP.H4-006

Executive Summary

Work Assignment (WA) 3-11 of the Environmental Protection Agency's Endocrine Disruptor Screening Program (EDSP) describes a reproductive and developmental study on frogs raised in water spiked with one of six study chemicals. The Chemical Repository (Repository) has the responsibility for carrying out the purity, stability, and in-life concentration determinations of the selected study chemicals for EDSP studies.

The chemical of interest for this portion of the study is Dexamethasone. The chemical purity of Dexamethasone, as determined by the manufacturer, was 99%. The analytical method developed in-house for the Dexamethasone stability study and in-life samples utilized High Performance Liquid Chromatography (HPLC) with UV/Vis detection. The Battelle-Sequim purity result by HPLC was 99.54%.

The stability of the water dosed with Dexamethasone was analyzed at 2 concentrations, one representing the stock feed water (9.9 mg/L) the other representing one of the amphibian exposure concentrations (15.84 ug/L). These concentrations were to be tested over 21 days. Due to rapid degradation, the study was terminated after 20 days. Based on the target of less than 10% degradation over the study period, the Dexamethasone in the stock feed water was demonstrated to remain greater than or equal to 90% of the target concentration for up to an estimated 7 days. The Dexamethasone in the diluted water was less than the 90% target prior to the 1st sampling at 3 days.

Notes:

The Dexamethasone appears to be unstable in a diluted form, particularly at the lower concentrations. Several theories exist, a likely candidate is that the concentrations evaluated are quite low and are very close to the sensitivity limits of the detection method. Some of what appears to be degradation may simply be the result of "noise" resulting in significant quantitation variability at the low end of the calibration curve. There are other potential sources of the apparent instability, such as the compound adsorbing to the walls of the container and/or changing forms in solution. A decision was made with the In'Life Study Director to proceed with the exposure study with the recommendation that the in-life samples be collected and shipped immediately via overnight courier to the Repository lab. Analysis will be carried out within 24 hours of sample receipt.

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5.0 INTRODUCTION

The goal of the Battelle-Sequim, Marine Sciences Laboratory (MSL) Repository for the Endocrine Disruptor Screening Program is to provide the participating laboratory with requested chemicals of documented quality at required concentrations and in a matrix appropriate for different toxicological tests. The EDSP Chemical Repository supplies the manufacturer's information regarding purity and stability, the material safety data sheet (MSDS) chemical information, and independent analysis of purity and stability in a matrix specified by the Study Protocol: Purity and Stability of Test Substances for Work Assignment 3-11 [EDSP Study Number: EDSP.311-01], made in collaboration with the requesting Principal Investigator. Under Work Assignment (WA) 3-11, Fort Environmental Laboratories requested purity and stability testing of Dexamethasone (figure 1).

6.0 GENERAL METHODS

Methods of standard operation of the Repository are currently addressed in MSL SOPs numbered R-001 through R-017. These procedures address chemical procurement including procurement of controlled substances, when applicable, which have unique permitting, ordering, handling, inventory, and storage requirements; chemical receipt and chain of custody, chemical log-in and labeling, inventory, chemical storage; stock solution preparation, documentation and archiving; test solution preparation, documentation and shipping; chemical disposal, and repository maintenance over time. The quality assurance (QA) requirements for procurement of chemicals for use in the Repository are addressed in Version 2 of the Quality Assurance Project Plan for EDSP Chemical Repository (currently in circulation for approval signatures).

6.1 CHEMICAL PROCUREMENT

As requested (Figure 1) by Doug Fort, the Principal Investigator for the Study, Dexamethasone, (CAS No. 50-02-2), was purchased for purity and stability analysis and used as the test chemical, in a one generational study on frogs. The Dexamethasone was purchased from BioMol, Inc. and the first shipment of lot number P4311 was received on 5/11/04 with a expiration date of 5/11/08 (Table 1). The chemical was logged in to the Chemical Management System (CMS) and given a CMS barcode and unique log-in (central file) number (CF-2207-1b) as per the QA Project Plan (QAPP) for the EDSP Chemical Repository. The chemical was stored in the Repository in a clean, dry location at room temperature, away from direct sunlight.

Table 1. Chemical Procurement Information

Parameter	Chemical
Compound Name	Dexamethasone
CAS #	50-02-2
Central File No.	2207-1b
Initial Receipt Date	05/11/2004
Expiration Date	05/11/2008
Manufacturer	BioMol
Lot Number	P4311
Battelle Study #	WA 3-11
Method	EDSP.H4-006

EDSP Chemical Request Form

For EPA WA: 3-11

Study Director:
Name: Douglas J. Fort, Ph.D.
Affiliation: Fort Environmental Laboratories
Location: Stillwater, Oklahoma 74074
Telephone number: 405-624-6771

Bioassay Information:

Proposed Bioassay: Frog Metamorphosis Assay Demonstration

Test Chemical: Dexamethasone

Carrier(s): NA

Concentrations/Dilution Series: 1.0, 3.9, 15.6, 62.5, 250.0 μg/L

*Consider if analysis method detection limit which may be determined in Purity analysis is above or below desired test concentrations?

In vitro or *in vivo* tests? *In vivo*

Organism to be tested: *Xenopus laevis* (South African Clawed Frog)

Method of test solution administration: Flow-through exposure/diluter system

Planned/proposed test duration: 14 and 21 days for assay

Estimated neat chemical required: 5 g

Chemical Information:

Chemical Name: Dexamethasone

CAS: 50-02-2

Any known purity information: may refer to attached documentation

Any known stability information: Contact Dr. Joe Tietge, EPA, Duluth, MN

Desired purity (%) for test? ≥98%

Is there a known calibration standard available?
Name:
CAS:
Manufacturer:

Figure 1. EDSP Chemical Request Form for Dexamethasone

6.2 CHEMICAL PURITY

Chemical purity was verified by high performance liquid chromatographic analysis to determine areas under peaks other than the principal peak and compared to the manufacturer's certificate of analysis/purity (Appendix A). No statistical analyses were performed for the verification of chemical purity. Purity verification was conducted by making a solution of Dexamethasone in 50:50 acetonitrile:water of about 0.5 µg/mL. This matrix was then run on an HPLC with a UV/Vis detector. An acetonitrile:water blank was also analyzed on the system. The purity was determined by first identifying the peaks in the Dexamethasone chromatogram that were the same as the peaks in the analysis of the blank. The areas associated with these common peaks were then eliminated by inhibiting integration and the remaining peaks were reported as a percentage of the total peak area. The percentage associated with the largest peak represented the purity of Dexamethasone. The HPLC was set up with an autosampler and a 250X4.6 mm reversed-phase HPLC column. The HPLC system was run isocratically, with a 50% acetonitrile/50% water eluent, the detector was set to a wavelength of 240 nm. One replicate was analyzed.

6.3 STABILITY TESTING PLAN DESIGN AND DETAIL

A general study plan for stability testing based on the WA 3-11 Chemical Request from Doug Forte (Figure 1) was developed as the *EDSP Stability Analysis and Purity Testing Plan* and is presented in Appendix C. A stock solution and one diluter solution were prepared to arrive at the chemical concentrations requested for stability analysis (Table 2). All samples were analyzed in triplicate for calculation of a mean concentration and relative standard deviation (RSD).

Dexamethasone stock matrices were prepared on 5/20/04 and 5/21/04 for testing as described in Table 2. Briefly, for the 9.9 mg/L Dexamethasone 0.0099 g was weighed into a 1 liter amber bottle and 1.0 L of well water was added using a 1 liter class A graduated cylinder. For the 15.84 ug/L 1.6 mL of the above 9.9 mg/L stock was measured into another 1 L amber bottle and diluted to 1 Liter with well water. All measurements were done with Class A graduated cylinders. The solution was agitated to insure uniformity. Solutions were stored on the counter at room temperature for the duration of the test.

Table 2. Stock Matrix Composition for Stability Testing

Study, Duration	Test Chemical	Target Conc.	Sample ID	Nominal Conc.	Stock Matrix
WA 3-11, 21 days	Dexamethasone	10 mg/L	Stock	9.9 mg/L	.0099 g in 1 L Water
		15.6 ug/L	Diluter	15.84 ug/L	1.6 ml of 9.9 mg/L in 1 L water

6.4 ANALYTICAL CHEMISTRY FOR STABILITY TESTING

Chemical stability was evaluated under storage conditions and matrix specifications as requested by the participating laboratory. At initiation and at each time period throughout the duration of the test, the chemical concentration was determined by chromatographic analysis. Triplicate aliquots were tested. The frequency of determinations and the duration of testing were determined by the requesting principal investigator and the chemists based on *a priori* knowledge about chemical stability.

Dexamethasone stock solution was sampled by taking 0.025 mL of the stock using a positive displacement pipette and diluting it to 1 mL with 0.975 mL of mobile phase, using a 1

mL syringe into a 1.8 mL vial. A transfer pipette was used to mix this solution and transfer it to an autosampler vial compatible with the HPLC autosampler.

Dexamethasone diluter solution was sampled by taking 0.5 mL of the diluter sample using a syringe and diluting it to 1 mL with 0.5 mL of Acetonitrile, using a 1 mL syringe into a 1.8 mL vial. A transfer pipette was used to mix this solution and transfer it to an autosampler vial compatible with the HPLC autosampler. Water blanks were prepared the same way. Spike solution, prepared similarly to the stability solutions, but stored at 4 deg. C. was used as a QA/QC sample, this sample was prepared for analysis in the same way as the stability solutions. This solution was then analyzed on the HPLC/UV/Vis for quantification. Initial calibration verification and continuing calibration verification (ICV/CCV) samples were analyzed to demonstrate on-going calibration accuracy.

Table 3. HPLC Conditions

Detector	UV/Vis, Wavelength, 240 nm
Flow Rate	1 ml/minute
Injection Volume	450 ul
Eluent; flow pattern	50% Acetonitrile:50% water, Isocratic
Column	Reverse Phase, Phenominex Synergi 4u Hydro RP 80A

7.0 RESULTS

7.1 CHEMICAL PURITY

Battelle-Sequim conducted an HPLC-UV/Vis purity scan on Dexamethasone. The chromatogram, after blank correction, showed only one large peak that had the appropriate retention time for Dexamethasone with several smaller peaks. The area of the Dexamethasone peak was 99.54% of the total area of all peaks in the chromatogram. Chemical purity of Dexamethasone determined by the manufacturer via TLC was 99% (Appendix A).

7.2 ANALYTICAL CHEMISTRY FOR STABILITY TESTING

Chemical stability testing was initiated on 05/21/04. Chemical concentrations were determined 6 times over a period of 20 days. Concentrations measured in the stock Stability test ranged from 102% to 74% of the target concentration. Concentrations measured in the diluter Stability test ranged from 94% to 60% of the target concentration. The analytical and quality control (QC) results are presented in Appendix C. 5 point calibration curves all met the R^2 criteria of 0.995, see table 4. Blanks and matrix spikes were analyzed with every batch for QC purposes. All but one blank were less than 3 times the detection limit of Dexamethasone of 1.36 ug/L for diluter samples and 54.53 ug/L stock and spike solutions. ICV/CCV results ranged from 93% to 111%. Some of the QC data were outside acceptance criteria, one blank was elevated, data was not blank corrected. Several matrix spikes were outside acceptance criteria of +/- 15%. This may indicate that Dexamethasone is unstable when stored at 4 deg. C. in well water, similar to room temperature instability. The MDL was determined to be 1.36 ug/L.

Table 4. Calibration Acceptance

Calibration Date	R2 Value (≥ .995)	Curve Fit
5/18/04	0.999898	Linear
5/21/04	0.999935	Linear
5/25/04	0.999982	Linear
5/28/04	0.999919	Linear
6/1/04	0.999921	Linear
6/4/04	0.999944	Linear
6/10/04	0.999970	Linear

8.0 CONCLUSIONS

Stability of the stock solution of Dexamethasone remained within 90% of the day zero concentration on day seven of the study. Though the diluted concentration dropped to 80% of the nominal day zero value by day three, it was decided to proceed with the in-life portion of the study with the caveat that a new stock solution would be prepared every 5 days over the duration of the in-life studies, and samples would be collected immediately and shipped via overnight courier to the lab in Sequim for analysis. All analyses will be carried out within 24 hours of sample receipt.

APPENDIX A

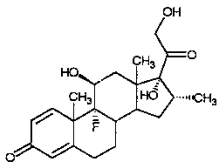
MANUFACTURER'S CERTIFICATE OF ANALYSIS/PURITY

PRODUCT DATA

PRODUCT: Dexamethasone **CAS NO:** 50-02-2

CATALOG NO.: EI-126 **LOT NO.:** P4311

STRUCTURE:



PHYSICAL APPEARANCE: White solid (m.p. 262-264°C)

MOLECULAR FORMULA: $C_{22}H_{29}FO_5$ **MOLECULAR WEIGHT:** 392.5

PURITY: 99% (TLC)

SOLUBILITY: Soluble in ethanol (25 mg/mL) and DMSO (25 mg/mL)

STORAGE: Store, as supplied, at room temperature for up to 5 years. Store solutions at -20°C for up to 3 months.

APPLICATION NOTES: Antiinflammatory glucocorticoid. Inhibits induction of NO synthase in a variety of cell lines and tissues (IC_{50} = 5nM).[1] Inhibits the production of PLA_2 inhibitory protein(lipocortin).[2] Induces apoptosis[3] in eosinophils, but inhibits it in neutrophils; both effects blocked by RU-486.[4] The product is not sterile.

REFERENCES:

1. S.Moncada *et al. Trends Pharmacol.Sci.* 1991 **12** 130
2. M.A.Clark *et al. Prostaglandins* 1986 **32** 703
3. H.O.Fearnhead *et al. Biochem.Pharmacol.* 1994 **48** 1073
4. L.C.Meagher *et al. J.Immunol.* 1996 **156** 4422

The pharmacological and toxicological properties of this product have not been fully investigated. Exercise caution in use and handling. This product must not be used in humans.

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APPENDIX B

PURITY AND STABILITY TESTING PROTOCOL

Study Protocol:
Purity and Stability of Test Substances for Work Assignment 3-11
EDSP Study Number: EDSP.311-01

Study Objective:

To verify the purity of 6 test substances (listed in Table 1), the solubility, (at the concentrations specified in Table 2), and to determine the stability of same dissolved in well water over a 21 day period. This study is in support of EPA contract number 68-W-01-023, MSL Work Assignment Number 3-11, Task 3, Amphibian Metamorphosis Assay Demonstration Study.

Address of Testing Facility and Sponsor's Representative:

Battelle – Marine Science Laboratory
1529 West Sequim Bay Road
Sequim, Washington

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propyl-2-thiouracil	PTU
L-Thyroxine	T4
Phenobarbital	ΦBarb
Pregnenolone-16-Alpha-carbonitrile	P16αc
Dexamethosone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	SOP MSL-O-	TBA	TBA	TBA	TBA	TBA

TABLE 3
Reference Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	TCI America	Fluka	TBA	TBA	TBA	Aldrich
CAS #	51-52-5	51-48-9	TBA	TBA	TBA	60-56-0
Lot #	0630144	411874/1	TBA	TBA	TBA	06109DP
Purity req.	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%
Mfg. Purity claim	> 99%	99.6%	TBA	TBA	TBA	99.7%

Proposed experimental start and termination dates:

Start Date – November 1, 2003
Termination Date – June 22, 2004

Experimental Design:

- Solubility of the substances will be determined as per SOP EDSP.C-0XX-01 (EDSP Chemical Characterization-TBA) in the diluent (well water) at a concentration approximately 10% above the stock solution concentration (see Table 2).
- If the solubility of the test substance is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP EDSP.C-0XX-01. Note: exposure concentrations won't change, so stability of an exposure solution will be carried out at the target concentration.
- Substance purity will be verified using HPLC, GC/MS or IC/ICP/MS as appropriate, and should be 98% or greater and be within ±2% of the value provided on the Certificate of Analysis by the manufacturer
- Stock solution – made up to concentrations delineated in Table 2 (approximate levels) in well water (obtained from MSL).
- Stability test solutions – Stability testing will be carried out on stock solutions and at a second level near or within the in-life exposure dose range. Nominal concentrations to be tested are delineated in Table 2 in well water (obtained from MSL).
- Storage and Labeling Requirements of Test Solution – stored in the dark (i.e., same storage conditions of solutions employed in the in-life studies of WA 3-11) at room temperature (20-25˚C). Minimally, containers will be labeled with the name of the test substance, the date of preparation, the solution concentration, and the study number.
- Test Schedule – Samples will be analyzed the day of collection from the test solution
- Replicates – 3 per sample tested at each analysis time point
- Sampling Timing – Sampling will be carried out on the first day and then as appropriate for statistical analysis for duration of the 21 day study
- For details of the analytical methods see the appropriate SOP associated with the analytical method employed for the specific substance: MSL-O-018-00 (HPLC), MSL-X-XXX-XX (GC/MS)-TBA, MSL-X-XXX-XX (ICP/MS)-TBA.

Statistical Analysis:

Log linear degradation curves will be fit to the stability data to describe the trend of the chemical concentration over time. Lack of fit and residual plots will be evaluated to determine the form of

the regression. Power calculations based on the observed variability will be used to determine the sensitivity of the test to detect degraded concentrations.

Regulatory requirements:

This study will be conducted in compliance with EPA FIFRA Good Laboratory Practices (40CFR, Part 160).

Records to be maintained:

All records and the final report generated as a result of this study will be transported to and maintained in the EDSP Data Coordination Center for archival purposes at the following address:

EDSP Data Coordination Center
Battelle Columbus Operations (PNL)
505 King Ave
Columbus, OH 43201

Sponsor Representative_____ _____
Michael Blanton **Date**

Study Director_____ _____
Michael Cobb **Date**

PROTOCOL AMENDMENT
STUDY NUMBER: EDSP.311-01
AMENDMENT NUMBER: A-1
DATE: FEBRUARY 4, 2004
Page 1 of 2

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01			DATE: February 4, 2004		
AMENDMENT NUMBER: A-1			WAL/STUDY DIRECTOR:		
NOTEBOOK NUMBER: N/A			Michael Blanton/Michael Cobb		
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

1. Solubility of the substances will be determined as per SOP EDSP.C-0XX-01 (EDSP Chemical Characterization-TBA)…
   If the solubility of the test substance is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP EDSP.C-0XX-01.
2. For details of the analytical methods see the appropriate SOP associated with the analytical method employed for the specific substance: MSL-O-018-00 (HPLC), MSL-X-XXX-XX (GC/MS)-TBA, MSL-X-XXX-XX (ICP/MS)-TBA.
3. All records and the final report generated as a result of this study will be transported to and maintained in the EDSP Data Coordination Center for archival purposes at the following address:
   EDSP Data Coordination Center
   Battelle Columbus Operations (PNL)
   505 King Ave
   Columbus, OH 43201

4. Sponsor Representative_____ _____
   Michael Blanton Date

AMENDMENT:

1. Sentences amended to read: “Solubility of the substances will be determined as per SOP MSL-R-009 (Chemical Characterization)”…
   “If the solubility of the test substances is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP MSL-R-009.”
   Last several SOPs identified in #2 below.
   Sentence amended to read: “For details of the analytical methods, see the method identified in Table 2 and provided in each specific chemical characterization notebook. These methods are supported by specific operation and maintenance SOPs appropriate to the analytical method employed for the particular substance under study: MSL-R-012 (Operation and Maintenance of HPLC Systems), MSL-O-008-02 (Operation and Maintenance of Gas Chromatographs (GC) and Gas Chromatograph/Mass
2. Spectrometer (GC/MS) Systems), MSL-R-014 (Operation & Maintenance of IC ICP/MS Systems – currently being drafted).

PROTOCOL AMENDMENT
STUDY NUMBER: EDSP.311-01
AMENDMENT NUMBER: A-1
DATE: FEBRUARY 4, 2004
Page 2 of 2

3. Sentence amended to read: "All records and the final report generated as a result of this study will be maintained and archived per SOP MSL-D-003 (Archiving of Records, Data and Retired SOPs). In addition, the documentation will be transported in electronic form to the EDSP Data Coordination Center for archival purposes at the following address:

   EDSP Data Coordination Center
   Battelle Columbus Operations
   505 King Ave
   Columbus, OH 43201

4. Sponsor Representative_____ _____
   David Houchens Date

REASON FOR CHANGE:

1. The SOP numbers were not available (in the "Experimental Design" section) when the protocol was written. These SOP numbers are now available and are provided in this amendment.
2. Our new documentation approach involves generation of a specific "Analytical Method" document developed for the chemical characterization and provided in the final report. Each specific "Method" will correspond to a generic SOP.
3. Original document section in "Records to be maintained" section requires clarification. Battelle Columbus Operations incorrectly labeled as PNL.
4. Correct Sponsor Representative is EDSP Program Manager.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01				DATE: February 24, 2004	
AMENDMENT NUMBER: A-2				WAL/STUDY DIRECTOR:	
NOTEBOOK NUMBER: N/A				Michael Blanton/Michael Cobb	
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

Table entries that are earmarked for change in this amendment are highlighted in Yellow

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	SOP MSL-0-018-00 (HPLC)	TBA	TBA	TBA	TBA	TBA

AMENDMENT:
New table entries are highlighted in **Orange**

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

REASON FOR CHANGE:

Information was not available when protocol was originally generated.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01			DATE: March 30, 2004		
AMENDMENT NUMBER: A-3			WAL/STUDY DIRECTOR:		
NOTEBOOK NUMBER: N/A			Michael Blanton/Michael Cobb		
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

Table entries that are earmarked for change in this amendment are presented underlined and italicized in ***Georgia Font***

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propyl-*2*-thiouracil	PTU
L-Thyroxine	T4
Phenobarbital	ΦBarb
Pregnenolone-16-Alpha-carbonitrile	P16αc
Dexameth***o***sone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	*0*, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, *100* mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

TBA = To Be Amended

AMENDMENT:

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propylthiouracil	PTU
L-Thyroxine	T4
Phenobarbital	ΦBarb
Pregnenolone-16-Alpha-carbonitrile	P16αc
Dexamethasone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

REASON FOR CHANGE:

Information was not available when protocol was originally generated.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

NOTE: A signed copy of this protocol and all relevant Amendments are available in the files of the Chemical Repository Study Director in MSL 5.

Protocol Deviation

Deviation EDSP.311-01-D4 was implemented to address the change from the statistical evaluation of stability using Log linear degradation curves to comparing the percent deviation of stability test samples (mean values) from the mean day zero results.

APPENDIX C

ANALYTICAL RESULTS OF STABILITY TESTING

Table C1. Dexamethasone concentration in Well Water (ug/L)

Target Conc. (ug/L)	Sample ID	Date	Dexamethasone (ug/L)	Average (ug/L)	Recovery	RSD
9900	stock 1 R-1	05/21/04	9371.24			
9900	stock 1 R-2	05/21/04	10470.56	10094.68	101.97%	6.21%
9900	stock 1 R-3	05/21/04	10442.25			
9900	stock 2 R-1	05/25/04	9284.78			
9900	stock 2 R-2	05/25/04	9373.49	9291.97	93.86%	0.84%
9900	stock 2 R-3	05/25/04	9217.63			
9900	stock 3 R-1	05/28/04	8521.44			
9900	stock 3 R-2	05/28/04	9435.12	8861.37	89.51%	5.64%
9900	stock 3 R-3	05/28/04	8627.55			
9900	stock 4 R-1	06/01/04	8394.96			
9900	stock 4 R-2	06/01/04	8426.11	8398.31	84.83%	0.31%
9900	stock 4 R-3	06/01/04	8373.87			
9900	stock 5 R-1	06/04/04	8367.89			
9900	stock 5 R-2	06/04/04	8488.97	8399.25	84.84%	0.94%
9900	stock 5 R-3	06/04/04	8340.90			
9900	stock 6 R-1	06/10/04	7125.32			
9900	stock 6 R-2	06/10/04	7416.83	7295.13	73.69%	2.08%
9900	stock 6 R-3	06/10/04	7343.25			
15.84	dil 1 R-1	05/21/04	12.24			
15.84	dil 1 R-2	05/21/04	17.50	14.94	94.31%	17.62%
15.84	dil 1 R-3	05/21/04	15.08			
15.84	dil 2 R-1	05/25/04	13.30			
15.84	dil 2 R-2	05/25/04	13.38	12.71	80.26%	8.51%
15.84	dil 2 R-3	05/25/04	11.46			
15.84	dil 3 R-1	05/28/04	7.47			
15.84	dil 3 R-2	05/28/04	14.17	10.80	68.18%	31.01%
15.84	dil 3 R-3	05/28/04	10.76			
15.84	dil 4 R-1	06/01/04	11.52			
15.84	dil 4 R-2	06/01/04	14.06	12.32	77.79%	12.24%
15.84	dil 4 R-3	06/01/04	11.38			
15.84	dil 5 R-1	06/04/04	13.79			
15.84	dil 5 R-2	06/04/04	15.91	14.18	89.53%	11.07%
15.84	dil 5 R-3	06/04/04	12.84			
15.84	dil 6 R-1	06/10/04	8.05			
15.84	dil 6 R-2	06/10/04	11.84	9.54	60.22%	21.20%
15.84	dil 6 R-3	06/10/04	8.73			

Table C2. ICV/CCV Data for Dexamethasone in Well Water

Date	Sample Name	Expected Dexamethasone (ug/L)	Measured Dexamethasonel (ug/L)	Recovery
Dex6 40ug-ml IVC	05/21/04	40	40.38	100.9%
dex3C CCV	05/21/04	39.8	42.45	106.7%
dex3C CCV	05/21/04	39.8	38.96	97.9%
dex3C CCV	05/21/04	39.8	40.49	101.7%
Dex6 40ug-ml IVC	05/25/04	40	43.16	107.9%
dex3C CCV	05/25/04	39.8	42.17	106.0%
dex3C CCV	05/25/04	39.8	40.28	101.2%
dex3C CCV	05/25/04	39.8	37.53	94.3%
Dex6 40ug-ml IVC	05/28/04	40	40.77	101.9%
dex3C CCV	05/28/04	39.8	38.46	96.6%
dex3C CCV	05/28/04	39.8	39.00	98.0%
dex3C CCV	05/28/04	39.8	36.92	92.8%
Dex6 40ug-ml IVC	06/01/04	40	44.48	111.2%
dex3C CCV	06/01/04	39.8	39.77	99.9%
dex3C CCV	06/01/04	39.8	39.23	98.6%
dex3C CCV	06/01/04	39.8	39.00	98.0%
Dex6 40ug-ml IVC	06/04/04	40	43.06	107.6%
dex3C CCV	06/04/04	39.8	40.29	101.2%
dex3C CCV	06/04/04	39.8	40.48	101.7%
dex3C CCV	06/04/04	39.8	39.58	99.5%
Dex6 40ng-ml IVC	06/10/04	40	42.98	107.5%
dex11 40ng-ml cal v	06/10/04	40.01	41.43	103.6%
dex3C CCV	06/10/04	39.8	38.15	95.9%
dex3C CCV	06/10/04	39.8	39.59	99.5%
dex3C CCV	06/10/04	39.8	39.14	98.4%

Table C3. Spike Recovery Data for Dexamethasone in Well Water

Target Conc. (ug/L)	Sample ID	Date	Dexamethasone (ug/L)	Average (ug/L)	Recovery	RSD
2700	Spike 1 R-1	5/20/2004	2936.14			
2700	Spike 1 R-2	5/20/2004	2921.81	2999.45	111.1%	4.08%
2700	Spike 1 R-3	5/20/2004	3140.38			
2700	Spike 2 R-1	5/25/2004	2439.05			
2700	Spike 2 R-2	5/25/2004	2426.03	2474.76	91.7%	2.97%
2700	Spike 2 R-3	5/25/2004	2559.19			
2700	Spike 3 R-1	5/28/2004	2157.87			
2700	Spike 3 R-2	5/28/2004	2339.16	2271.09	84.1%	4.35%
2700	Spike 3 R-3	5/28/2004	2316.23			
2700	Spike 4 R-1	6/1/2004	2435.51			
2700	Spike 4 R-2	6/1/2004	2432.28	2478.66	91.8%	3.13%
2700	Spike 4 R-3	6/1/2004	2568.19			
2700	Spike 5 R-1	6/4/2004	2360.00			
2700	Spike 5 R-2	6/4/2004	2394.55	2374.61	87.9%	0.75%
2700	Spike 5 R-3	6/4/2004	2369.27			
2700	Spike 6 R-1	6/10/2004	2152.31			
2700	Spike 6 R-2	6/10/2004	2280.73	2217.04	82.1%	2.90%
2700	Spike 6 R-3	6/10/2004	2218.08			

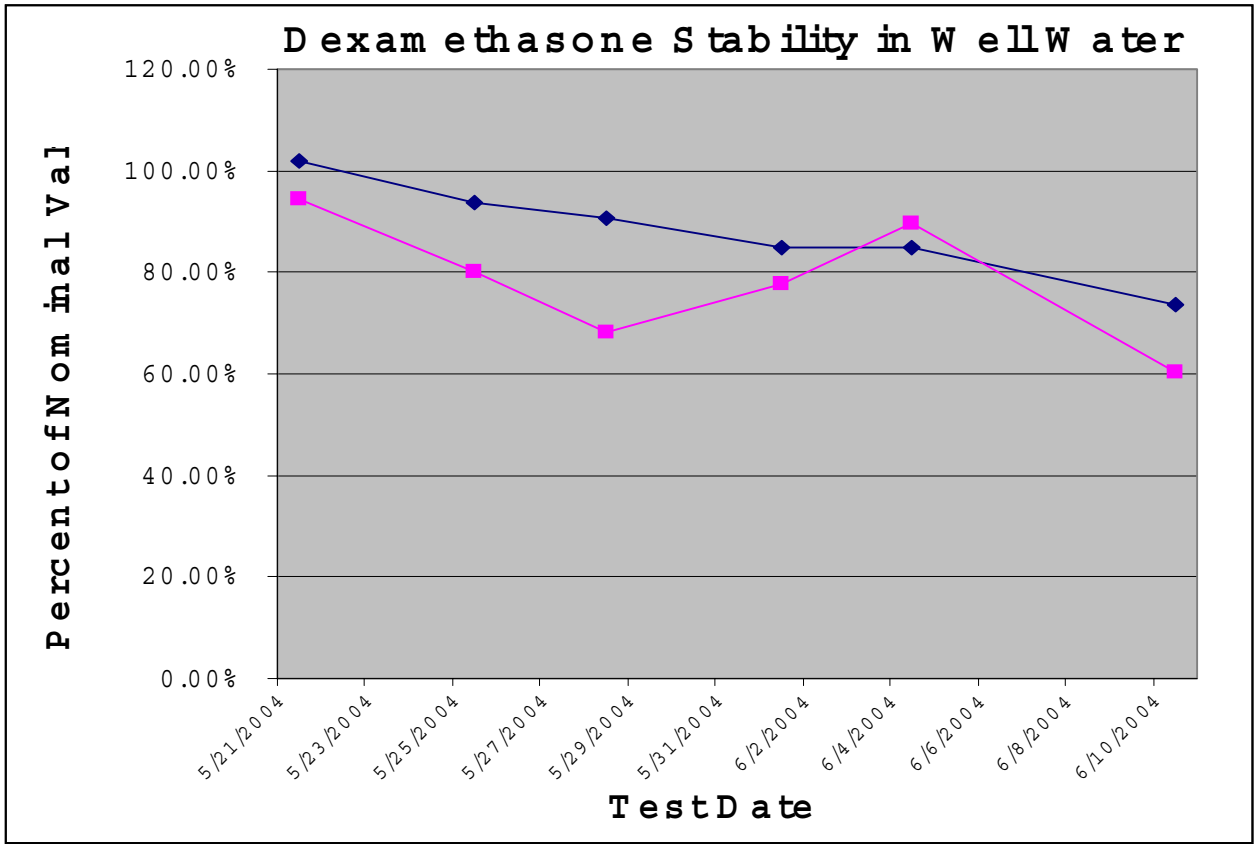


Figure 2. Observed concentration of Dexamethasone with a target concentration of 9.9 mg/L (upper) and 15.84 µg/L (lower) plotted against time

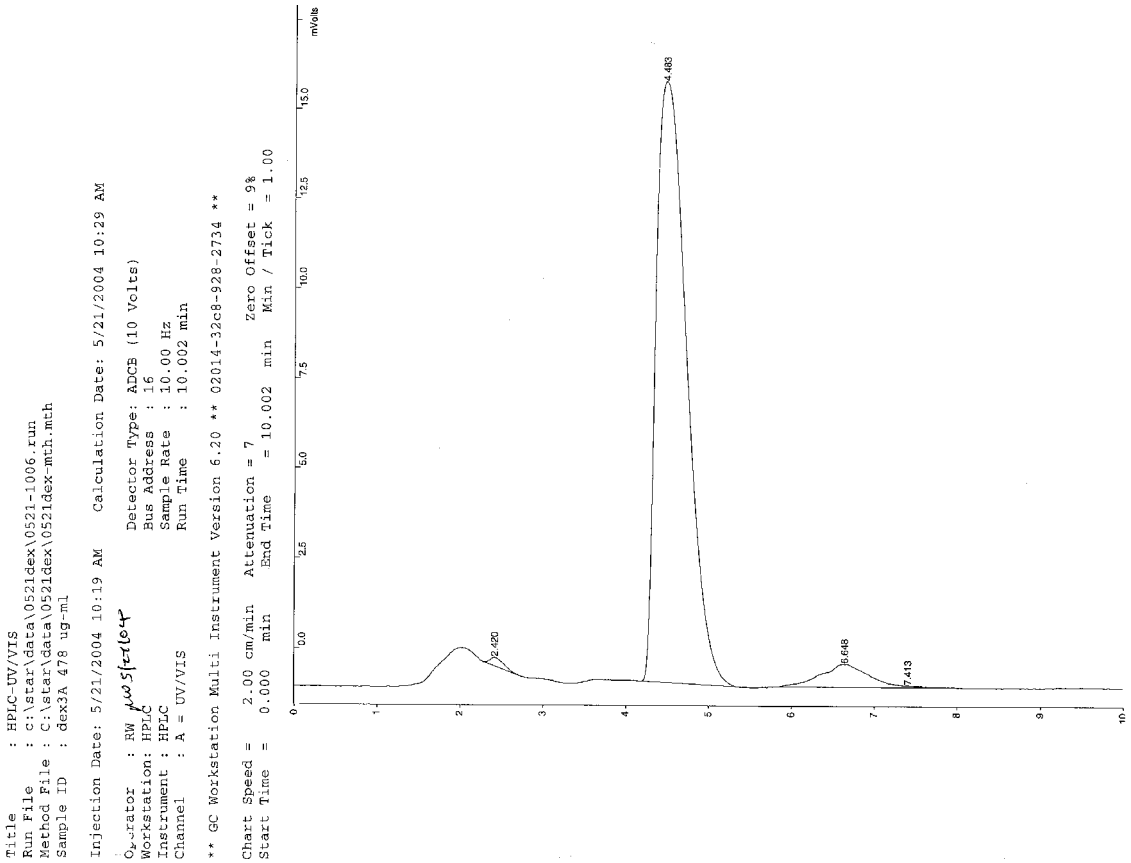


Figure 3. Typical Chromatogram, 478 ng/ml Dexamethasone

Dexamethasone
Test Chemical Analytical Data from Battelle:
In-Life Test Chemical Analysis Report

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

Client:	B. Helle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Noll
P.O. #:	

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road

Stillwater, OK 74074

405-624-6771 Fax 405-377-3388

mail@fortlabs.com

Lab Use Only

Lab Project # - WO #

BA1702 - 00063

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only: Ice?	Temp.	pH
002	$DeCl_2$ Tap H_2O	7/15/04 1600	1	G	Gluc Ice	Dexamethazone			
003	62.5 mg/L Dexamethasone	↓ ↓	1	↓	↓	↓			
004	250 mg/L Dexamethasone		1						
005	20 mg/L Dexamethasone		2						

SPECIAL INSTRUCTIONS:

DATE:

INITIALS:

			Date/Time
Andrea Noll	7/15/04 1700		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3386
mail@fortlabs.com

Client: Battelle
Submit Report To: Doug Fort
Sampler's Signature: Andra Noll / [signature]
P.O. #

Lab Use Only
Client Project # WO #
BATTP2-00003

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only ID#	Temp	pH
007	DeCl₂ Trap H₂O	7/21/04 1600	1	G	Blue Ice	Dexamethasone			
008	62.5 mg/L Dexamethasone	↓ ↓	1	↓	↓	↓			
009	150 mg/L Dexamethasone	↓ ↓	2	↓	↓	↓			
0060	30 mg/L Dexamethasone	7/20/04 1600	1	↓	↓	↓			

DATE:
INITIALS:

SPECIAL INSTRUCTIONS:

			Date/Time
Andra Noll	7/21/04 1900		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Noll
P.O. #:	

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road

Stillwater, OK 74074

405-624-6771 Fax 405-377-3388

mail@fortlabs.com

BATT02 - 00063

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
010	20 mg/L Dexamethasone	7/26/04 1600	1	G	Blue ice	Dexamethasone
011	$DeCl_2$ Tap H_2O	7/29/04 1600	1			
012	62.5 mg/L Dexamethasone		1			
013	250 mg/L Dexamethasone		1			
014	20 mg/L Dexamethasone		2			

SPECIAL INSTRUCTIONS:

DATE:

INITIALS:

Andrea Noll	7/29/04 1700	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	BA Helk.
Submit Report To:	Doug Fort
Sampler's Signature:	John H. Fort
P.O. #:	

Lab Use Only
Client/Project # - WO #
BA1102 - 00063
Lab Use Only

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	ID#	Temp	pH
015	20 ng/L Dexamethasone	8/2/04 - 1600	1	G	HgCl2	Dexamethasone			
016	DeCl2 Tap H2O	8/4/04 - 1600	1	G					
017	62.5 ng/L Dexamethasone	↓ ↓	1	G					
018	250 ng/L Dexamethasone	↓ ↓	2	G	↓	↓			

DATE:
INITIALS:

SPECIAL INSTRUCTIONS:

			Date-Time
John H. Fort	8/4/04	1700	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

Dexamethasone
Thyroid Histology Data from EPL:
Sample Identification Key
Thyroid Pathology Report

EPL Sample Key for Dexamethasone

FEL Sample Number	Individual Specimen Number[1]	Conc. Replicate Number	Initial Specimen Stage	Chemical Conc. (µg/L)
019	242, 250, 253, 255, 256 263, 271, 273, 277, 280	A B	51	0
020	282, 283, 289, 291, 299 304, 312, 314, 317, 318	A B	51	1.0
021	323, 325, 332, 333, 339 343, 346, 352, 355, 360	A B	51	3.9
022	366, 369, 375, 377, 379 385, 386, 395, 397, 399	A B	51	15.6
023	403, 405, 415, 417, 419 422, 425, 433, 435, 436	A B	51	62.5
024	465, 467, 474, 476, 478 441, 445, 452, 455, 457	A B	51	250
025	002, 004, 009, 013, 019 022, 030, 036, 037, 039	C D	54	0
026	041, 045, 051,057, 059 063, 067, 069, 072, 080	C D	54	1.0
027	084, 086, 088, 093, 097 102, 104, 107, 111, 115	C D	54	3.9
028	123, 126, 131, 136, 138 141, 143, 149, 152, 159	C D	54	15.6
029	163, 166, 170, 175, 179 182, 187, 196, 198, 199	C D	54	62.5
030	202, 207, 210, 214, 218 222, 229, 231, 236, 239	C D	54	250

[1] Individual specimens for thyroid histology were randomly selected using *Principles and Procedures of Statistics, A Biometrical Approach*, 2nd Ed., by Robert G.D. Steel and James H. Torrie, McGraw-Hill Book Co., USA, 1980.

EPL®

EXPERIMENTAL PATHOLOGY LABORATORIES, INC.

BATTELLE
STUDY NUMBER BATT02-00063
EPL PROJECT NUMBER 237-014

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

DRAFT PATHOLOGY REPORT

Submitted by:

Experimental Pathology Laboratories, Inc.
P.O. Box 474
Herndon, VA 20172-0474
(703) 471-7060

Submitted to:

Battelle
Columbus, OH 43201

March 4, 2005

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DRAFT PATHOLOGY SUMMARY

BATTELLE
STUDY NUMBER BATT02-00063
EPL PROJECT NUMBER 237-014

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

DRAFT PATHOLOGY SUMMARY

A histomorphologic evaluation was performed on the thyroid glands of *Xenopus laevis* (South African clawed frog) that were exposed to various concentrations of dexamethasone to determine the differential sensitivity of exposing Stage 51 and Stage 54 larvae and to establish the necessary duration of exposure. Stage 51 larval frogs were exposed to dexamethasone for 21 days and Stage 54 larval frogs were exposed to dexamethasone for 14 days. The experimental designs were as follows:

Stage 51 Frog Larvae Exposed for 21 Days

Group/ Replicate	Dexamethasone (µg/L)	No. of Frogs/Group	Individual Frog Numbers
019A	0	5	242, 250, 253, 255, 256
019B	0	5	263, 271, 273, 277, 280
020A	1.0	5	282, 283, 289, 291, 299
020B	1.0	5	304, 312, 314, 317, 318
021A	3.9	5	323, 325, 332, 333, 339
021B	3.9	5	343, 346, 352, 355, 360
022A	15.6	5	366, 369, 375, 377, 379
022B	15.6	5	385, 386, 395, 397, 399
023A	62.5	5	403, 405, 415, 417, 419
023B	62.5	5	422, 425, 433, 435, 436
024A	250	5	465, 467, 474, 476, 478
024B	250	5	441, 445, 452, 455, 457

Stage 54 Frog Larvae Exposed for 14 Days

Group/ Replicate	Dexamethasone (µg/L)	No. of Frogs/Group	Individual Frog Numbers
025C	0	5	002, 004, 009, 013, 019
025D	0	5	022, 030, 036, 037, 039
026C	1.0	5	041, 045, 051, 057, 059
026D	1.0	5	063, 067, 069, 072, 080
027C	3.9	5	084, 086, 088. 093, 097
027D	3.9	5	102, 104, 107, 111, 115
028C	15.6	5	123, 126, 131, 136, 138
028D	15.6	5	141, 143, 149, 152, 159
029C	62.5	5	163, 166, 170, 175, 179
029D	62.5	5	182, 187, 196, 198, 199
030C	250	5	202, 207, 210, 214, 218
030D	250	5	222, 229, 231, 236, 239

MATERIALS AND METHODS

Slides were prepared by Experimental Pathology Laboratories, Inc. (EPL®). Head samples were trimmed from the frog larvae from just behind the eyes. The heads were placed in cassettes with the cut side down so that the heads were sectioned in a transverse plane from caudal to rostral. Five step sections (30 microns apart) were taken from each block at the level of the thyroid glands and were mounted on one slide. A duplicate slide was made of the five steps. The slides were examined by a technician to assure that a sufficient amount of thyroid tissue was present bilaterally in the sections. If necessary to meet these criteria, additional step sections were cut from the block. One slide was stained with hematoxylin and eosin (H&E) and the second slide remained unstained.

Microscopic findings for thyroid glands from each frog are listed in the Histopathology Incidence Tables. The changes are graded from 1 to 5 depending upon severity (1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high). All lesions are summarized by group on the Summary Incidence Tables. The sexes of the frogs were not reported.

RESULTS

Stage 51 X*enopus laevis* exposed to dexamethasone for 21 days

There were exposure-related changes in the thyroid gland which included a decrease in size of the thyroid follicles and a decrease in the amount of colloid present within the follicles. These changes occurred among frogs exposed to 15.6, 62.5, and 250 μg/L dexamethasone and the prevalence was exposure concentration related. These changes did not occur in controls or frogs exposed to 1.0 or 3.9 μg/L dexamethasone. Decreased follicular size and decreased colloid were minimal in frogs exposed to 15.6 μg/L dexamethasone and minimal to mild in frogs exposed to 62.5 μg/L dexamethasone. In frogs exposed to 250 μg/L dexamethasone, the severity of decreased follicular size varied from minimal to mild and the severity of decreased colloid varied from minimal to moderate.

Interfollicular pigment was present in the thyroid glands of only three frogs, one each in Groups 022A, 023B, and 024A exposed to 15.6, 62.5, and 250 μg/L dexamethasone, respectively. The severity of the change was minimal in two frogs and mild in the frog from Group 023B. See Table 1 below for a tabulation of the prevalence of the thyroid changes described above in Stage 51 frogs.

Table 1: Prevalence with Replicates Combined of Selected Changes in Thyroid Glands of Stage 51 Frogs after Exposure to Various Concentrations of Dexamethasone for 21 Days

Group	19A&B	20A&B	21A&B	22A&B	23A&B	24A&B
Exposure Conc. Dexamethasone	0 μg/L	1.0μg/L	3.9 μg/L	15.6 μg/L	62.5 μg/L	250 μg/L
Thyroid (No. examined)	(10)	(10)	(10)	(10)	(10)	(10)
Colloid, Decreased	0	0	0	2 (20%)	7 (70%)	10 (100%)
Thyroid Follicles, Decreased Size	0	0	0	2 (20%)	7 (70%)	10 (100%)
Interfollicular Pigment	0	0	0	1	1	1

See Appendix A for photographs of thyroid glands in frogs from each of the exposure concentrations. Each frog is illustrated by photographs taken at low magnification (1.25x) and high magnification (10x). The photographs are annotated to illustrate various features of thyroid glands and thyroid gland changes.

Stage 54 *Xenopus laevis* exposed to dexamethasone for 14 days

There were no exposure-related changes in the thyroid glands of Stage 54 frog larvae exposed to dexamethasone at any of the exposure concentrations. Two frogs, one in Group 026C (1.0 µg/L dexamethasone) and one in Group 030C (250 µg/L dexamethasone), had interfollicular pigment in the thyroid glands. The severity of the change was minimal in both frogs.

See Appendix B for photographs of thyroid glands in frogs from each of the exposure concentrations. Each frog is illustrated by photographs taken at low magnification (1.25x) and high magnification (10x). The photographs are annotated to illustrate various features of thyroid glands. A representative frog or several frogs from each exposure concentration were photographed to illustrate that the thyroid glands were similar regardless of exposure concentration.

DISCUSSION

The corticoids corticosterone and aldosterone are the main steroids produced by the interrenal gland in amphibians. Corticoid production is regulated by the hypothalamic-hypophyseal-interrenal axis through the actions of adrenocorticotrophic hormone (ACTH), corticotrophin-releasing hormone (CRH), and arginine vasotocin (AVT).[1] Although thyroid hormone is the primary hormone that controls metamorphosis in amphibians, it is thought that the corticoids may work together with thyroid hormone to accelerate metamorphosis.[1] The influence of corticoids on growth and development, however, appears to be more complex

than that of thyroid hormone. When exogenous corticosteroids are administered to larval frogs, their effect on growth and metamorphosis is variable depending upon the stage of development of the frog when the exogenous hormone is administered, the dose of the corticoid, and whether or not the corticoid is administered along with thyroid hormone.[1]

Dexamethasone is a synthetic glucocorticoid used in the study described above in which larval frogs were exposed to various concentrations at Stage 51 and Stage 54 of metamorphosis for 21 days and 14 days duration, respectively. In frog larvae exposed to dexamethasone at Stage 51 of development, the effects on the thyroid glands were similar to those observed in frog larvae exposed to exogenous thyroid hormone.[2] There was a decrease in the amount of colloid in the thyroid follicles and a decrease in the size of the thyroid follicles that was dose-related at the three highest exposure concentration of dexamethasone. These effects suggest that thyroid hormone produced by the thyroid glands was utilized, but was not replaced. This would occur when there is increased utilization of thyroid hormone and/or no stimulation of the thyroid gland by thyroid stimulating hormone (TSH) to produce additional thyroid hormone. Perhaps dexamethasone in some way enhanced the utilization and thus the release of thyroid hormone in its role of accelerating metamorphosis in synergy with thyroid hormone. Under the conditions of this study, neither the exposure concentration of dexamethasone nor the duration of exposure may have been sufficient to induce a reduction in circulating thyroid hormone such that TSH was released from the pituitary gland. Increased TSH likely would have resulted in histomorphologic thyroid changes characterized by thyroid follicular cell hyperplasia and increased colloid that were not observed in this dexamethasone study.

The thyroid glands of Stage 54 frogs in this study appeared to be unaffected by exposure to dexamethasone at any exposure concentration.

One difference between the studies of exposure to T_4 and exposure to dexamethasone was the amount of interfollicular pigment present in the thyroid glands. In the T_4 exposure study the majority of the frogs (including the control frogs) had interfollicular pigment present in the thyroid glands, whereas only a few frogs in the dexamethasone exposure study had interfollicular pigment present. The significance, if any, of this difference in occurrence of interfollicular pigment is not known.

CONCLUSIONS

In the thyroid gland, decreased colloid and decreased follicular size occurred in Stage 51 frogs exposed to 15.6, 62.5, and 250 μg/L dexamethasone, and the prevalence was related to exposure concentration. There were no exposure-related changes in the thyroid glands of Stage 54 frog larvae exposed to dexamethasone at any of the exposure concentrations.

MARILYN J. WOLFE, DVM, PhD, Diplomate, ACVP — Date
Veterinary Pathologist

MJW/cb

REFERENCES

[1] Denver, Robert J., Glennemeier, Karen A., Boorse, Graham C. "Endocrinology of Complex Life Cycles: Amphibians." Chapter 28 in v. 2 Non-Mammalian Hormone-Behavior Systems of *Hormones, Brain and Behavior* (D. W. Pfaff, A. P. Arnold, A. E. Etgen, S. E. Fahrbach, and R. T. Rubin, eds.) Elsevier Science, 2002, pp. 484-486.

[2] Pathology Summary in Battelle Study Number BATT02-00062 (EPL Project Number 237-013) Amphibian Metamorphosis Assay Demonstration Study (Exposure of Stage 51 and Stage 54 *Xenopus laevis* larvae to various concentrations of L-Thyroxine).

Battelle Study Number BATT02-00063

3/4/05 DRAFT

COMPLIANCE STATEMENT

Client Name	Battelle	EPL Project Coordinator	Marilyn J. Wolfe
Client Study	BATT02-00063	EPL Pathologist	Marilyn J. Wolfe
Species	*Xenopus laevis* (South African clawed frog)	EPL Project Number	237-014
Study Title	Amphibian Metamorphosis Assay Demonstration Study		
Test Article	Dexamethasone		

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 160.

EPL Project Coordinator

Date

3/4/05 DRAFT

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Amphibian Metamorphosis Assay Demonstration Study

Client Study: BATT02-00063 EPL Project Coordinator: Marilyn J. Wolfe

EPL Project Number: 237-014 EPL Pathologist: Marilyn J. Wolfe

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 Project Coordinator and Management are indicated below.

	Dates	
Area Inspected	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management XXX

Date of last quarterly facility inspection 1/05

EPL Quality Assurance Unit Date

SUMMARY INCIDENCE TABLES

STAGE 51 FROGS

SUMMARY INCIDENCE TABLE

BATT02-00063
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 019A	GROUP 019B	GROUP 020A	GROUP 020B	GROUP 021A	GROUP 021B
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Colloid, Decreased						
Interfollicular Pigment						
Thyroid Follicles, Decreased Size						

SUMMARY INCIDENCE TABLE

BATT02-00063
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 022A	GROUP 022B	GROUP 023A	GROUP 023B	GROUP 024A	GROUP 024B
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Colloid, Decreased		2	3	4	5	5
Interfollicular Pigment	1			1	1	
Thyroid Follicles, Decreased Size		2	3	4	5	5

HISTOPATHOLOGY INCIDENCE TABLES

STAGE 51 FROGS

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00063
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 019A						GROUP 019B						GROUP 020A							
ANIMAL	51-242	51-250	51-253	51-255	51-256		51-263	51-271	51-273	51-277	51-280		51-282	51-283	51-289	51-291	51-299			
THYROID	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X			
Colloid, Decreased																				
Interfollicular Pigment																				
Thyroid Follicles, Decreased Size																				

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Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00063
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 020B						GROUP 021A						GROUP 021B							
ANIMAL	51-304	51-312	51-314	51-317	51-318		51-323	51-325	51-332	51-333	51-339		51-343	51-346	51-352	51-355	51-360			
THYROID	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X			
Colloid, Decreased																				
Interfollicular Pigment																				
Thyroid Follicles, Decreased Size																				

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P=Present B=Benign M=Malignant
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HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00063
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 022A						GROUP 022B						GROUP 023A							
ANIMAL	51-336 6	51-336 9	51-337 5	51-337 7	51-337 9		51-338 5	51-338 6	51-339 5	51-339 7	51-339 9		51-340 3	51-340 5	51-411 5	51-411 7	51-411 9			
THYROID	X	X		X	X			X	X		X		X	X						
Colloid, Decreased							1			1					1	2	2			
Interfollicular Pigment			1																	
Thyroid Follicles, Decreased																				
Size							1			1					1	2	2			

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m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00063
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 023B						GROUP 024A						GROUP 024B								
ANIMAL	51-442	51-425	51-433	51-435	51-436		51-446	51-466	51-474	51-476	51-478		51-441	51-445	51-452	51-455	51-457				
THYROID																					
Colloid, Decreased	2	2		2	1		2	3	3	2	2		2	1	1	3	1				
Interfollicular Pigment			2				1														
Thyroid Follicles, Decreased Size	1	2		2	1		1	2	2	1	1		1	1	1	2	1				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
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P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

SUMMARY INCIDENCE TABLES

STAGE 54 FROGS

SUMMARY INCIDENCE TABLE

BATT02-00063
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 025C	GROUP 025D	GROUP 026C	GROUP 026D	GROUP 027C	GROUP 027D
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Interfollicular Pigment			1			

SUMMARY INCIDENCE TABLE

BATT02-00063
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 028C	GROUP 028D	GROUP 029C	GROUP 029D	GROUP 030C	GROUP 030D
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Interfollicular Pigment					1	

HISTOPATHOLOGY INCIDENCE TABLES

STAGE 54 FROGS

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00063
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 025C						GROUP 025D						GROUP 026C							
ANIMAL	54-002	54-004	54-009	54-013	54-019		54-022	54-030	54-036	54-037	54-039		54-041	54-045	54-051	54-057	54-059			
THYROID	X	X	X	X	X		X	X	X	X	X		X	X	X		X			
Interfollicular Pigment																1				

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m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00063
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 026D						GROUP 027C						GROUP 027D							
ANIMAL	54-063	54-067	54-069	54-072	54-080		54-084	54-086	54-088	54-093	54-097		54-102	54-104	54-107	54-111	54-115			
THYROID	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X			
Interfollicular Pigment																				

EPL
Experimental Pathology Laboratories, Inc.

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P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00063
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 028C						GROUP 028D						GROUP 029C							
ANIMAL	54-123	54-126	54-131	54-136	54-138		54-141	54-143	54-149	54-152	54-159		54-163	54-166	54-170	54-175	54-179			
THYROID	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X			
Interfollicular Pigment																				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00063
Stage 54/14 Day Sacrifice
Xenopus laevis

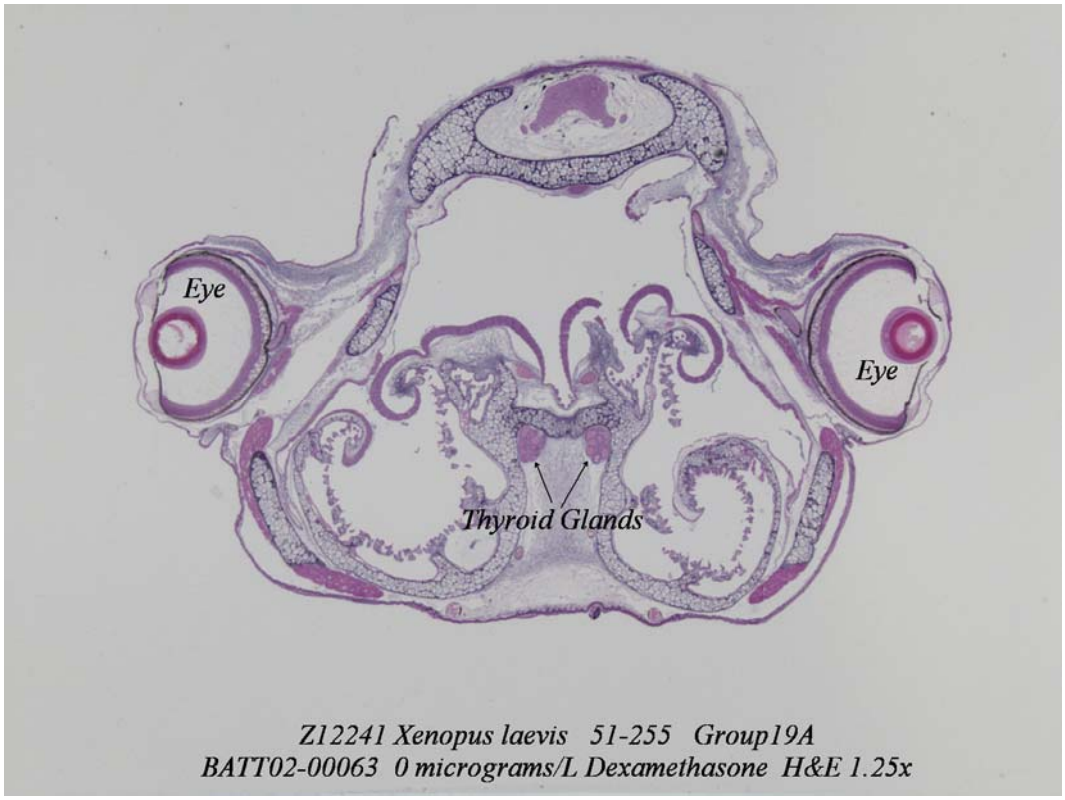
	GROUP 029D						GROUP 030C						GROUP 030D							
ANIMAL	54-182	54-187	54-196	54-198	54-199		54-202	54-207	54-210	54-214	54-218		54-222	54-229	54-231	54-236	54-239			
THYROID	X	X	X	X	X		X		X	X	X		X	X	X	X	X			
Interfollicular Pigment								1												

EPL
Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

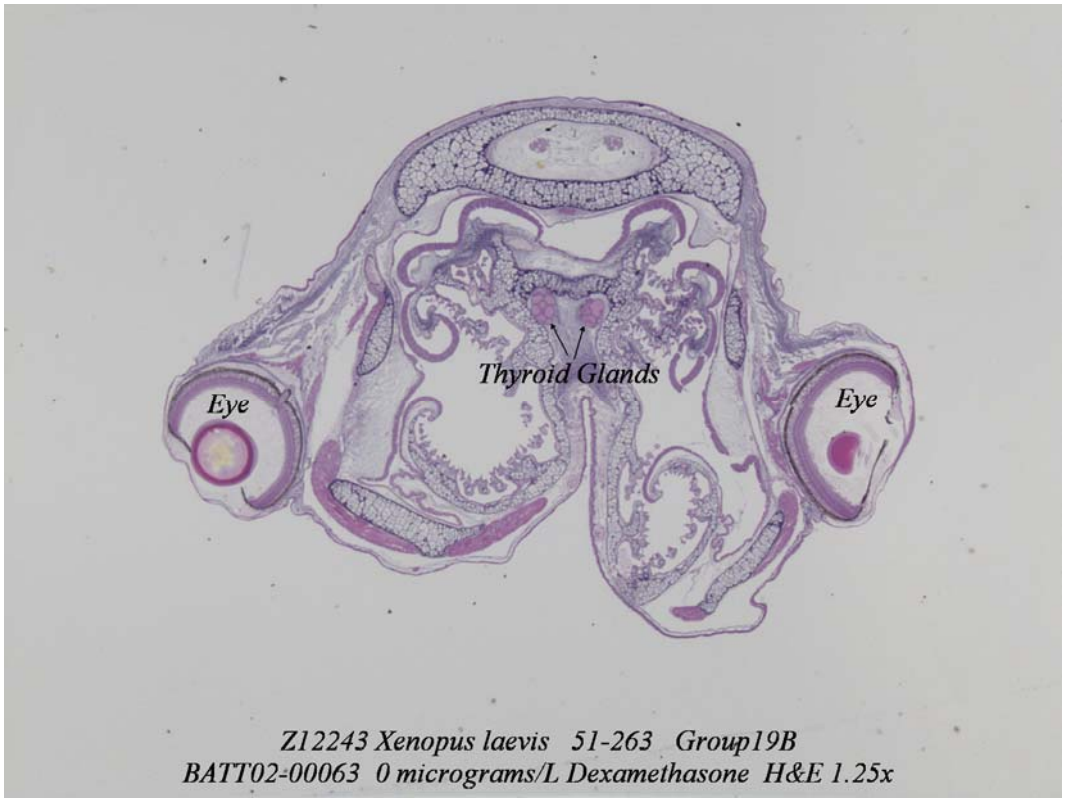
Appendix A:

Stage 51 Photo Legends





Z12241 (1.25x) and Z12242 (10x). Thyroid glands of control frog 51-255. There is a variation in size of the normal colloid-filled thyroid follicles.



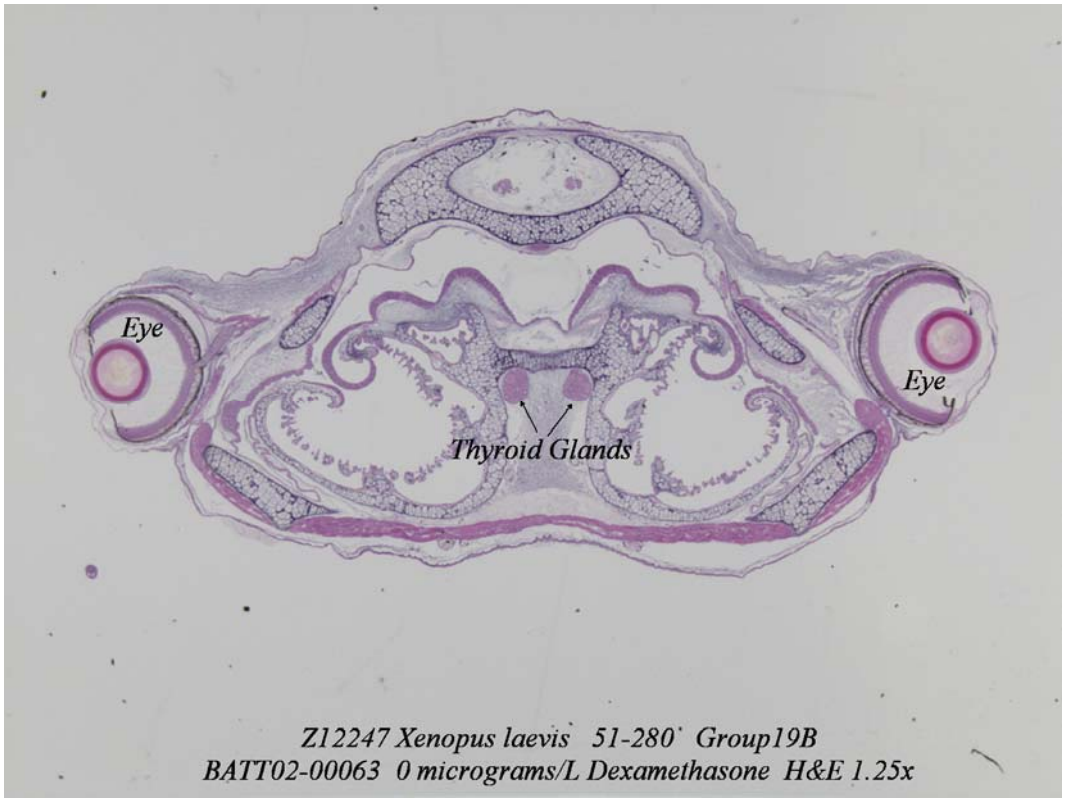


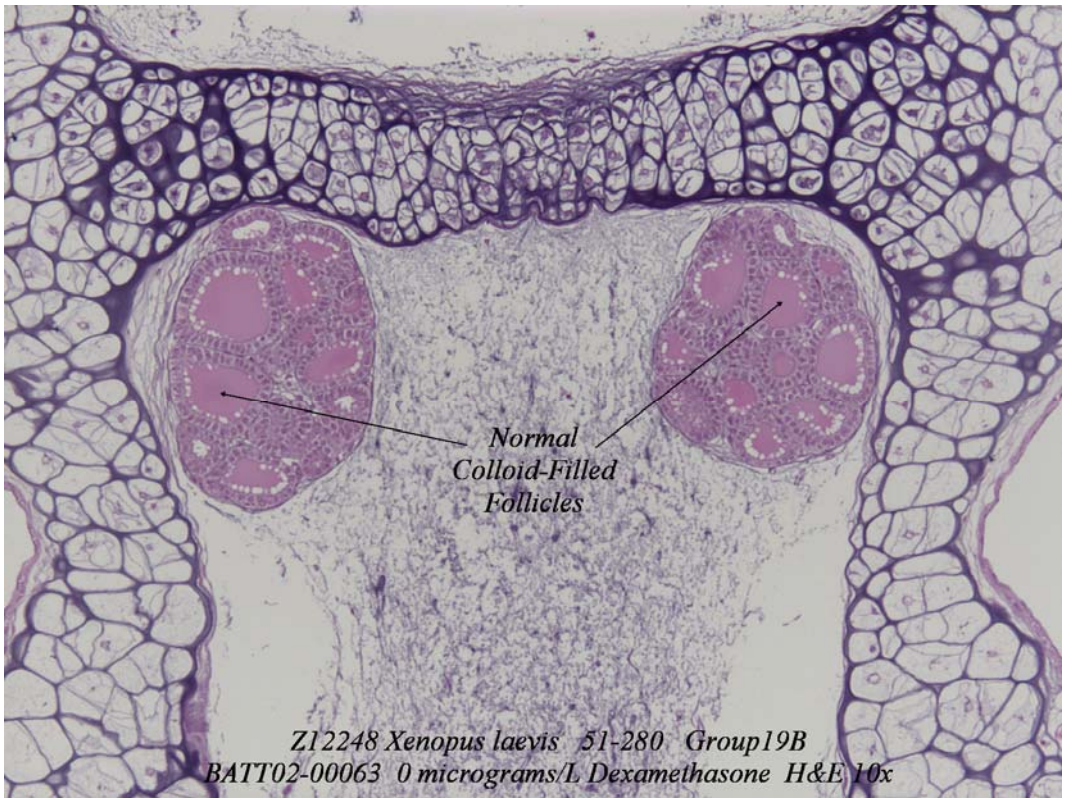
Z12243 (1.25x) and Z12244 (10x). Thyroid glands of control frog 51-263. There is a variation in size of the normal colloid-filled thyroid follicles.



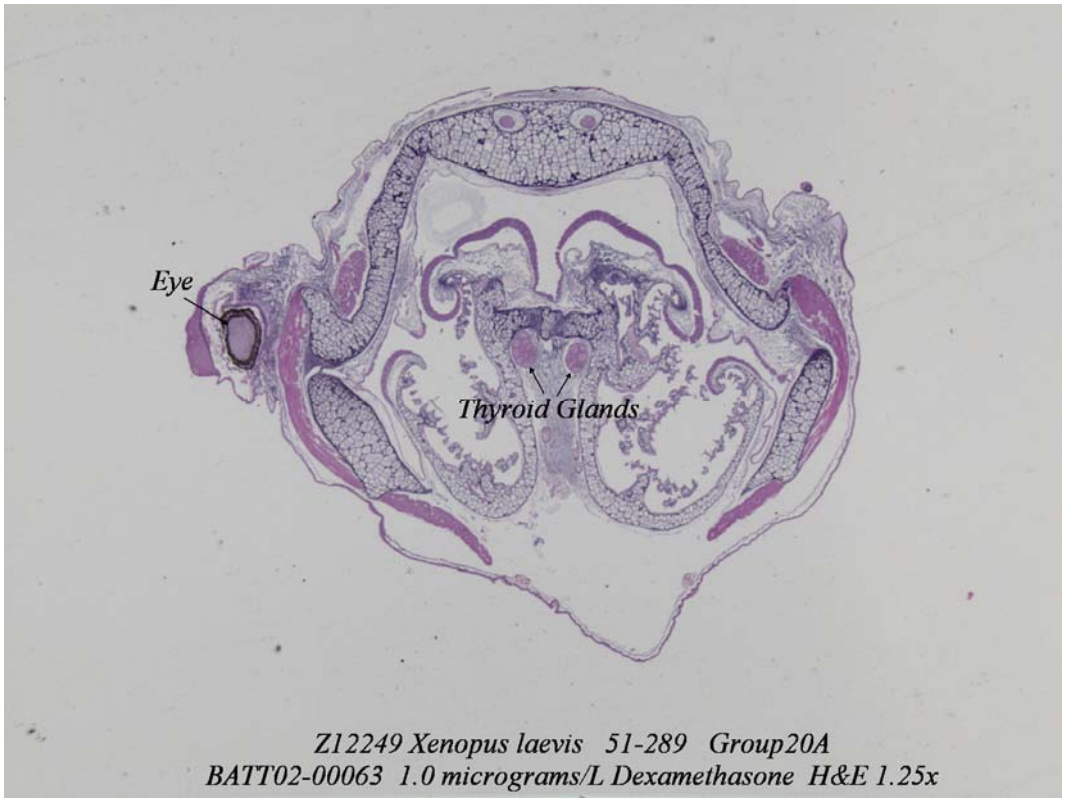


Z12245 (1.25x) and Z12246 (10x). Thyroid glands of control frog 51-273. There is a variation in size of the normal colloid-filled thyroid follicles.



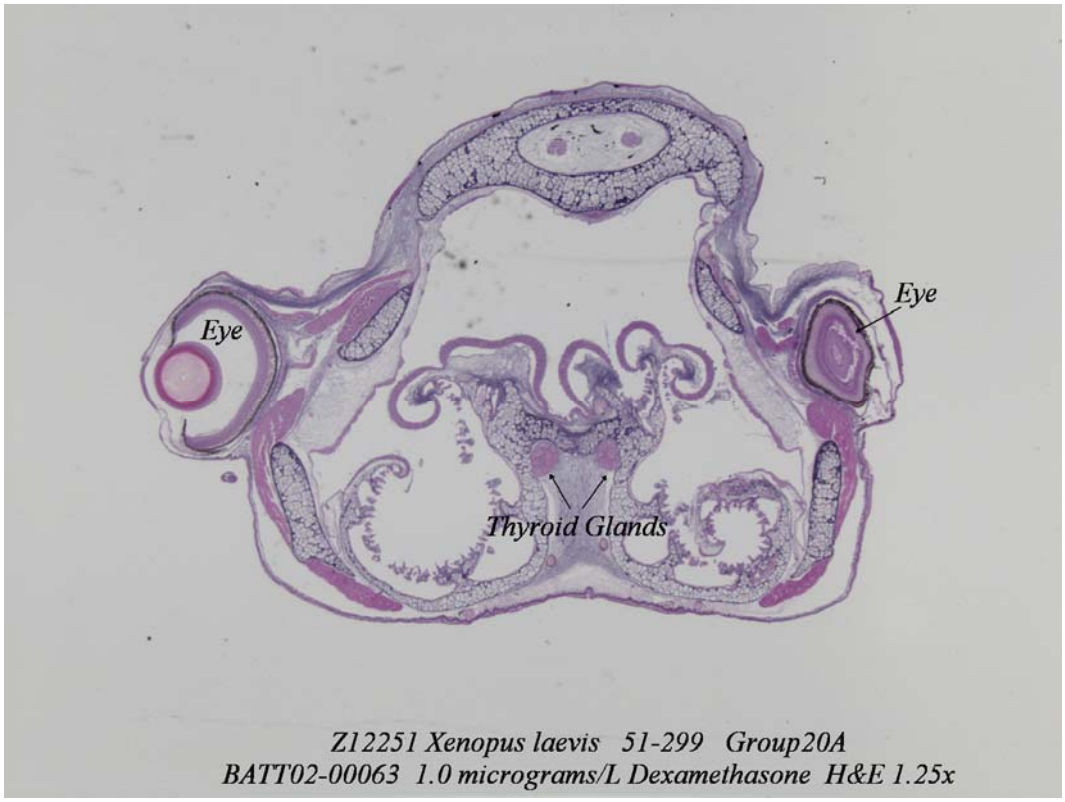


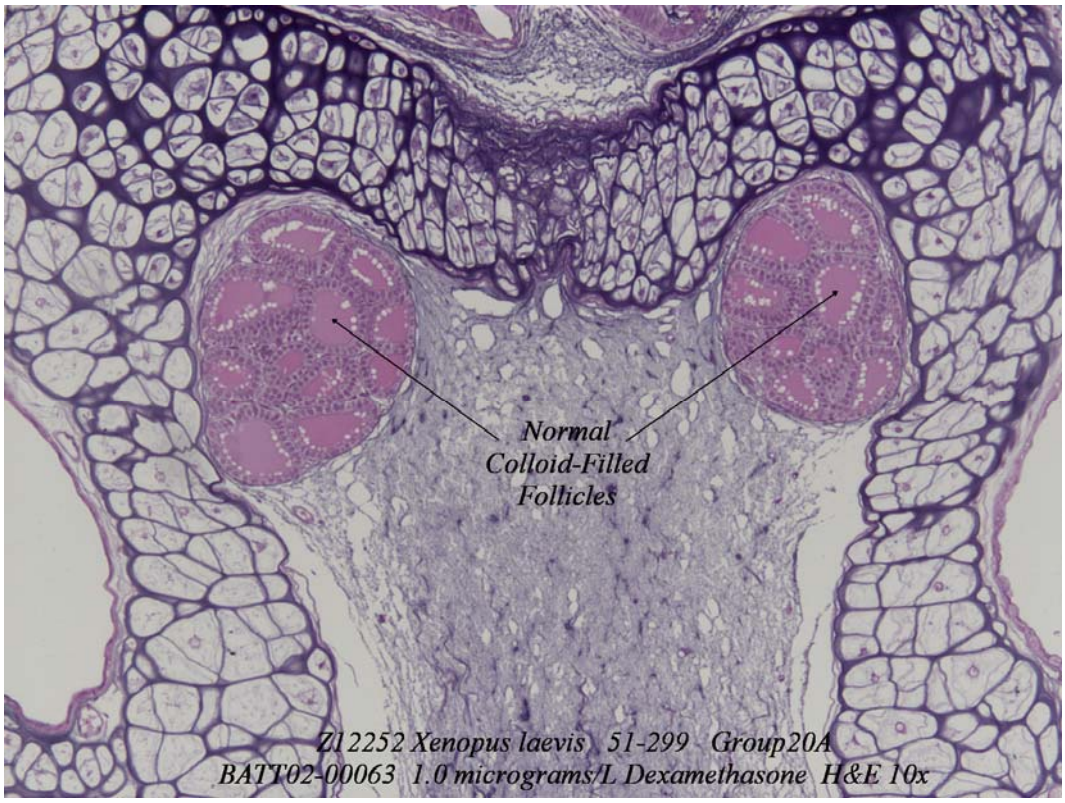
Z12247 (1.25x) and Z12248 (10x). Thyroid glands of control frog 51-280. There is a variation in size of the normal colloid-filled thyroid follicles.





Z12249 (1.25x) and Z12250 (10x). Thyroid glands of frog 51-289 exposed to 1.0 µg/L dexamethasone. There is a variation in size of the normal colloid-filled thyroid follicles.



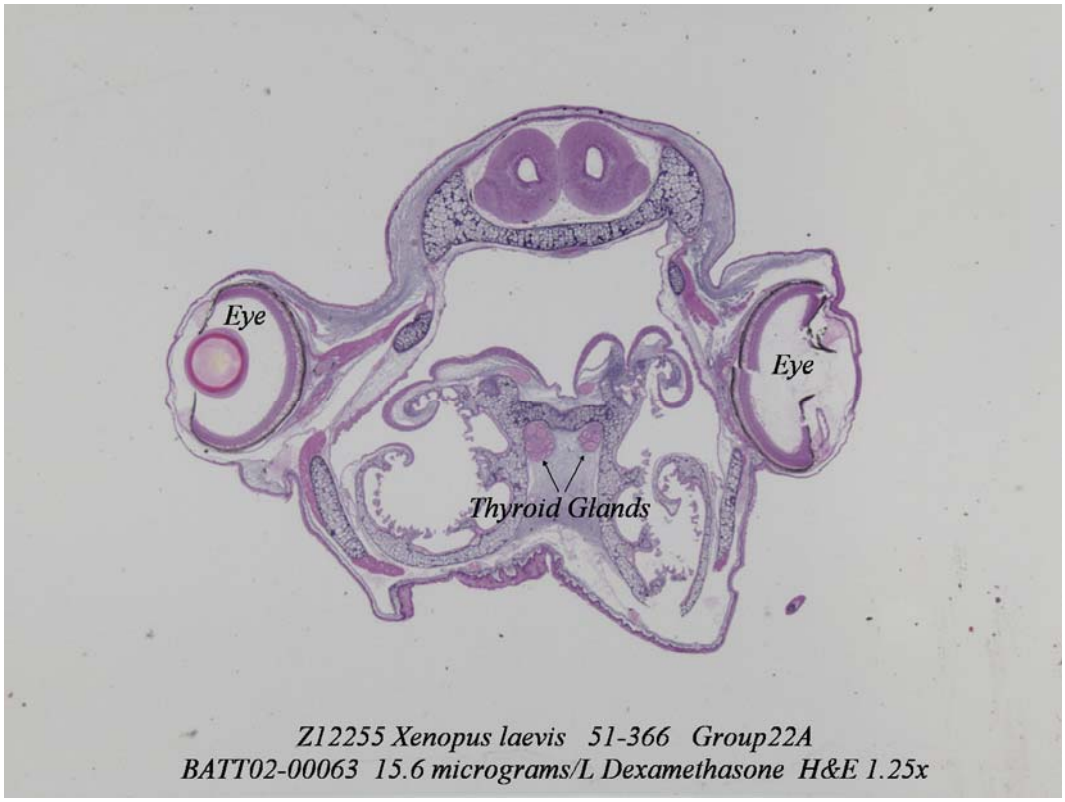


Z12251 (1.25x) and Z12252 (10x). Thyroid glands of frog 51-299 exposed to 1.0 µg/L dexamethasone. There is a variation in size of the normal colloid-filled follicles.





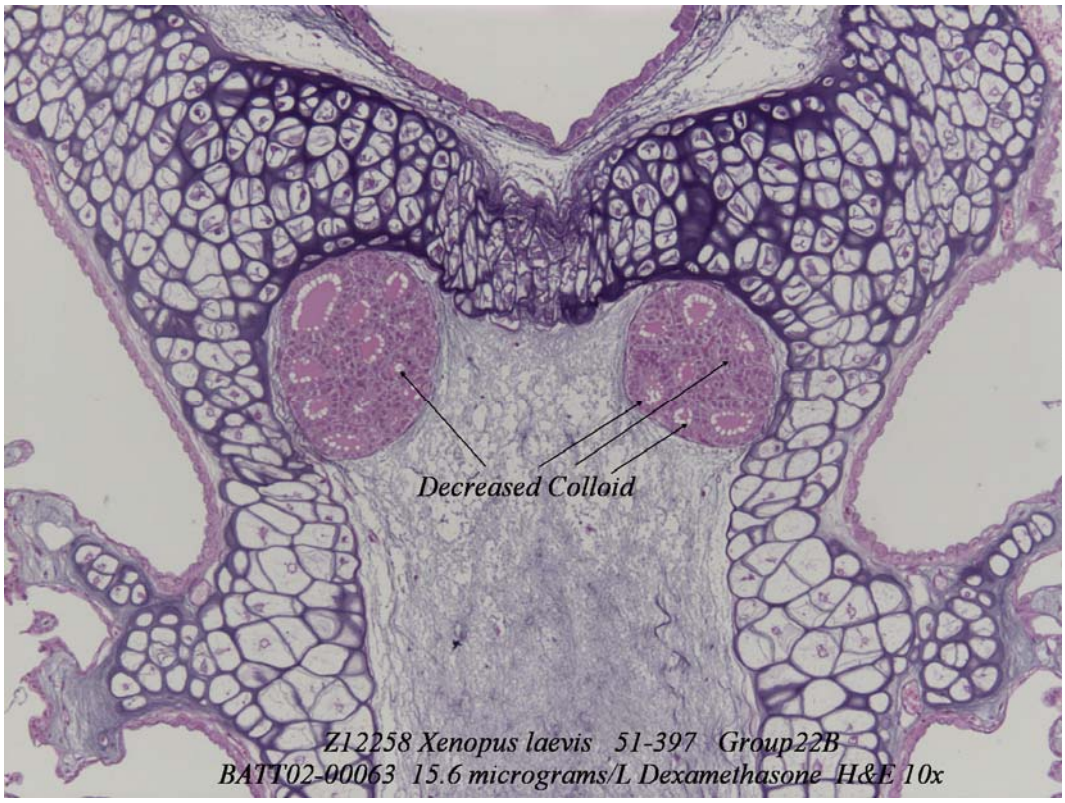
Z12253 (1.25x) and Z12254 (10x). Thyroid glands of frog 51-325 exposed to 3.9 µg/L dexamethasone. There is a variation in size of the normal colloid-filled thyroid follicles.



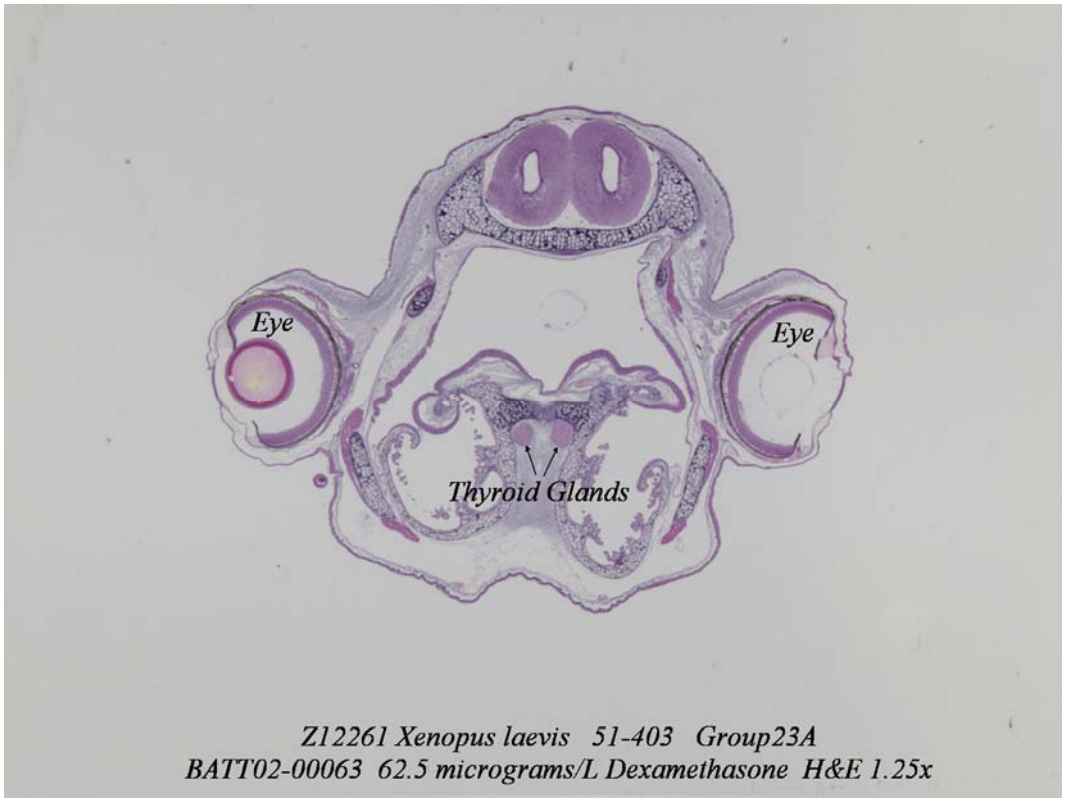


Z12255 (1.25x) and Z12256 (10x). Thyroid glands of frog 51-366 exposed to 15.6 µg/L dexamethasone. There is a variation in size of the normal colloid-filled follicles.





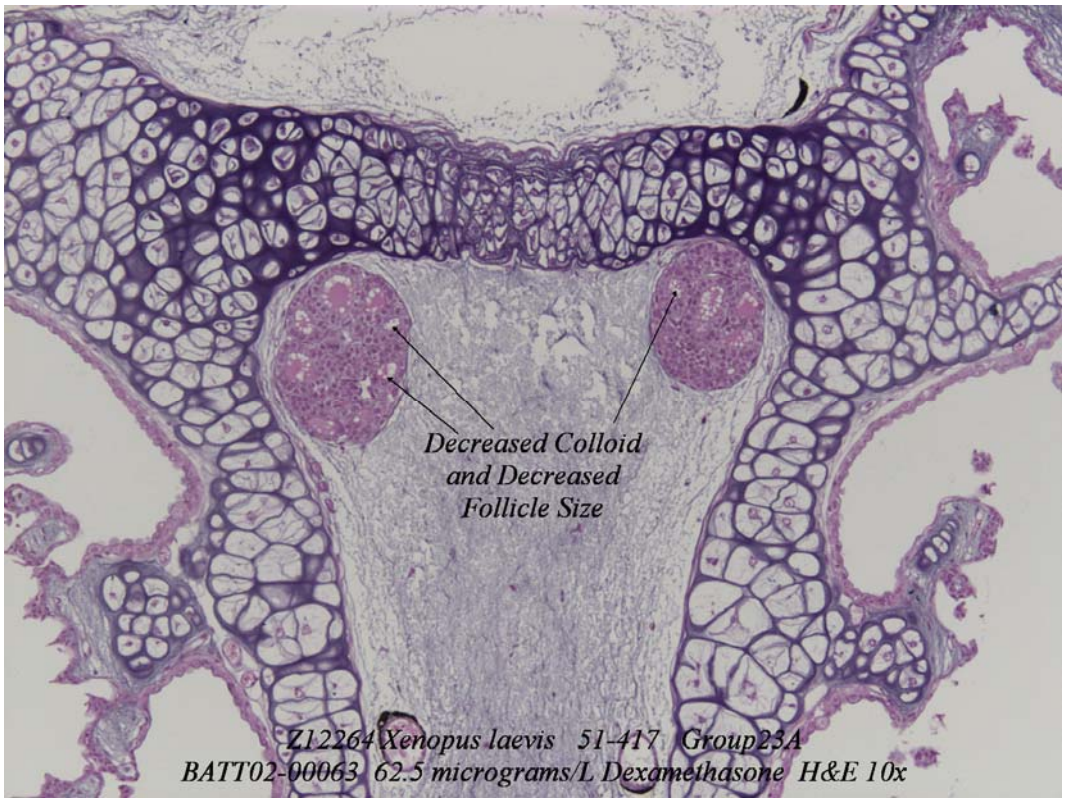
Z12257 (1.25x) and Z12258 (10x). Thyroid glands of frog 51-397 exposed to 15.6 µg/L dexamethasone. There is a minimal decreased quantity of colloid in the thyroid follicles.





Z12261 (1.25x) and Z12262 (10x). Thyroid glands of frog 51-403 exposed to 62.5 µg/L dexamethasone. There is a variation in the size of normal colloid-filled thyroid follicles.





Z12263 (1.25x) and Z12264 (10x). Thyroid glands of frog 51-417 exposed to 62.5 µg/L dexamethasone. There is a mild decreased quantity of colloid and a mild decreased size of thyroid follicles.





Z12267 (1.25x) and Z12268 (10x). Thyroid glands of frog 51-467 exposed to 250 µg/L dexamethasone. There is a moderate decreased quantity of colloid and a mild decreased size of thyroid follicles.

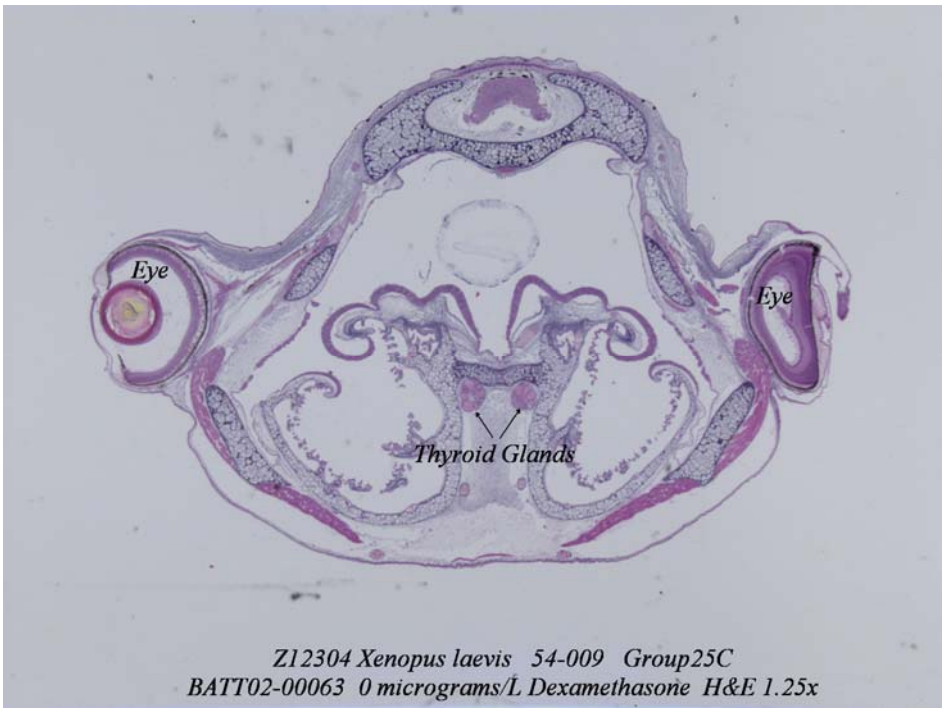




Z12269 (1.25x) and Z12270 (10x). Thyroid glands of frog 51-455 exposed to 250 µg/L dexamethasone. There is a moderate decreased quantity of colloid and a mild decreased size of thyroid follicles.

Appendix B:

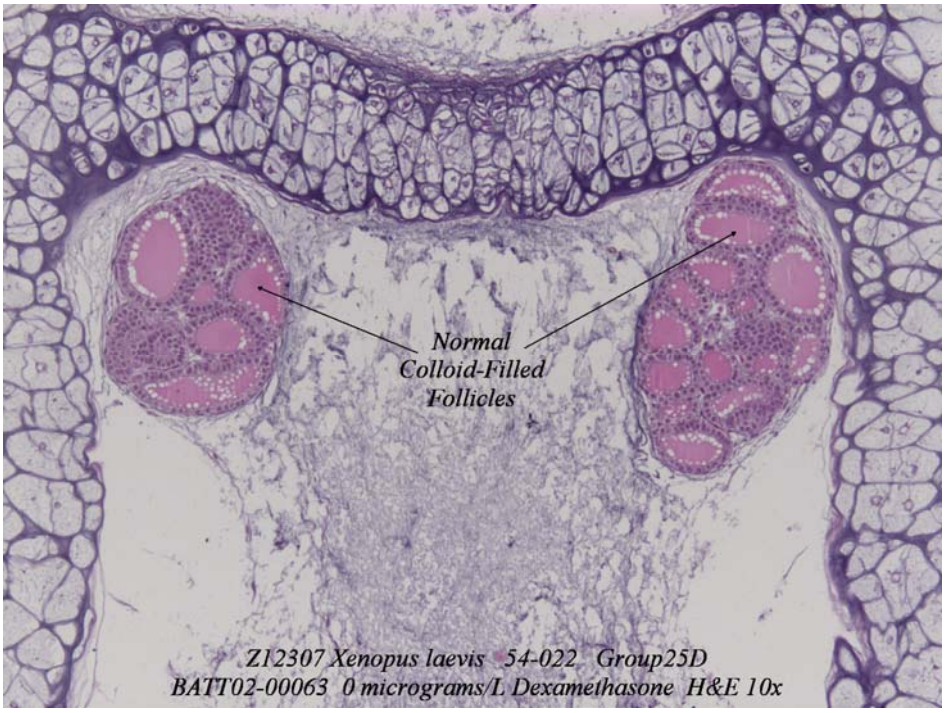
Stage 54 Photo Legends



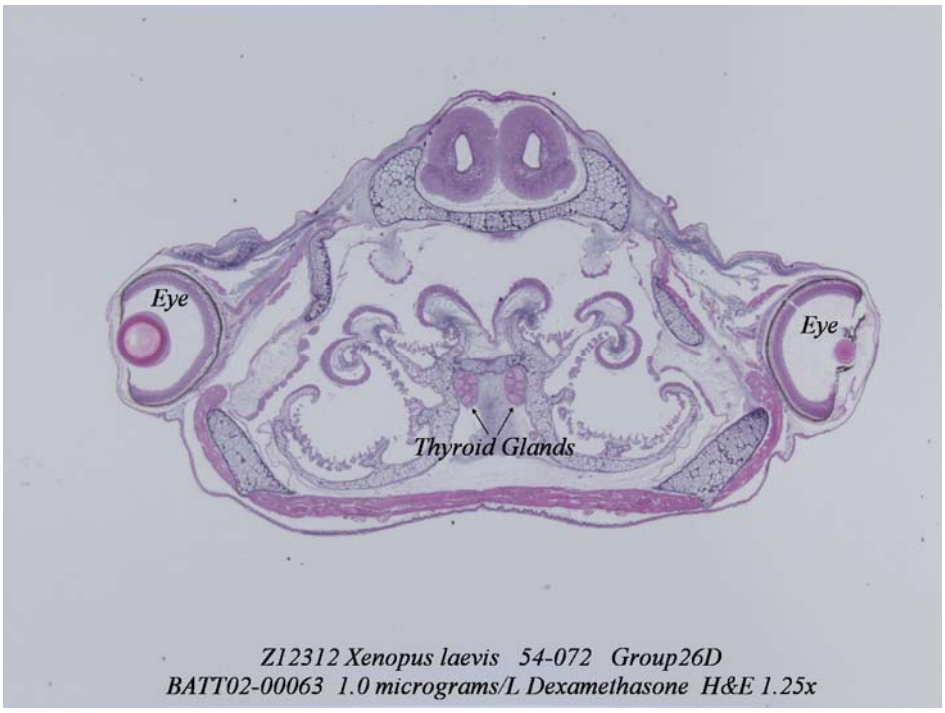


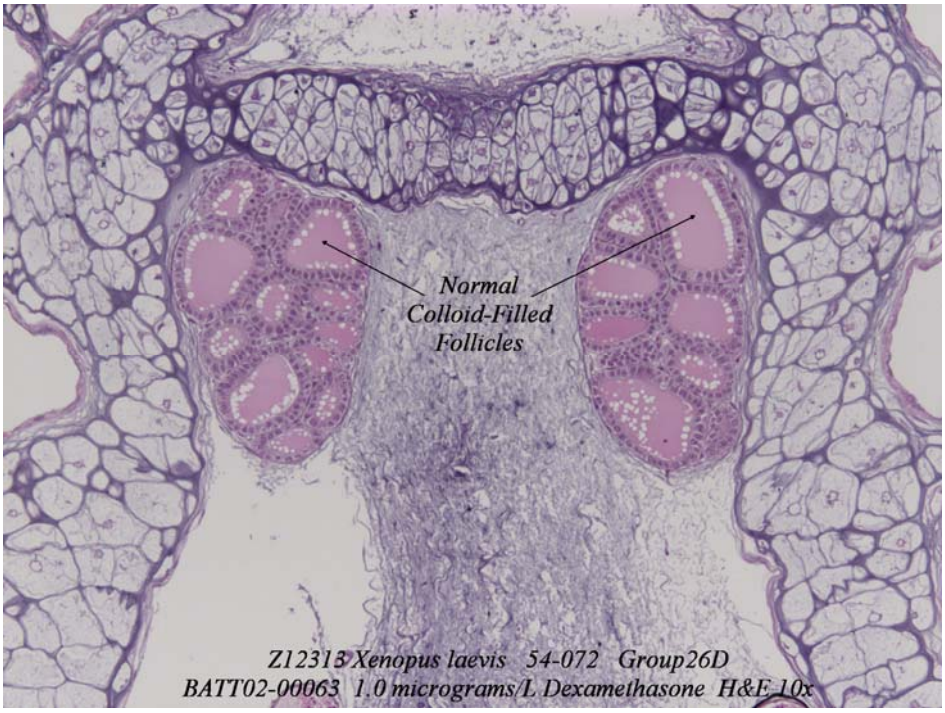
Z12304 (1.25x) and Z12305 (10x). Thyroid glands of control frog 54-009. There is a variation in size of the normal colloid-filled thyroid follicles.





Z12306 (1.25x) and Z12307 (10x). Thyroid glands of control frog 54-022. There is a variation in size of the normal colloid-filled thyroid follicles.



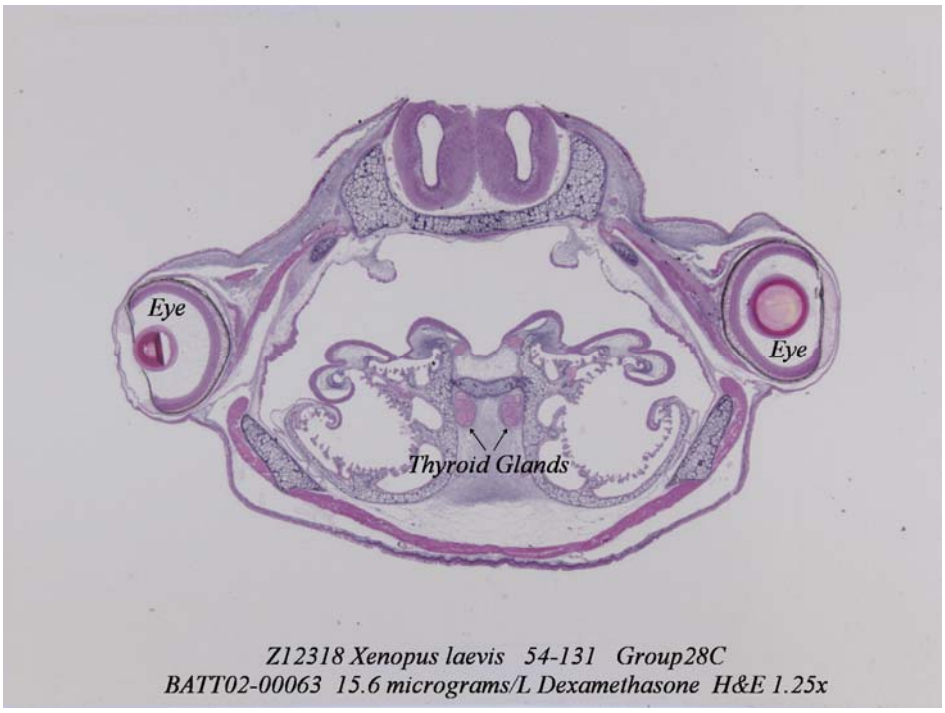


Z12312 (1.25x) and Z12313 (10x). Thyroid glands of frog 54-072 exposed to 1.0 µg/L dexamethasone. There is a variation in size of the normal colloid-filled thyroid follicles.



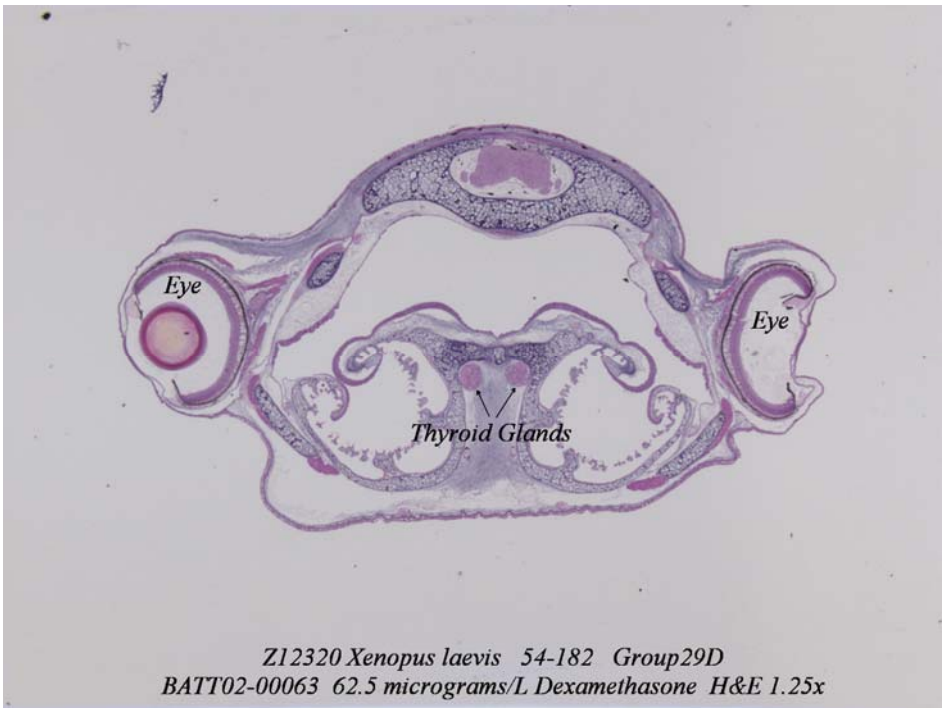


Z12316 (1.25x) and Z12317 (10x). Thyroid glands of frog 54-097 exposed to 3.9 µg/L dexamethasone. There is a variation in size of the normal colloid-filled thyroid follicles.



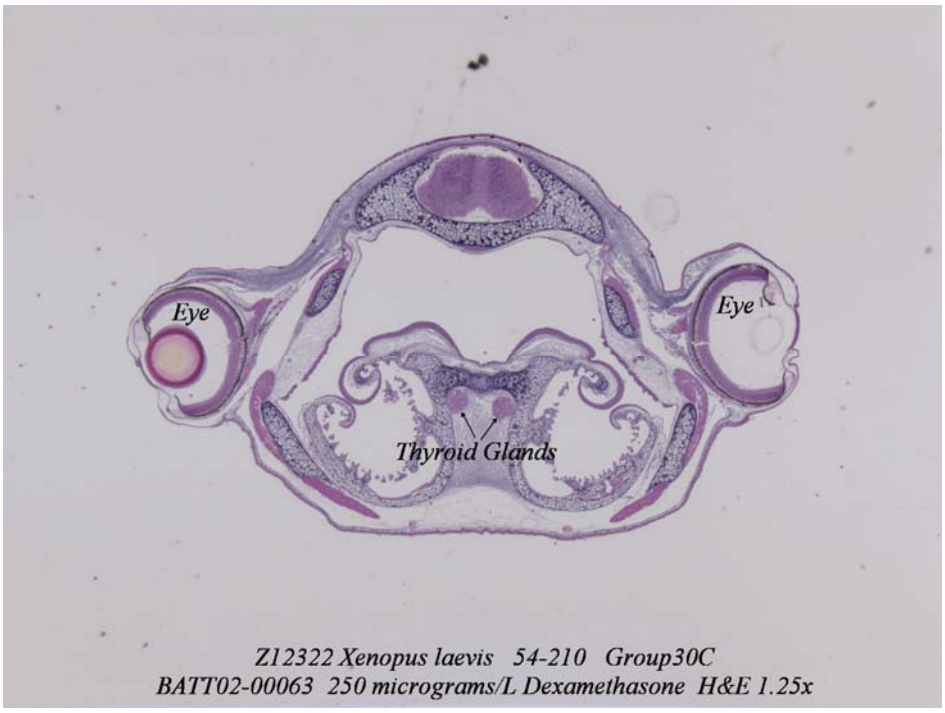


Z12318 (1.25x) and Z12319 (10x). Thyroid glands of frog 54-131 exposed to 15.6 µg/L dexamethasone. There is a variation in size of the normal colloid-filled thyroid follicles.





Z12320 (1.25x) and Z12321 (10x). Thyroid glands of frog 54-182 exposed to 62.5 µg/L dexamethasone. There is a variation in size of normal colloid-filled thyroid follicles.





Z12322 (1.25x) and Z12323 (10x). Thyroid glands of frog 54-210 exposed to 250 µg/L dexamethasone. There is a variation in size of normal colloid-filled thyroid follicles.





Z12324 (1.25x) and Z12325 (10x). Thyroid glands of frog 54-231 exposed to 250 µg/L dexamethasone. There is a variation in size of normal colloid-filled thyroid follicles.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

Client:	Ba Helle
Submit Report To:	Doug Fort
Sampler's Signature:	
P.O. #:	

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

BATT02 - 00063

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
019	Specimen #'s: 247, 250, 253,	11/30/04 1000	10	G	10% Formalin	Histological examination of Thyroid Gland ~~as per protocol # BATT02~~ ①
↓	255, 256, 263, 271, 273,	↓			↓	
↓	277, 280					
020	Specimen #'s: 282, 293, 289,		10	G		
↓	291, 299, 304, 312, 314,					
↓	317, 318					
021	Specimen #'s: 323, 325, 332,		10	G		
↓	332, 339, 343, 346, 352,					
↓	355, 360					
022	Specimen #'s: 366, 369, 375,		10	G		
↓	377, 379, 385, 386, 395					
↓	397, 399					
023	Specimen #'s: 402, 405, 415,		10	G		
↓	417, 419, 422, ~~425~~ 423,					
↓	425, 436					

SPECIAL INSTRUCTIONS: Organisms exposed to Dexamethasone

DATE:
INITIALS:

Andria Walker	12/2/04 1000	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

① mistake. AW 11/30/04

② N/A - JT - 12/2/04

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

Client:	Battelle
Submit Report To:	Doug Forty
Sampler's Signature:	
P.O. #:	

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

BATTS2 - 00063

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
024	Specimen#'s: 465, 467, 474,	11/30/04 1000	10	G	10% Formalin	Histological examination of Thyroid Gland
↓	476, 478, 441, 445, 452,	↓ ↓			↓	~~as per protocol #BATTS2-1~~ ①
↓	455, 457					↓
025	Specimen#'s: 002, 004, 009,		10	G		
↓	013, 019, 022, 030, 036,					
↓	037, 039					
026	Specimen#'s: 041, 045, 051,		10	G		
↓	057, 059, 063, 067, 069,					
↓	072, 080					
027	Specimen #'s: 084, 086, 088,		10	G		
↓	093, 097, 102, 104, 107,					
↓	111, 115					
028	Specimen #'s: 123, 126, 131,		10	G		
↓	136, 138, 141, 143, 149,					
↓	152, 159	↓ ↓			↓	↓

DATE:
INITIALS:

SPECIAL INSTRUCTIONS: Organisms exposed to Dexamethasone

Andria Wallace	12/2/04 1000	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

① N/A - JT - 12/2/04

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3386
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	
P.O. #:	

BATT02-00063

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
029	Specimen #'s: 163, 166, 170,	11/30/04 1000	10	G	10% Formalin	Histological examination of Thyroid Gland as per protocol #BATT02-1 ①
↓	175, 179, 182, 187, 196,	↓			↓	↓
↓	198, 199					
030	Specimen #'s 202, 207, 210,		10	G		
↓	214, 218, 222, 229, 231,					
↓	234, 239	↓			↓	↓

SPECIAL INSTRUCTIONS: Organisms exposed to Dexamethasone

DATE:
INITIALS:

Andia Walker	12/2/04 1000	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

① N/A - JT - 12/2/04

Appendix G
Phenobarbital

Metamorphosis Assay – Raw Data:

Daily Operational Data
Test Termination Data – Stage and Growth
Statistical Data – Stage and Growth

Test Chemical Analytical Data from Battelle:

Purity / Stability Report
In-Life Test Chemical Analysis Report

Thyroid Histology Data from EPL:

Sample Identification Key
Thyroid Pathology Report

Phenobarbital Metamorphosis Assay: Daily Operational Data

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA RENEW

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 0.0 mg/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Conc[1] (mg/L)
		Initial No.= 20							**Initial No.= 20**												
0	08/26/04			100.0	20.7	7.0	4.9	1067			100.0	20.7	7.1	4.8	1208	100.0	20.7	7.1	4.9	1138	0.30
1	08/27/04		20	100.0	22.0	7.1	5.4	1070		20	100.0	22.0	7.1	5.2	1190	100.0	22.0	7.1	5.3	1130	
2	08/28/04		20	100.0	20.2	7.0	5.2	1085		20	100.0	20.3	7.0	4.8	1134	100.0	20.3	7.0	5.0	1110	
3	08/29/04		20	100.0	20.2	7.1	5.0	1080		20	100.0	20.2	7.1	5.2	1127	100.0	20.2	7.1	5.1	1104	
4	08/30/04		20	100.0	20.2	7.1	5.1	1110		20	100.0	20.1	7.1	5.0	1099	100.0	20.2	7.1	5.1	1105	
5	08/31/04		20	100.0	21.9	7.2	5.6	1090		20	100.0	22.0	7.4	5.5	1140	100.0	22.0	7.3	5.6	1115	
6	09/01/04		20	100.0	20.8	7.3	5.6	-		20	100.0	20.8	7.4	6.3	-	100.0	20.8	7.4	6.0	-	0.25
7	09/02/04		20	100.0	20.9	7.2	5.7	1177		20	100.0	20.8	7.2	6.2	1180	100.0	20.9	7.2	6.0	1179	
8	09/03/04		20	100.0	20.7	7.1	5.5	1066		20	100.0	20.7	7.2	6.2	1146	100.0	20.7	7.2	5.9	1106	
9	09/04/04		20	100.0	20.5	7.2	5.7	1029		20	100.0	20.6	7.2	5.9	1103	100.0	20.6	7.2	5.8	1066	
10	09/05/04		20	100.0	20.6	7.2	5.6	1002		20	100.0	20.6	7.3	6.0	1209	100.0	20.6	7.3	5.8	1106	
11	09/06/04		20	100.0	20.7	7.0	4.9	1081		20	100.0	20.6	7.0	5.3	1105	100.0	20.7	7.0	5.1	1093	
12	09/07/04		20	100.0	20.5	7.0	6.1	1150		20	100.0	20.4	7.1	6.7	1172	100.0	20.5	7.1	6.4	1161	
13	09/08/04		20	100.0	20.5	7.0	6.4	1070		20	100.0	20.4	7.1	7.1	1113	100.0	20.5	7.1	6.8	1092	
14	09/09/04		20	100.0	20.8	7.2	6.2	1088		20	100.0	20.7	7.3	7.0	1163	100.0	20.8	7.3	6.6	1126	0.24
15	09/10/04		20	100.0	20.4	7.3	6.6	1129		20	100.0	20.4	7.3	6.9	1190	100.0	20.4	7.3	6.8	1160	
16	09/11/04		20	100.0	20.3	7.3	6.3	1092		20	100.0	20.5	7.2	6.7	1154	100.0	20.4	7.3	6.5	1123	
17	09/12/04		20	100.0	20.4	7.1	7.5	1084		20	100.0	20.3	7.0	7.4	1131	100.0	20.4	7.1	7.5	1108	
18	09/13/04		20	100.0	21.5	7.2	6.5	1124		20	100.0	21.5	7.2	7.0	1153	100.0	21.5	7.2	6.8	1139	
19	09/14/04		20	100.0	21.5	7.1	5.8	1185		20	100.0	21.5	7.2	6.3	1165	100.0	21.5	7.2	6.1	1175	
20	09/15/04		20	100.0	21.5	7.2	6.2	1015		20	100.0	21.5	7.2	6.7	991	100.0	21.5	7.2	6.5	1003	0.06
21	09/16/04		20	100.0						20	100.0					100.0					0.38
	Median:				**20.7**	**7.1**	**5.7**	**1085**				**20.6**	**7.2**	**6.2**	**1150**		**20.7**	**7.2**	**6.0**	**1112**	
	Mode:				**20.7**	**7.2**	**5.6**	**1070**				**20.7**	**7.2**	**6.7**	**1190**		**21.5**	**7.1**	**6.8**	**#N/A**	
	Mean:			**100.0**	**20.8**	**7.1**	**5.8**	**1090**			**100.0**	**20.8**	**7.2**	**6.1**	**1144**	**100.0**	**20.8**	**7.2**	**6.0**	**1117**	**0.246**
	SEM:			**0.00**	**0.12**	**0.02**	**0.14**	**10.58**			**0.00**	**0.12**	**0.03**	**0.18**	**10.98**	**0.00**	**0.12**	**0.02**	**0.16**	**8.83**	**0.05**
	% CV:			**0.00**	**12.13**	**3.83**	**27.13**	**143.30**			**0.00**	**12.42**	**4.40**	**33.12**	**145.16**	**0.00**	**12.24**	**3.89**	**29.50**	**118.14**	**23.63**

[1]All measured test concentrations reported by Battelle as less than method detection limit (MDL) [0.1112 mg/L] or none detected (ND) are expressed as 0.06 mg/L (1/2 of MDL).
0.06 mg/L was used in calculating the average measured concentration for 9/9/04.

DAILY DATA RENEW

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 125 mg/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc (mg/L)
		Initial No.= 20							**Initial No.= 20**												
0	08/26/04			100.0	20.1	7.8	6.4	1001			100.0	20.3	7.9	6.4	1180	100.0	20.2	7.9	6.4	1091	126.80
1	08/27/04		20	100.0	20.8	7.8	7.1	1240		20	100.0	20.7	7.9	7.2	1000	100.0	20.8	7.9	7.2	1120	
2	08/28/04		20	100.0	20.4	7.6	7.2	1162		20	100.0	20.4	7.7	7.3	970	100.0	20.4	7.7	7.3	1066	
3	08/29/04		20	100.0	20.5	7.7	7.2	1184		20	100.0	20.5	7.7	7.2	1010	100.0	20.5	7.7	7.2	1097	
4	08/30/04		20	100.0	20.0	7.7	7.9	1190		20	100.0	20.1	7.7	8.0	1032	100.0	20.1	7.7	8.0	1111	
5	08/31/04		20	100.0	22.0	7.7	6.1	1180		20	100.0	22.0	7.7	5.9	990	100.0	22.0	7.7	6.0	1085	
6	09/01/04		20	100.0	20.6	7.7	7.1	-		20	100.0	20.6	7.7	7.4	-	100.0	20.6	7.7	7.3	-	
7	09/02/04		20	100.0	20.8	7.6	6.9	1195		20	100.0	20.7	7.7	7.3	992	100.0	20.8	7.7	7.1	1094	
8	09/03/04		20	100.0	21.0	7.7	6.8	1200		20	100.0	21.0	7.8	7.2	1011	100.0	21.0	7.8	7.0	1106	
9	09/04/04		20	100.0	20.4	7.7	7.6	1134		20	100.0	20.6	7.6	7.2	870	100.0	20.5	7.7	7.4	1002	
10	09/05/04		20	100.0	20.6	7.6	7.8	1092		20	100.0	20.6	7.8	7.8	897	100.0	20.6	7.7	7.8	995	
11	09/06/04		20	100.0	20.5	7.8	7.8	1120		20	100.0	20.6	7.8	8.0	917	100.0	20.6	7.8	7.9	1019	
12	09/07/04		20	100.0	20.3	7.6	7.2	1010		20	100.0	20.4	7.8	7.7	1201	100.0	20.4	7.7	7.5	1106	
13	09/08/04	1	19	95.0	20.3	7.6	7.7	1239		20	100.0	20.3	7.7	7.3	938	97.5	20.3	7.7	7.5	1089	
14	09/09/04		19	95.0	20.7	7.7	7.1	1228		20	100.0	20.7	7.7	7.3	999	97.5	20.7	7.7	7.2	1114	
15	09/10/04		19	95.0	20.7	7.6	7.2	1180		20	100.0	20.7	7.6	7.1	980	97.5	20.7	7.6	7.2	1080	
16	09/11/04		19	95.0	20.6	7.7	7.2	1227		20	100.0	20.6	7.7	7.0	975	97.5	20.6	7.7	7.1	1101	
17	09/12/04		19	95.0	20.8	7.7	7.0	1210		20	100.0	20.8	7.7	6.9	967	97.5	20.8	7.7	7.0	1089	
18	09/13/04		19	95.0	21.3	7.5	6.3	1127		20	100.0	21.3	7.5	6.9	962	97.5	21.3	7.5	6.6	1045	
19	09/14/04		19	95.0	21.3	7.5	6.3	1168		20	100.0	21.3	7.5	6.7	942	97.5	21.3	7.5	6.5	1055	
20	09/15/04		19	95.0	21.4	7.3	6.0	1167		20	100.0	21.4	7.5	6.5	984	97.5	21.4	7.4	6.3	1076	
21	09/16/04		19	95.0						20	100.0					97.5					104.50
	Median:				**20.6**	**7.7**	**7.1**	**1180**				**20.6**	**7.7**	**7.2**	**982**		**20.6**	**7.7**	**7.2**	**1089**	
	Mode:				**20.8**	**7.7**	**7.2**	**1180**				**20.6**	**7.7**	**7.2**	**#N/A**		**20.6**	**7.7**	**7.2**	**1106**	
	Mean:			**98.0**	**20.7**	**7.6**	**7.0**	**1163**			**100.0**	**20.7**	**7.7**	**7.2**	**991**	**99.0**	**20.7**	**7.7**	**7.1**	**1077**	**115.650**
	SEM:			**0.54**	**0.10**	**0.03**	**0.12**	**14.96**			**0.00**	**0.10**	**0.02**	**0.11**	**17.69**	**0.27**	**0.10**	**0.02**	**0.11**	**8.15**	**11.15**
	% CV:			**25.42**	**10.39**	**4.22**	**21.20**	**196.27**			**0.00**	**9.71**	**4.11**	**19.12**	**251.26**	**12.65**	**10.02**	**3.90**	**19.43**	**111.08**	**146.63**

DAILY DATA RENEW

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 250 mg/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc (mg/L)
		Initial No.= 20							**Initial No.= 20**												
0	08/26/04			100.0	20.5	8.0	6.3	1455			100.0	20.5	8.0	5.7	1507	100.0	20.5	8.0	6.0	1481	239.40
1	08/27/04		20	100.0	20.5	8.0	7.0	1460		20	100.0	20.5	8.0	6.5	1472	100.0	20.5	8.0	6.8	1466	
2	08/28/04		20	100.0	20.5	7.8	7.5	1155		20	100.0	20.5	7.8	7.0	1191	100.0	20.5	7.8	7.3	1173	
3	08/29/04		20	100.0	20.4	7.8	7.0	1205		20	100.0	20.6	7.8	7.1	1224	100.0	20.5	7.8	7.1	1215	
4	08/30/04		20	100.0	20.1	7.8	8.1	1525		20	100.0	20.1	7.8	8.1	1444	100.0	20.1	7.8	8.1	1485	
5	08/31/04		20	100.0	22.0	7.8	6.1	1463		20	100.0	22.0	7.9	6.1	1419	100.0	22.0	7.9	6.1	1441	
6	09/01/04		20	100.0	20.7	7.8	6.9	-		20	100.0	20.8	7.8	7.4	-	100.0	20.8	7.8	7.2	-	243.00
7	09/02/04		20	100.0	20.6	7.8	7.2	1445		20	100.0	20.5	7.9	7.5	1453	100.0	20.6	7.9	7.4	1449	
8	09/03/04		20	100.0	20.9	7.8	6.4	1463		20	100.0	20.9	7.8	7.0	1309	100.0	20.9	7.8	6.7	1386	
9	09/04/04		20	100.0	20.6	7.8	7.1	1401		20	100.0	20.7	7.7	7.1	1374	100.0	20.7	7.8	7.1	1388	
10	09/05/04		20	100.0	20.5	7.9	7.4	1425		20	100.0	20.6	7.8	7.3	1390	100.0	20.6	7.9	7.4	1408	
11	09/06/04		20	100.0	20.6	7.7	7.7	1401		20	100.0	20.6	7.8	7.7	1273	100.0	20.6	7.8	7.7	1337	
12	09/07/04		20	100.0	20.6	7.7	7.3	1427		20	100.0	20.8	7.7	7.0	1443	100.0	20.7	7.7	7.2	1435	
13	09/08/04		20	100.0	20.4	7.7	7.0	1267		20	100.0	20.5	7.7	6.9	1368	100.0	20.5	7.7	7.0	1318	
14	09/09/04		20	100.0	20.8	7.8	7.1	1421		20	100.0	20.8	7.8	6.4	1334	100.0	20.8	7.8	6.8	1378	197.90
15	09/10/04		20	100.0	20.7	7.7	6.6	1373		20	100.0	20.8	7.7	6.7	1339	100.0	20.8	7.7	6.7	1356	
16	09/11/04		20	100.0	20.8	7.8	7.1	1272		20	100.0	20.7	7.8	6.9	1370	100.0	20.8	7.8	7.0	1321	
17	09/12/04		20	100.0	20.7	7.8	7.1	1415		20	100.0	20.8	7.8	7.0	1332	100.0	20.8	7.8	7.1	1374	
18	09/13/04		20	100.0	21.1	7.6	6.1	1323		20	100.0	21.1	7.6	6.3	1286	100.0	21.1	7.6	6.2	1305	
19	09/14/04		20	100.0	21.4	7.7	6.2	1428		20	100.0	21.3	7.6	6.3	1403	100.0	21.4	7.7	6.3	1416	
20	09/15/04		20	100.0	21.5	7.5	5.9	1508		20	100.0	21.4	7.6	6.2	1440	100.0	21.5	7.6	6.1	1474	203.60
21	09/16/04		20	100.0						20	100.0					100.0					204.30
	Median:				**20.6**	**7.8**	**7.0**	**1423**				**20.7**	**7.8**	**7.0**	**1372**		**20.7**	**7.8**	**7.0**	**1387**	
	Mode:				**20.5**	**7.8**	**7.1**	**1463**				**20.5**	**7.8**	**7.0**	**#N/A**		**20.5**	**7.8**	**6.8**	**#N/A**	
	Mean:			**100.0**	**20.8**	**7.8**	**6.9**	**1392**			**100.0**	**20.8**	**7.8**	**6.9**	**1369**	**100.0**	**20.8**	**7.8**	**6.9**	**1380**	**217.640**
	SEM:			**0.00**	**0.09**	**0.02**	**0.13**	**22.01**			**0.00**	**0.09**	**0.02**	**0.13**	**18.70**	**0.00**	**0.09**	**0.02**	**0.12**	**18.97**	**9.70**
	% CV:			**0.00**	**9.46**	**4.07**	**21.85**	**263.84**			**0.00**	**8.81**	**4.03**	**22.07**	**226.06**	**0.00**	**9.08**	**3.91**	**20.97**	**228.38**	**147.01**

DAILY DATA RENEW

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 500 mg/L**

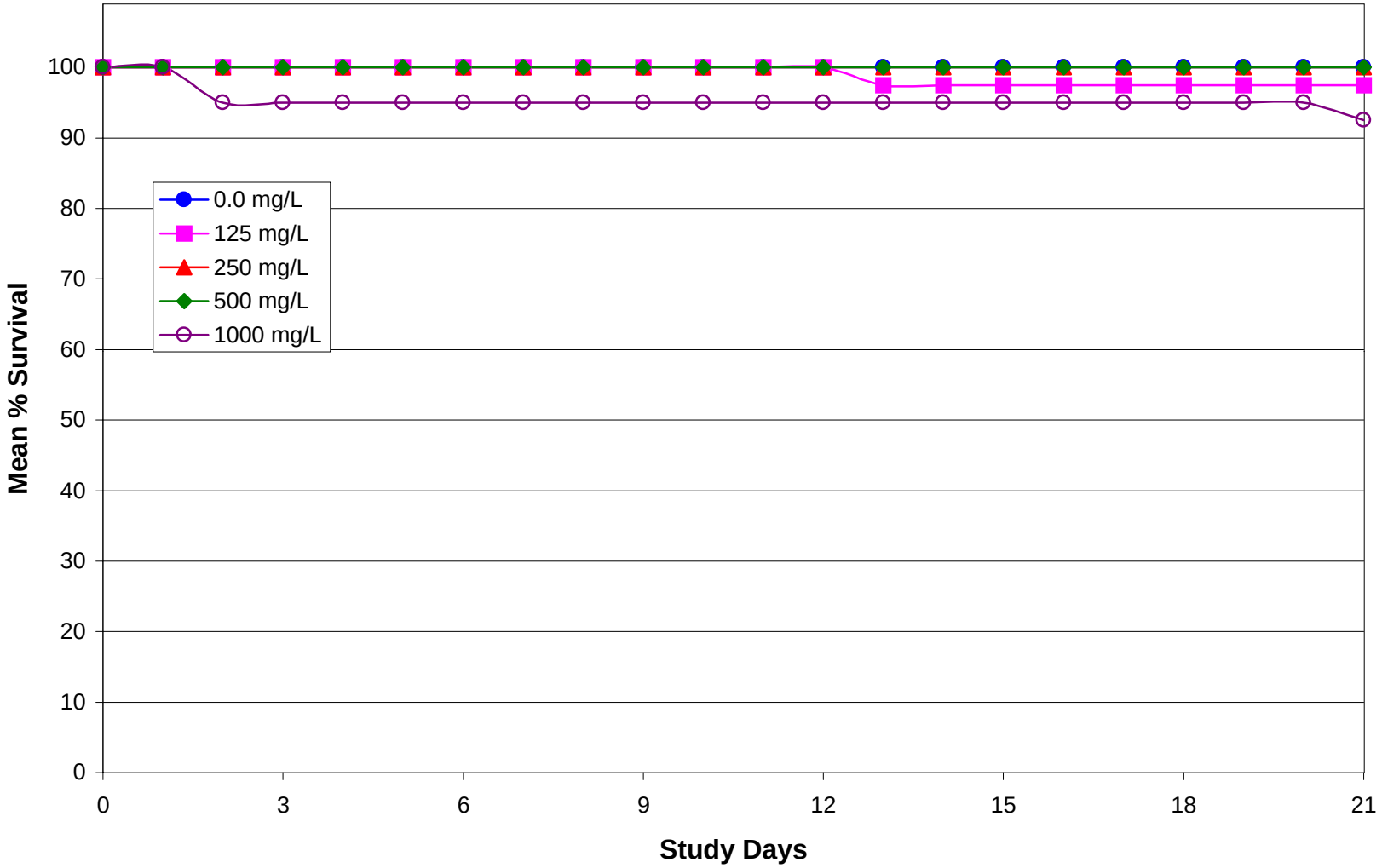
Study Day	Study Date	INITIAL STAGE 51 REPLICATE A: Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	INITIAL STAGE 51 REPLICATE B: Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	INITIAL STAGE 51 DAILY MEANS OF REPLICATES: % Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Average Measured Test Conc (mg/L)
		Initial No.= 20							**Initial No.= 20**													
0	08/26/04			100.0	20.6	8.2	7.0	1388			100.0	20.7	8.2	7.0	1260	100.0	20.7	8.2	7.0	1324	469.90
1	08/27/04		20	100.0	20.7	8.1	7.4	1233		20	100.0	20.7	8.1	7.4	1393	100.0	20.7	8.1	7.4	1313	
2	08/28/04		20	100.0	20.6	8.0	7.3	1130		20	100.0	20.6	8.0	7.2	1351	100.0	20.6	8.0	7.3	1241	
3	08/29/04		20	100.0	20.5	8.0	7.4	1150		20	100.0	20.6	7.9	7.7	1410	100.0	20.6	8.0	7.6	1280	
4	08/30/04		20	100.0	20.1	7.9	7.8	1254		20	100.0	20.1	7.9	7.7	1337	100.0	20.1	7.9	7.8	1296	
5	08/31/04		20	100.0	22.0	8.0	5.5	1374		20	100.0	22.0	7.9	5.4	1360	100.0	22.0	8.0	5.5	1367	
6	09/01/04		20	100.0	20.8	7.9	6.5	-		20	100.0	20.8	8.0	5.7	-	100.0	20.8	8.0	6.1	-	496.80
7	09/02/04		20	100.0	20.6	8.0	7.0	1253		20	100.0	20.6	7.9	7.2	1405	100.0	20.6	8.0	7.1	1329	
8	09/03/04		20	100.0	20.9	8.0	6.5	1216		20	100.0	20.9	8.0	7.0	1362	100.0	20.9	8.0	6.8	1289	
9	09/04/04		20	100.0	20.5	7.9	7.1	1048		20	100.0	20.6	7.9	6.9	1248	100.0	20.6	7.9	7.0	1148	
10	09/05/04		20	100.0	20.8	7.8	7.3	1276		20	100.0	20.7	8.1	7.2	1324	100.0	20.8	8.0	7.3	1300	
11	09/06/04		20	100.0	20.5	7.7	7.0	1168		20	100.0	20.4	7.8	7.7	1333	100.0	20.5	7.8	7.4	1251	
12	09/07/04		20	100.0	21.4	7.8	6.2	1229		20	100.0	20.6	7.9	6.0	1337	100.0	21.0	7.9	6.1	1283	
13	09/08/04		20	100.0	20.4	7.8	7.1	1279		20	100.0	20.4	7.8	7.4	1352	100.0	20.4	7.8	7.3	1316	
14	09/09/04		20	100.0	20.9	7.9	7.0	1224		20	100.0	20.8	7.9	7.2	1254	100.0	20.9	7.9	7.1	1239	404.75
15	09/10/04		20	100.0	20.8	7.8	6.8	1271		20	100.0	20.9	7.9	6.9	1340	100.0	20.9	7.9	6.9	1306	
16	09/11/04		20	100.0	20.7	7.9	7.3	1288		20	100.0	20.8	7.9	7.3	1348	100.0	20.8	7.9	7.3	1318	
17	09/12/04		20	100.0	20.9	7.9	6.7	1278		20	100.0	20.9	7.8	6.5	1354	100.0	20.9	7.9	6.6	1316	
18	09/13/04		20	100.0	20.9	7.8	6.6	1149		20	100.0	21.0	7.8	6.7	1318	100.0	21.0	7.8	6.7	1234	
19	09/14/04		20	100.0	21.4	7.8	6.4	1286		20	100.0	21.3	7.8	6.5	1345	100.0	21.4	7.8	6.5	1316	
20	09/15/04		20	100.0	21.4	7.7	6.2	1050		20	100.0	21.4	7.8	6.8	1132	100.0	21.4	7.8	6.5	1091	407.50
21	09/16/04		20	100.0						20	100.0					100.0					402.50
	Median:				**20.8**	**7.9**	**7.0**	**1243**				**20.7**	**7.9**	**7.0**	**1343**		**20.8**	**7.9**	**7.0**	**1298**	
	Mode:				**20.9**	**7.9**	**7.0**	**#N/A**				**20.6**	**7.9**	**7.2**	**1337**		**20.6**	**8.0**	**7.3**	**1316**	
	Mean:			**100.0**	**20.8**	**7.9**	**6.9**	**1227**			**100.0**	**20.8**	**7.9**	**6.9**	**1328**	**100.0**	**20.8**	**7.9**	**6.9**	**1278**	**436.290**
	SEM:			**0.00**	**0.09**	**0.03**	**0.11**	**20.15**			**0.00**	**0.09**	**0.02**	**0.14**	**14.22**	**0.00**	**0.09**	**0.02**	**0.12**	**14.41**	**19.69**
	% CV:			**0.00**	**9.30**	**4.50**	**20.07**	**257.18**			**0.00**	**8.72**	**3.99**	**23.76**	**174.56**	**0.00**	**8.77**	**3.92**	**21.12**	**180.33**	**210.82**

DAILY DATA RENEW

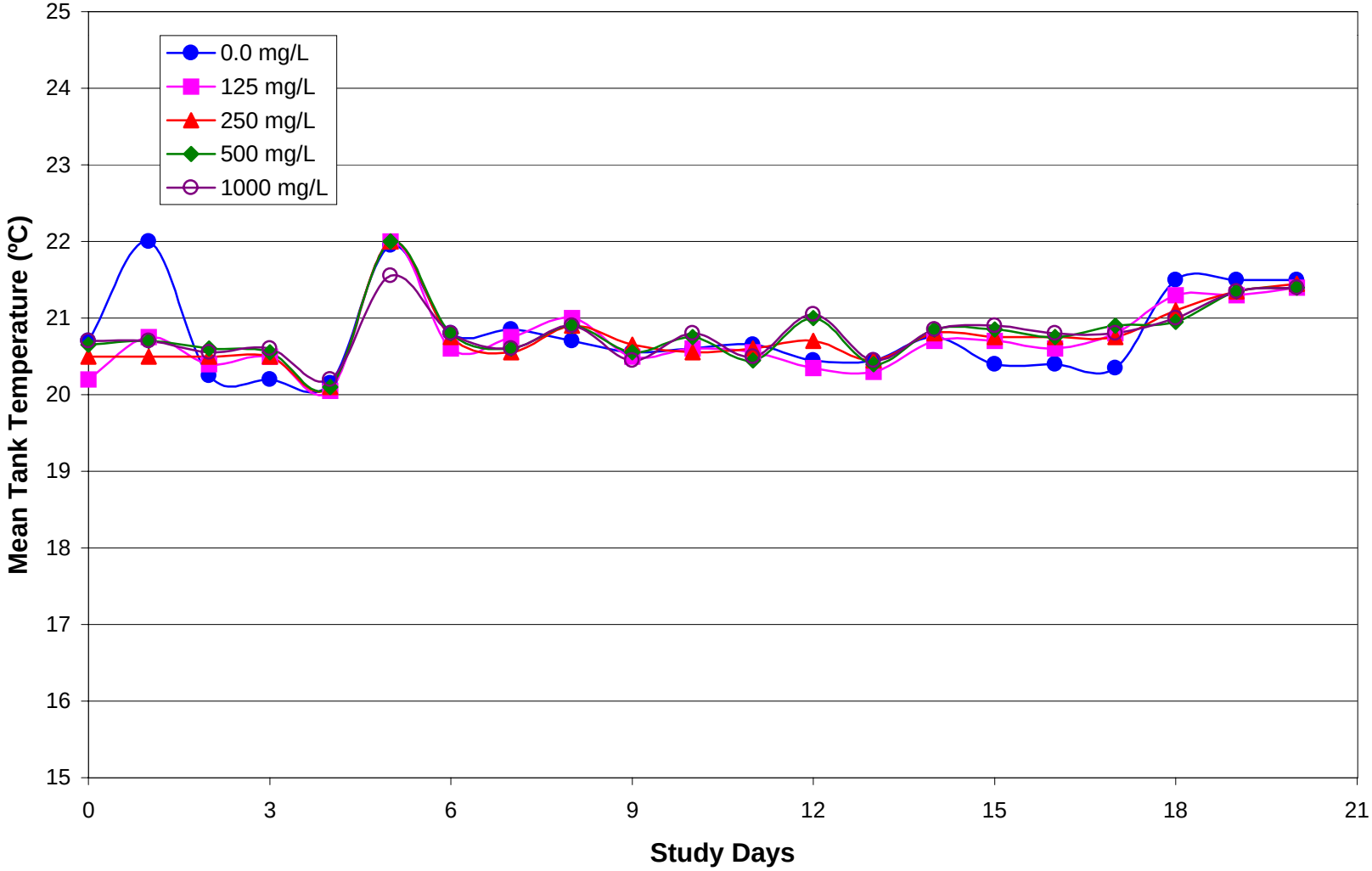
Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 1000 mg/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc (mg/L)
		Initial No.= 20							**Initial No.= 20**												
0	08/26/04			100.0	20.7	7.9	6.8	1095			100.0	20.7	7.8	7.1	1073	100.0	20.7	7.9	7.0	1084	952.20
1	08/27/04		20	100.0	20.7	7.8	6.9	1013		20	100.0	20.7	7.8	7.7	1112	100.0	20.7	7.8	7.3	1063	
2	08/28/04		20	100.0	20.6	7.8	6.9	1057	2	18	90.0	20.5	7.7	7.6	1095	95.0	20.6	7.8	7.3	1076	
3	08/29/04		20	100.0	20.6	7.8	7.4	1080		18	90.0	20.6	7.9	7.3	1107	95.0	20.6	7.9	7.4	1094	
4	08/30/04		20	100.0	20.2	7.7	7.2	1070		18	90.0	20.2	7.8	8.0	1123	95.0	20.2	7.8	7.6	1097	
5	08/31/04		20	100.0	21.5	8.0	6.1	1110		18	90.0	21.6	8.0	6.5	1153	95.0	21.6	8.0	6.3	1132	
6	09/01/04		20	100.0	20.8	8.1	6.5	-		18	90.0	20.8	8.1	7.3	-	95.0	20.8	8.1	6.9	-	1047.10
7	09/02/04		20	100.0	20.6	8.1	7.2	1050		18	90.0	20.6	8.1	7.8	1169	95.0	20.6	8.1	7.5	1110	
8	09/03/04		20	100.0	20.9	8.1	7.1	1050		18	90.0	20.9	8.1	7.6	1100	95.0	20.9	8.1	7.4	1075	
9	09/04/04		20	100.0	20.4	7.8	7.7	921		18	90.0	20.5	7.9	7.8	1010	95.0	20.5	7.9	7.8	966	
10	09/05/04		20	100.0	20.8	8.2	7.6	969		18	90.0	20.8	8.2	7.6	1037	95.0	20.8	8.2	7.6	1003	
11	09/06/04		20	100.0	20.5	8.0	7.8	986		18	90.0	20.5	8.0	8.1	981	95.0	20.5	8.0	8.0	984	
12	09/07/04		20	100.0	21.1	8.0	6.3	1061		18	90.0	21.0	8.0	7.4	1136	95.0	21.1	8.0	6.9	1099	
13	09/08/04		20	100.0	20.5	8.0	7.1	1031		18	90.0	20.4	8.0	7.6	1139	95.0	20.5	8.0	7.4	1085	
14	09/09/04		20	100.0	20.8	8.0	7.0	1236		18	90.0	20.9	8.1	7.4	1179	95.0	20.9	8.1	7.2	1208	
15	09/10/04		20	100.0	20.9	8.0	6.4	1033		18	90.0	20.9	8.0	6.8	1010	95.0	20.9	8.0	6.6	1022	
16	09/11/04		20	100.0	20.8	8.0	7.1	1033		18	90.0	20.8	8.0	6.5	1170	95.0	20.8	8.0	6.8	1102	
17	09/12/04		20	100.0	20.8	8.1	7.2	1050		18	90.0	20.8	8.0	6.8	1169	95.0	20.8	8.1	7.0	1110	
18	09/13/04		20	100.0	21.0	7.9	5.9	1030		18	90.0	21.0	7.9	7.1	1084	95.0	21.0	7.9	6.5	1057	
19	09/14/04		20	100.0	21.4	7.9	6.1	561		18	90.0	21.3	7.9	6.8	1001	95.0	21.4	7.9	6.5	781	
20	09/15/04		20	100.0	21.4	7.9	6.3	883		18	90.0	21.4	7.9	7.0	964	95.0	21.4	7.9	6.7	924	814.10
21	09/16/04	1	19	95.0						18	90.0					92.5					821.15
	Median:				**20.8**	**8.0**	**7.0**	**1042**				**20.8**	**8.0**	**7.4**	**1104**		**20.8**	**8.0**	**7.2**	**1080**	
	Mode:				**20.8**	**8.0**	**7.2**	**1050**				**20.8**	**8.0**	**7.6**	**1169**		**20.8**	**8.0**	**7.4**	**1110**	
	Mean:			**99.8**	**20.8**	**8.0**	**6.9**	**1016**			**90.9**	**20.8**	**8.0**	**7.3**	**1091**	**95.3**	**20.8**	**8.0**	**7.1**	**1053**	**908.638**
	SEM:			**0.23**	**0.07**	**0.03**	**0.12**	**28.82**			**0.63**	**0.07**	**0.03**	**0.10**	**15.29**	**0.34**	**0.07**	**0.03**	**0.10**	**20.14**	**56.02**
	% CV:			**10.67**	**7.27**	**4.56**	**20.88**	**404.30**			**30.86**	**7.35**	**4.41**	**17.25**	**207.11**	**16.38**	**7.28**	**4.36**	**17.12**	**277.59**	**371.70**

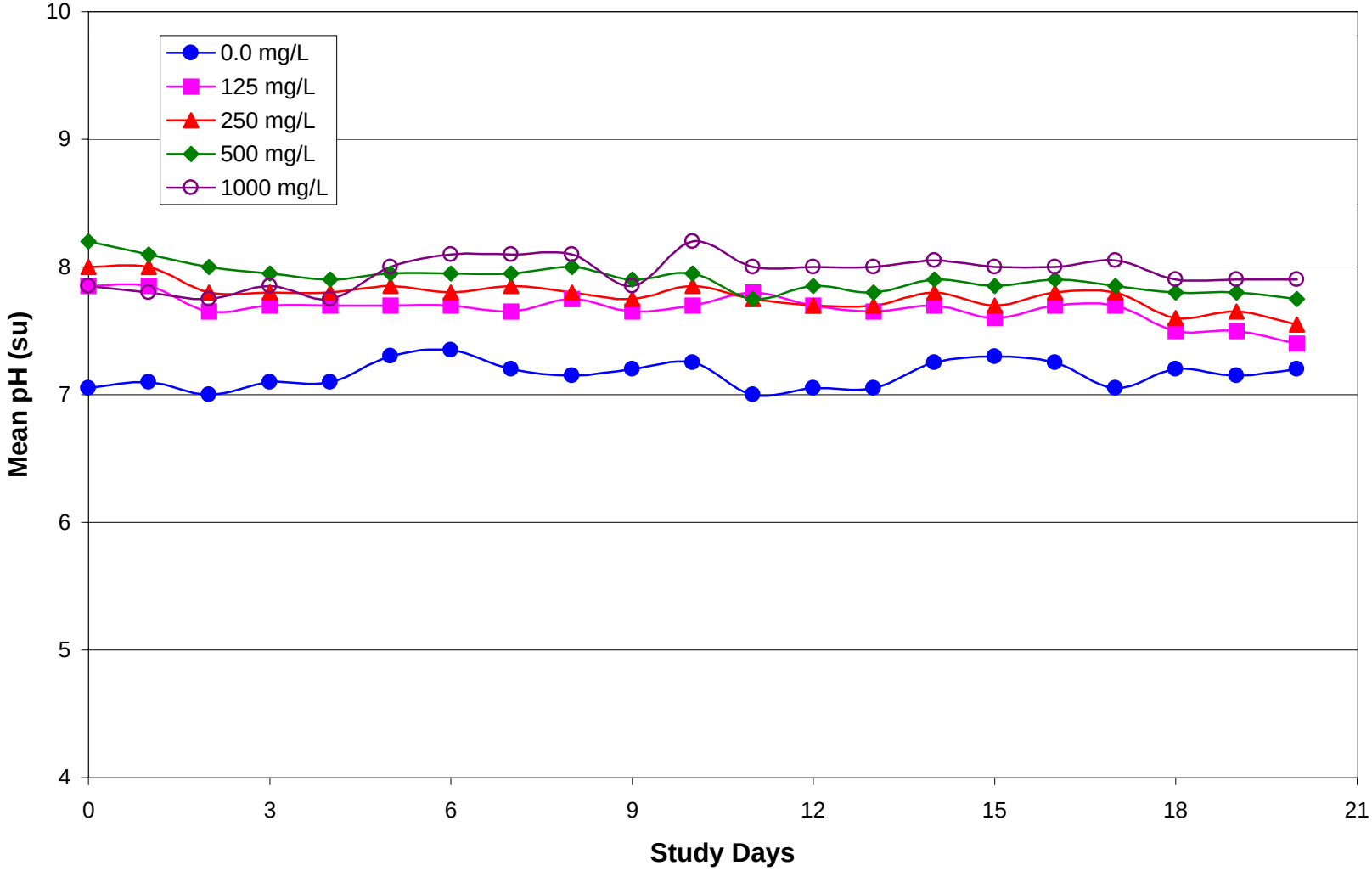
BATT02-00056
Phenobarbital - Renewal
Daily Average of Larval Survival for Initial Stage 51 Test



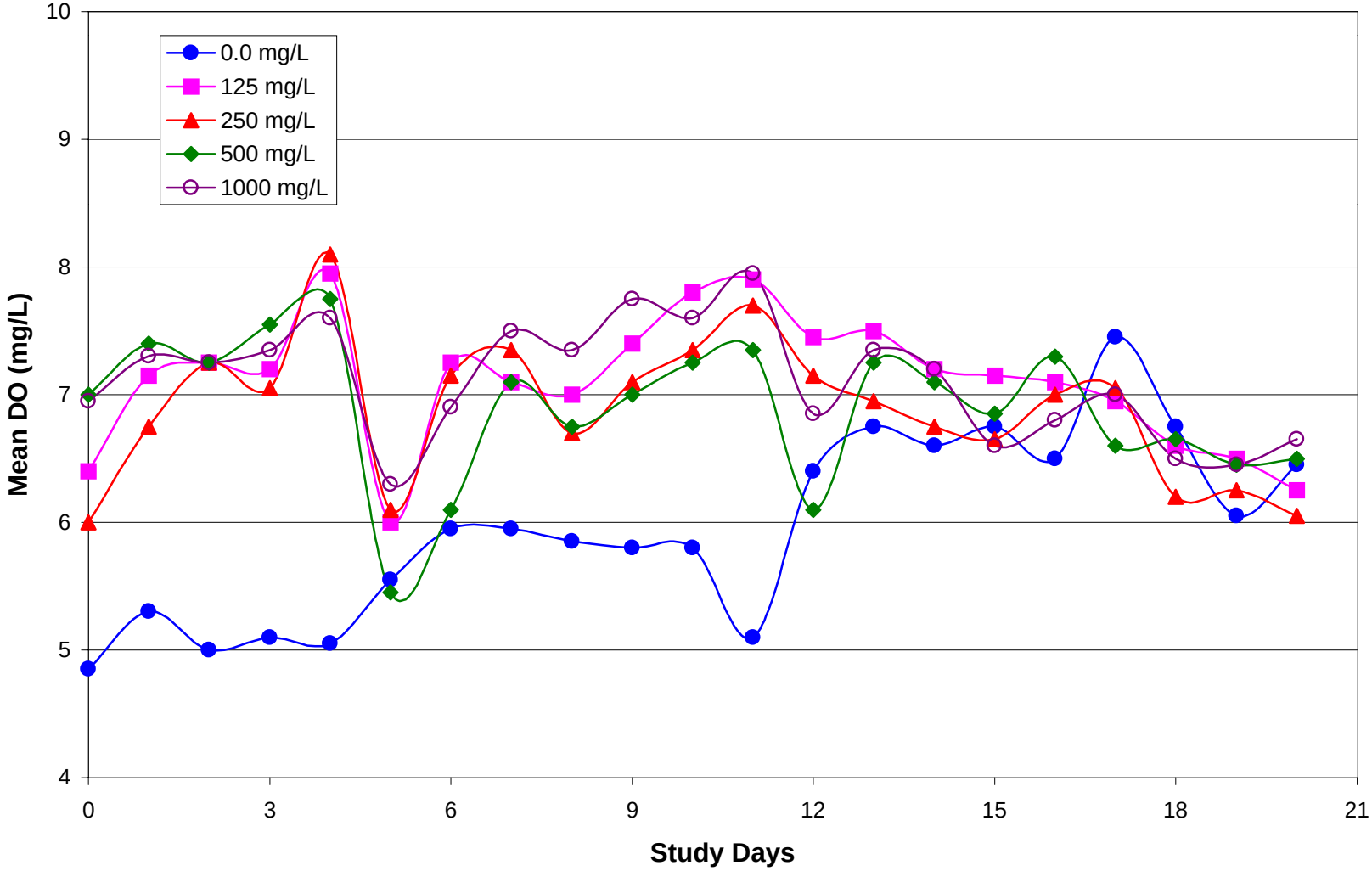
BATT02-00056
Phenobarbital - Renewal
Daily Average Tank Temperatures for Initial Stage 51 Test



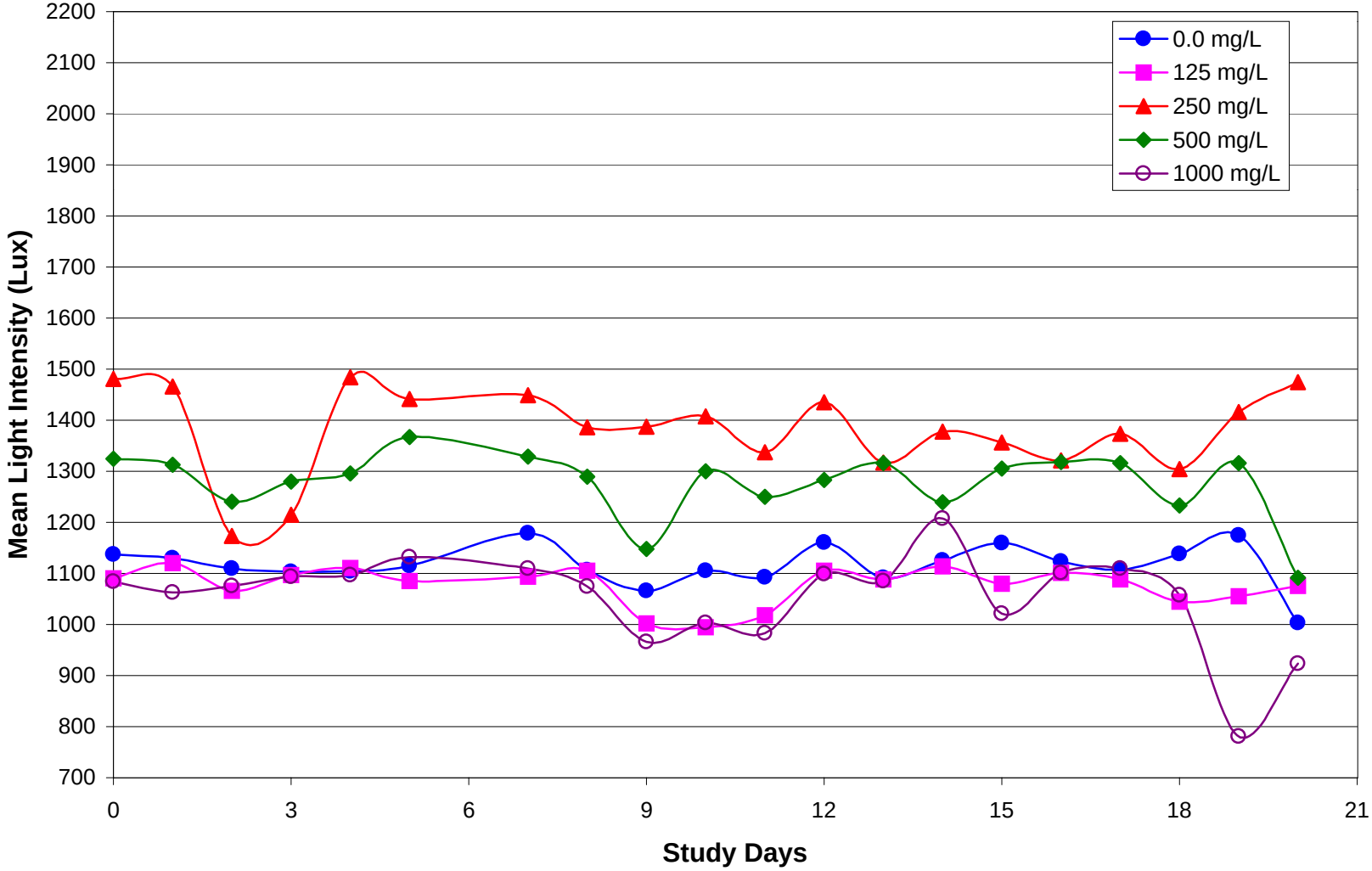
BATT02-00056
Phenobarbital - Renewal
Daily Average pH for Initial Stage 51 Test



BATT02-00056
Phenobarbital - Renewal
Daily Average Dissolved Oxygen (DO) for Initial Stage 51 Test



BATT02-00056
Phenobarbital - Renewal
Daily Average Light Intensity for Initial Stage 51 Test



DAILY DATA SPENT

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 0.0 mg/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	08/27/04		20	100.0	20.1	7.5	7.8	1070		20	100.0	20.1	7.5	7.7	1190	100.0	20.1	7.5	7.8	1130	
2	08/28/04		20	100.0	20.1	7.6	7.9	1085		20	100.0	20.2	7.6	7.7	1134	100.0	20.2	7.6	7.8	1110	
3	08/29/04		20	100.0	20.2	7.6	7.8	1080		20	100.0	20.2	7.6	8.0	1127	100.0	20.2	7.6	7.9	1104	
4	08/30/04		20	100.0	20.0	7.5	8.1	1110		20	100.0	20.0	7.5	7.9	1099	100.0	20.0	7.5	8.0	1105	
5	08/31/04		20	100.0	20.1	7.6	7.9	1090		20	100.0	20.1	7.6	8.1	1140	100.0	20.1	7.6	8.0	1115	
6	09/01/04		20	100.0	20.3	7.3	7.8	-		20	100.0	20.2	7.3	8.0	-	100.0	20.3	7.3	7.9	-	
7	09/02/04		20	100.0	20.0	7.3	7.7	1177		20	100.0	20.0	7.4	8.0	1180	100.0	20.0	7.4	7.9	1179	
8	09/03/04		20	100.0	20.2	7.2	7.6	1066		20	100.0	20.2	7.3	7.9	1146	100.0	20.2	7.3	7.8	1106	
9	09/04/04		20	100.0	20.1	7.2	7.9	1029		20	100.0	20.1	7.3	7.5	1103	100.0	20.1	7.3	7.7	1066	
10	09/05/04		20	100.0	20.2	7.2	7.9	1002		20	100.0	20.2	7.4	7.8	1209	100.0	20.2	7.3	7.9	1106	
11	09/06/04		20	100.0	20.3	7.5	7.5	1081		20	100.0	20.3	7.6	7.9	1105	100.0	20.3	7.6	7.7	1093	
12	09/07/04		20	100.0	20.1	7.4	7.2	1150		20	100.0	20.1	7.4	7.4	1172	100.0	20.1	7.4	7.3	1161	
13	09/08/04		20	100.0	20.0	7.3	7.4	1070		20	100.0	20.0	7.4	7.7	1113	100.0	20.0	7.4	7.6	1092	
14	09/09/04		20	100.0	20.0	7.4	7.4	1088		20	100.0	20.0	7.4	7.7	1163	100.0	20.0	7.4	7.6	1126	
15	09/10/04		20	100.0	20.0	7.4	7.4	1129		20	100.0	20.0	7.4	7.8	1190	100.0	20.0	7.4	7.6	1160	
16	09/11/04		20	100.0	20.0	7.4	7.5	1092		20	100.0	20.0	7.2	7.6	1154	100.0	20.0	7.3	7.6	1123	
17	09/12/04		20	100.0	20.0	7.4	7.4	1084		20	100.0	20.0	7.4	7.7	1131	100.0	20.0	7.4	7.6	1108	
18	09/13/04		20	100.0	20.6	7.3	6.6	1124		20	100.0	20.6	7.3	7.2	1153	100.0	20.6	7.3	6.9	1139	
19	09/14/04		20	100.0	20.9	7.3	6.9	1185		20	100.0	20.8	7.4	7.4	1165	100.0	20.9	7.4	7.2	1175	
20	09/15/04		20	100.0	20.9	7.4	6.7	1015		20	100.0	20.9	7.4	7.3	991	100.0	20.9	7.4	7.0	1003	
21	09/16/04		20	100.0	21.5	7.5	7.8	-		20	100.0	21.4	7.5	7.3	-	100.0	21.5	7.5	7.6	-	
	Median:				**20.1**	**7.4**	**7.6**	**1085**				**20.1**	**7.4**	**7.7**	**1146**		**20.1**	**7.4**	**7.7**	**1110**	
	Mode:				**20.0**	**7.4**	**7.8**	**1070**				**20.0**	**7.4**	**7.7**	**1190**		**20.0**	**7.4**	**7.6**	**#N/A**	
	Mean:			**100.0**	**20.3**	**7.4**	**7.5**	**1091**			**100.0**	**20.3**	**7.4**	**7.7**	**1140**	**100.0**	**20.3**	**7.4**	**7.6**	**1116**	
	SEM:			**0.00**	**0.09**	**0.03**	**0.09**	**11.08**			**0.00**	**0.08**	**0.02**	**0.06**	**11.01**	**0.00**	**0.08**	**0.03**	**0.07**	**9.23**	
	% CV:			**0.00**	**8.73**	**4.72**	**14.86**	**146.20**			**0.00**	**8.18**	**4.17**	**9.43**	**142.07**	**0.00**	**8.44**	**4.21**	**11.11**	**120.50**	

DAILY DATA SPENT

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 125 mg/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES				
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)
		Initial No.= 20							**Initial No.= 20**											
1	08/27/04		20	100.0	20.0	7.7	7.7	1240		20	100.0	20.0	7.6	7.4	1000	100.0	20.0	7.7	7.6	1120
2	08/28/04		20	100.0	20.1	7.7	7.8	1162		20	100.0	20.1	7.6	7.7	970	100.0	20.1	7.7	7.8	1066
3	08/29/04		20	100.0	20.2	7.6	7.5	1184		20	100.0	20.1	7.7	7.6	1010	100.0	20.2	7.7	7.6	1097
4	08/30/04		20	100.0	20.0	7.6	7.9	1190		20	100.0	20.0	7.6	8.0	1032	100.0	20.0	7.6	8.0	1111
5	08/31/04		20	100.0	20.1	7.6	7.8	1180		20	100.0	20.1	7.5	7.7	990	100.0	20.1	7.6	7.8	1085
6	09/01/04		20	100.0	20.0	7.6	7.9	-		20	100.0	20.1	7.6	8.3	-	100.0	20.1	7.6	8.1	-
7	09/02/04		20	100.0	20.0	7.6	7.3	1195		20	100.0	20.0	7.6	8.0	992	100.0	20.0	7.6	7.7	1094
8	09/03/04		20	100.0	20.2	7.6	7.2	1200		20	100.0	20.1	7.6	8.0	1011	100.0	20.2	7.6	7.6	1106
9	09/04/04		20	100.0	20.1	7.6	7.6	1134		20	100.0	20.1	7.6	6.7	870	100.0	20.1	7.6	7.2	1002
10	09/05/04		20	100.0	20.1	7.6	7.7	1092		20	100.0	20.1	7.7	7.5	897	100.0	20.1	7.7	7.6	995
11	09/06/04		20	100.0	20.0	7.6	7.7	1120		20	100.0	20.1	7.7	8.2	917	100.0	20.1	7.7	8.0	1019
12	09/07/04		20	100.0	20.0	7.5	7.2	1010		20	100.0	20.0	7.6	8.0	1201	100.0	20.0	7.6	7.6	1106
13	09/08/04	1	19	95.0	20.0	7.6	7.4	1239		20	100.0	20.0	7.7	8.0	938	97.5	20.0	7.7	7.7	1089
14	09/09/04		19	95.0	20.0	7.6	8.0	1228		20	100.0	20.0	7.7	8.0	999	97.5	20.0	7.7	8.0	1114
15	09/10/04		19	95.0	20.0	7.6	7.5	1180		20	100.0	20.0	7.6	7.9	980	97.5	20.0	7.6	7.7	1080
16	09/11/04		19	95.0	20.2	7.6	7.7	1227		20	100.0	20.1	7.6	7.9	975	97.5	20.2	7.6	7.8	1101
17	09/12/04		19	95.0	20.0	7.6	8.0	1210		20	100.0	20.0	7.7	7.7	967	97.5	20.0	7.7	7.9	1089
18	09/13/04		19	95.0	20.6	7.4	6.4	1127		20	100.0	20.6	7.5	7.5	962	97.5	20.6	7.5	7.0	1045
19	09/14/04		19	95.0	20.7	7.4	6.4	1168		20	100.0	20.8	7.5	7.2	942	97.5	20.8	7.5	6.8	1055
20	09/15/04		19	95.0	20.8	7.4	6.4	1167		20	100.0	20.8	7.5	7.2	984	97.5	20.8	7.5	6.8	1076
21	09/16/04		19	95.0	21.2	7.4	7.0	-		20	100.0	21.1	7.5	7.6	-	97.5	21.2	7.5	7.3	-
	Median:				**20.1**	**7.6**	**7.6**	**1180**				**20.1**	**7.6**	**7.7**	**980**		**20.1**	**7.6**	**7.7**	**1089**
	Mode:				**20.0**	**7.6**	**7.7**	**1180**				**20.1**	**7.6**	**8.0**	**#N/A**		**20.0**	**7.7**	**7.6**	**1106**
	Mean:			**97.9**	**20.2**	**7.6**	**7.4**	**1171**			**100.0**	**20.2**	**7.6**	**7.7**	**981**	**98.9**	**20.2**	**7.6**	**7.6**	**1076**
	SEM:			**0.55**	**0.07**	**0.02**	**0.11**	**12.97**			**0.00**	**0.07**	**0.02**	**0.08**	**15.41**	**0.28**	**0.07**	**0.02**	**0.08**	**8.56**
	% CV:			**25.63**	**7.39**	**3.32**	**18.68**	**165.26**			**0.00**	**7.21**	**2.68**	**13.73**	**214.45**	**12.75**	**7.27**	**2.70**	**13.63**	**113.72**

DAILY DATA SPENT

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 250 mg/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES				
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)
		Initial No.= 20							**Initial No.= 20**											
1	08/27/04		20	100.0	20.0	7.8	8.0	1460		20	100.0	20.0	7.8	6.7	1472	100.0	20.0	7.8	7.4	1466
2	08/28/04		20	100.0	20.2	7.8	7.8	1155		20	100.0	20.2	7.7	7.0	1191	100.0	20.2	7.8	7.4	1173
3	08/29/04		20	100.0	20.2	7.7	7.0	1205		20	100.0	20.1	7.8	7.3	1224	100.0	20.2	7.8	7.2	1215
4	08/30/04		20	100.0	20.0	7.7	8.2	1525		20	100.0	20.0	7.7	8.2	1444	100.0	20.0	7.7	8.2	1485
5	08/31/04		20	100.0	20.1	7.6	7.1	1463		20	100.0	20.1	7.6	7.8	1419	100.0	20.1	7.6	7.5	1441
6	09/01/04		20	100.0	20.1	7.6	7.4	-		20	100.0	20.2	7.7	8.1	-	100.0	20.2	7.7	7.8	-
7	09/02/04		20	100.0	20.0	7.6	7.3	1445		20	100.0	20.0	7.7	8.1	1453	100.0	20.0	7.7	7.7	1449
8	09/03/04		20	100.0	20.2	7.6	7.0	1463		20	100.0	20.2	7.7	8.0	1309	100.0	20.2	7.7	7.5	1386
9	09/04/04		20	100.0	20.2	7.6	6.9	1401		20	100.0	20.1	7.7	7.0	1374	100.0	20.2	7.7	7.0	1388
10	09/05/04		20	100.0	20.1	7.8	7.7	1425		20	100.0	20.3	7.8	6.9	1390	100.0	20.2	7.8	7.3	1408
11	09/06/04		20	100.0	20.1	7.6	7.6	1401		20	100.0	20.2	7.7	7.9	1273	100.0	20.2	7.7	7.8	1337
12	09/07/04		20	100.0	20.0	7.6	7.5	1427		20	100.0	20.1	7.6	7.1	1443	100.0	20.1	7.6	7.3	1435
13	09/08/04		20	100.0	20.0	7.7	7.6	1267		20	100.0	20.0	7.6	7.4	1368	100.0	20.0	7.7	7.5	1318
14	09/09/04		20	100.0	20.0	7.7	7.8	1421		20	100.0	20.0	7.7	7.6	1334	100.0	20.0	7.7	7.7	1378
15	09/10/04		20	100.0	20.0	7.7	7.5	1373		20	100.0	20.0	7.7	7.7	1339	100.0	20.0	7.7	7.6	1356
16	09/11/04		20	100.0	20.1	7.7	7.8	1272		20	100.0	20.1	7.8	7.3	1370	100.0	20.1	7.8	7.6	1321
17	09/12/04		20	100.0	20.0	7.7	7.5	1415		20	100.0	20.0	7.7	7.7	1332	100.0	20.0	7.7	7.6	1374
18	09/13/04		20	100.0	20.6	7.5	6.6	1323		20	100.0	20.6	7.5	7.1	1286	100.0	20.6	7.5	6.9	1305
19	09/14/04		20	100.0	20.8	7.5	6.4	1428		20	100.0	20.9	7.5	6.6	1403	100.0	20.9	7.5	6.5	1416
20	09/15/04		20	100.0	20.8	7.5	6.5	1508		20	100.0	20.9	7.5	6.7	1440	100.0	20.9	7.5	6.6	1474
21	09/16/04		20	100.0	21.3	7.4	6.7	-		20	100.0	21.3	7.5	6.7	-	100.0	21.3	7.5	6.7	-
	Median:				**20.1**	**7.6**	**7.5**	**1421**				**20.1**	**7.7**	**7.3**	**1370**		**20.2**	**7.7**	**7.5**	**1386**
	Mode:				**20.0**	**7.7**	**7.8**	**1463**				**20.0**	**7.7**	**6.7**	**#N/A**		**20.0**	**7.7**	**7.8**	**#N/A**
	Mean:			**100.0**	**20.2**	**7.6**	**7.3**	**1388**			**100.0**	**20.3**	**7.7**	**7.4**	**1361**	**100.0**	**20.2**	**7.7**	**7.4**	**1375**
	SEM:			**0.00**	**0.08**	**0.02**	**0.11**	**22.93**			**0.00**	**0.08**	**0.02**	**0.11**	**18.15**	**0.00**	**0.08**	**0.02**	**0.09**	**19.20**
	% CV:			**0.00**	**7.77**	**3.88**	**18.80**	**268.26**			**0.00**	**8.06**	**3.67**	**19.27**	**214.47**	**0.00**	**7.88**	**3.59**	**15.80**	**225.69**

DAILY DATA SPENT

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 500 mg/L**

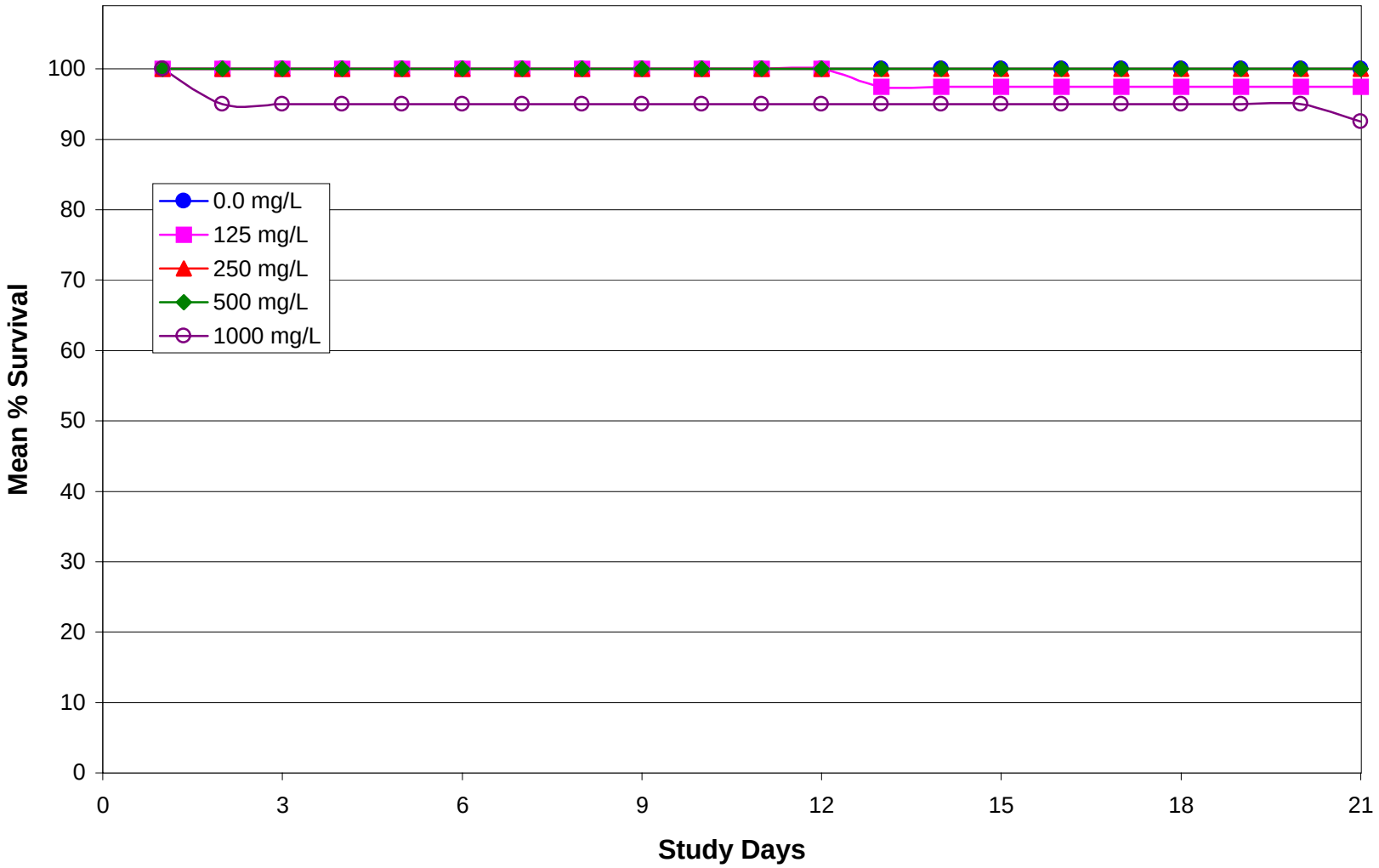
		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	08/27/04		20	100.0	20.1	8.0	7.7	1233		20	100.0	20.1	8.0	7.2	1393	100.0	20.1	8.0	7.5	1313	
2	08/28/04		20	100.0	20.2	7.9	7.4	1130		20	100.0	20.2	7.9	6.8	1351	100.0	20.2	7.9	7.1	1241	
3	08/29/04		20	100.0	20.1	7.8	7.7	1150		20	100.0	20.3	7.9	7.9	1410	100.0	20.2	7.9	7.8	1280	
4	08/30/04		20	100.0	20.0	7.7	7.5	1254		20	100.0	20.0	7.8	7.4	1337	100.0	20.0	7.8	7.5	1296	
5	08/31/04		20	100.0	20.1	7.7	7.3	1374		20	100.0	20.1	7.7	6.4	1360	100.0	20.1	7.7	6.9	1367	
6	09/01/04		20	100.0	20.2	7.8	7.3	-		20	100.0	20.2	7.7	5.4	-	100.0	20.2	7.8	6.4	-	
7	09/02/04		20	100.0	20.1	7.8	7.1	1253		20	100.0	20.1	7.8	6.9	1405	100.0	20.1	7.8	7.0	1329	
8	09/03/04		20	100.0	20.2	7.8	6.9	1216		20	100.0	20.1	7.8	7.9	1362	100.0	20.2	7.8	7.4	1289	
9	09/04/04		20	100.0	20.3	7.8	6.5	1048		20	100.0	20.2	7.8	6.6	1248	100.0	20.3	7.8	6.6	1148	
10	09/05/04		20	100.0	20.2	7.7	7.1	1276		20	100.0	20.2	7.8	7.6	1324	100.0	20.2	7.8	7.4	1300	
11	09/06/04		20	100.0	20.2	7.6	6.8	1168		20	100.0	20.2	7.8	7.7	1333	100.0	20.2	7.7	7.3	1251	
12	09/07/04		20	100.0	20.1	7.7	6.9	1229		20	100.0	20.1	7.7	7.0	1337	100.0	20.1	7.7	7.0	1283	
13	09/08/04		20	100.0	20.0	7.7	7.1	1279		20	100.0	20.0	7.7	7.1	1352	100.0	20.0	7.7	7.1	1316	
14	09/09/04		20	100.0	20.0	7.8	7.7	1224		20	100.0	20.0	7.8	8.0	1254	100.0	20.0	7.8	7.9	1239	
15	09/10/04		20	100.0	20.0	7.8	7.7	1271		20	100.0	20.0	7.8	8.0	1340	100.0	20.0	7.8	7.9	1306	
16	09/11/04		20	100.0	20.0	7.8	7.7	1288		20	100.0	20.2	7.8	7.9	1348	100.0	20.1	7.8	7.8	1318	
17	09/12/04		20	100.0	20.0	7.9	7.6	1278		20	100.0	20.0	7.8	7.6	1354	100.0	20.0	7.9	7.6	1316	
18	09/13/04		20	100.0	20.6	7.7	7.3	1149		20	100.0	20.6	7.7	7.5	1318	100.0	20.6	7.7	7.4	1234	
19	09/14/04		20	100.0	20.7	7.7	7.3	1286		20	100.0	20.8	7.7	7.1	1345	100.0	20.8	7.7	7.2	1316	
20	09/15/04		20	100.0	20.9	7.7	6.7	1050		20	100.0	20.9	7.7	7.2	1132	100.0	20.9	7.7	7.0	1091	
21	09/16/04		20	100.0	21.3	7.6	6.5	-		20	100.0	21.3	7.7	7.4	-	100.0	21.3	7.7	7.0	-	
	Median:				**20.1**	**7.8**	**7.3**	**1233**				**20.2**	**7.8**	**7.4**	**1345**		**20.2**	**7.8**	**7.3**	**1296**	
	Mode:				**20.0**	**7.8**	**7.7**	**#N/A**				**20.2**	**7.8**	**7.9**	**1337**		**20.1**	**7.7**	**7.0**	**1316**	
	Mean:			**100.0**	**20.3**	**7.8**	**7.2**	**1219**			**100.0**	**20.3**	**7.8**	**7.3**	**1332**	**100.0**	**20.3**	**7.8**	**7.2**	**1275**	
	SEM:			**0.00**	**0.07**	**0.02**	**0.09**	**19.27**			**0.00**	**0.08**	**0.02**	**0.14**	**14.51**	**0.00**	**0.07**	**0.02**	**0.09**	**14.97**	
	% CV:			**0.00**	**7.62**	**3.49**	**14.79**	**240.61**			**0.00**	**7.67**	**2.92**	**23.38**	**173.31**	**0.00**	**7.60**	**2.98**	**15.37**	**182.78**	

DAILY DATA SPENT

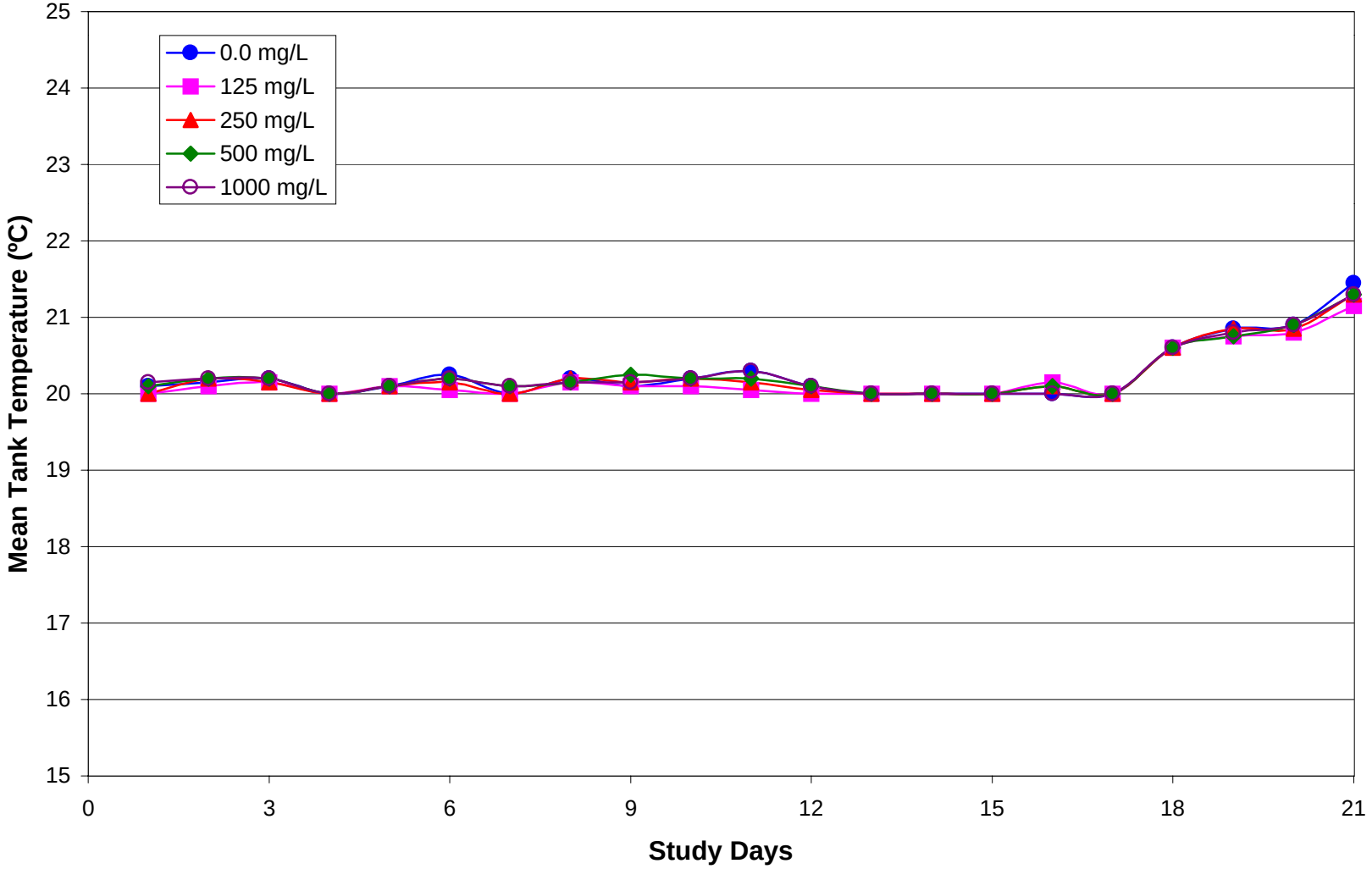
Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 1000 mg/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	08/27/04		20	100.0	20.1	7.7	6.1	1013		20	100.0	20.2	7.8	8.2	1112	100.0	20.2	7.8	7.2	1063	
2	08/28/04		20	100.0	20.2	7.7	6.3	1057	2	18	90.0	20.2	7.8	8.1	1095	95.0	20.2	7.8	7.2	1076	
3	08/29/04		20	100.0	20.2	7.9	7.6	1080		18	90.0	20.2	7.8	7.9	1107	95.0	20.2	7.9	7.8	1094	
4	08/30/04		20	100.0	20.0	7.6	6.4	1070		18	90.0	20.0	7.7	8.2	1123	95.0	20.0	7.7	7.3	1097	
5	08/31/04		20	100.0	20.1	7.6	7.2	1110		18	90.0	20.1	7.7	8.1	1153	95.0	20.1	7.7	7.7	1132	
6	09/01/04		20	100.0	20.2	7.8	7.3	-		18	90.0	20.2	7.9	8.2	-	95.0	20.2	7.9	7.8	-	
7	09/02/04		20	100.0	20.1	8.0	6.6	1050		18	90.0	20.1	8.0	8.2	1169	95.0	20.1	8.0	7.4	1110	
8	09/03/04		20	100.0	20.1	8.0	8.0	1050		18	90.0	20.2	8.0	8.2	1100	95.0	20.2	8.0	8.1	1075	
9	09/04/04		20	100.0	20.2	7.7	7.9	921		18	90.0	20.1	7.9	8.2	1010	95.0	20.2	7.8	8.1	966	
10	09/05/04		20	100.0	20.3	8.1	7.5	969		18	90.0	20.1	8.2	6.7	1037	95.0	20.2	8.2	7.1	1003	
11	09/06/04		20	100.0	20.3	7.9	7.8	986		18	90.0	20.3	7.9	8.2	981	95.0	20.3	7.9	8.0	984	
12	09/07/04		20	100.0	20.1	7.9	6.5	1061		18	90.0	20.1	7.9	7.9	1136	95.0	20.1	7.9	7.2	1099	
13	09/08/04		20	100.0	20.0	7.9	7.2	1031		18	90.0	20.0	7.9	7.9	1139	95.0	20.0	7.9	7.6	1085	
14	09/09/04		20	100.0	20.0	7.9	7.5	1236		18	90.0	20.0	7.9	8.0	1179	95.0	20.0	7.9	7.8	1208	
15	09/10/04		20	100.0	20.0	7.9	7.5	1033		18	90.0	20.0	7.9	7.9	1010	95.0	20.0	7.9	7.7	1022	
16	09/11/04		20	100.0	20.0	7.9	8.2	1033		18	90.0	20.0	7.8	7.6	1170	95.0	20.0	7.9	7.9	1102	
17	09/12/04		20	100.0	20.0	7.8	8.1	1050		18	90.0	20.0	7.8	7.8	1169	95.0	20.0	7.8	8.0	1110	
18	09/13/04		20	100.0	20.6	7.9	6.8	1030		18	90.0	20.6	7.9	7.3	1084	95.0	20.6	7.9	7.1	1057	
19	09/14/04		20	100.0	20.8	7.8	6.9	561		18	90.0	20.8	7.8	7.5	1001	95.0	20.8	7.8	7.2	781	
20	09/15/04		20	100.0	20.9	7.8	6.5	883		18	90.0	20.9	7.8	7.5	964	95.0	20.9	7.8	7.0	924	
21	09/16/04	1	19	95.0	21.3	7.8	7.5	-		18	90.0	21.3	7.8	7.7	-	92.5	21.3	7.8	7.6	-	
	Median:				**20.1**	**7.9**	**7.3**	**1033**				**20.1**	**7.9**	**7.9**	**1107**		**20.2**	**7.9**	**7.6**	**1076**	
	Mode:				**20.0**	**7.9**	**7.5**	**1050**				**20.0**	**7.8**	**8.2**	**1169**		**20.0**	**7.9**	**7.8**	**1110**	
	Mean:			**99.8**	**20.3**	**7.8**	**7.2**	**1012**			**90.5**	**20.3**	**7.9**	**7.9**	**1092**	**95.1**	**20.3**	**7.9**	**7.5**	**1052**	
	SEM:			**0.24**	**0.08**	**0.03**	**0.14**	**30.06**			**0.48**	**0.08**	**0.02**	**0.08**	**16.09**	**0.27**	**0.08**	**0.02**	**0.08**	**21.17**	
	% CV:			**10.92**	**7.77**	**4.58**	**23.67**	**411.87**			**22.94**	**7.78**	**3.96**	**13.72**	**212.30**	**12.76**	**7.75**	**4.09**	**13.02**	**284.50**	

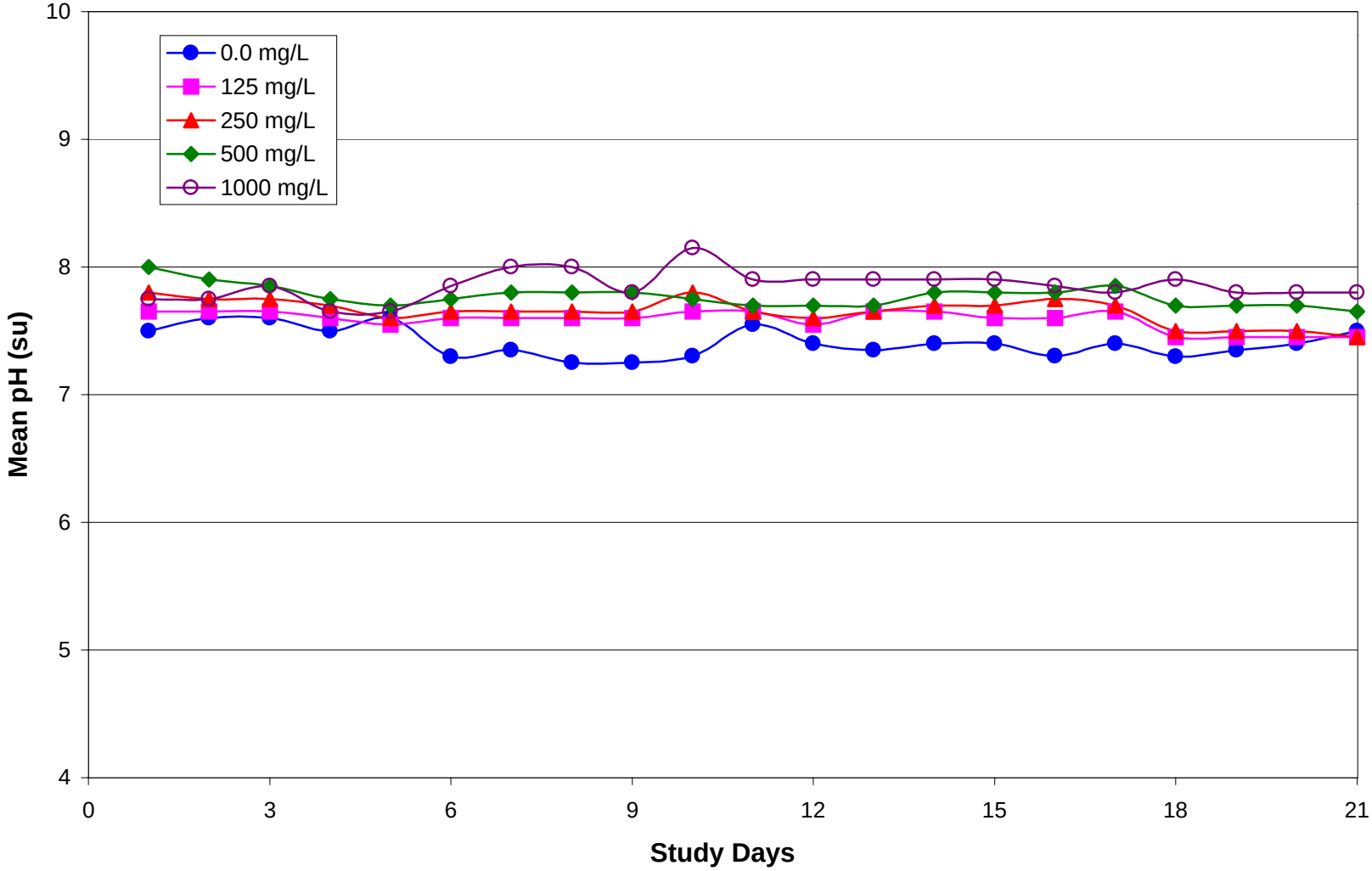
BATT02-00056
Phenobarbital - Spent
Daily Average of Larval Survival for Initial Stage 51 Test



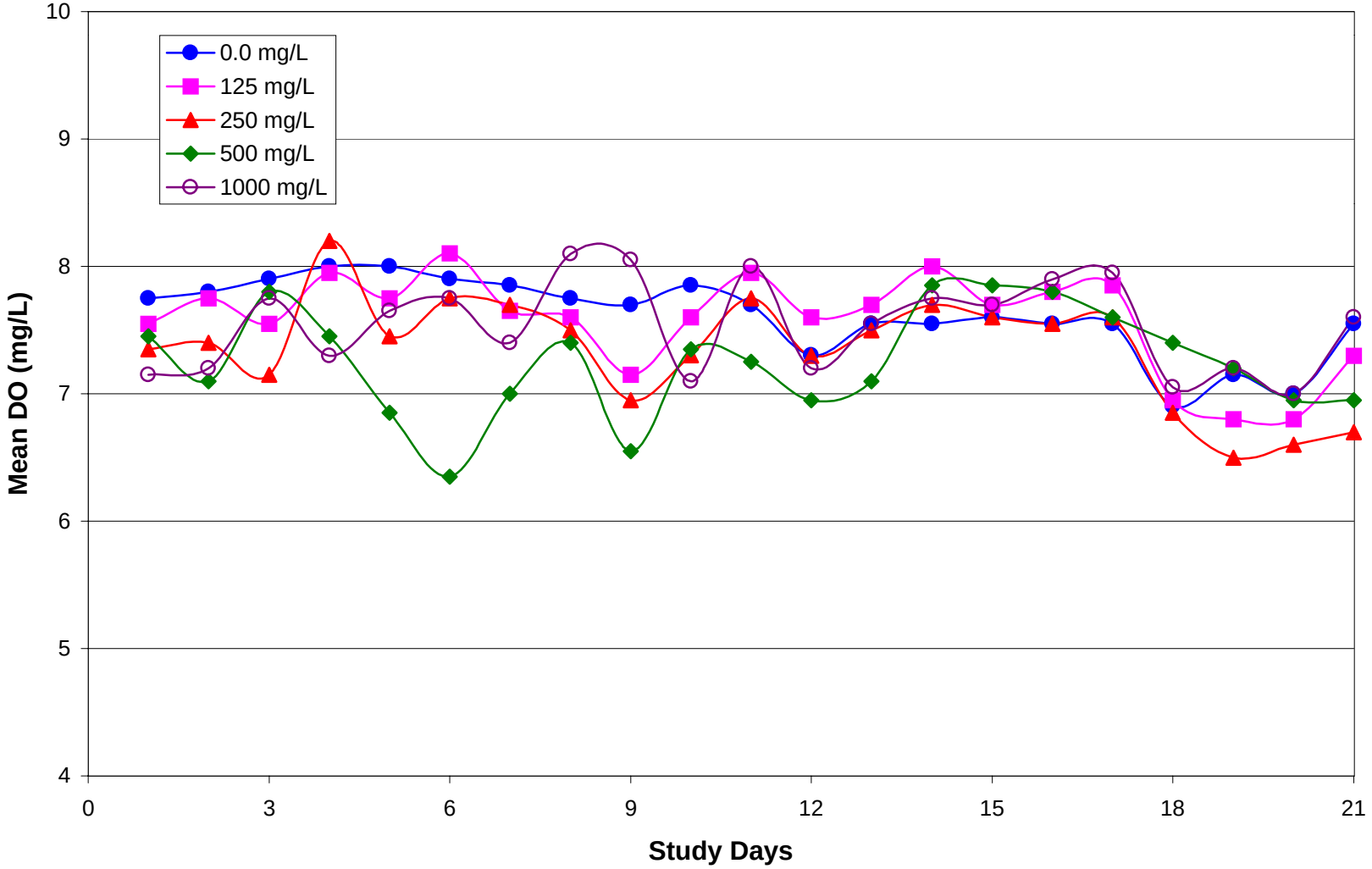
BATT02-00056
Phenobarbital - Spent
Daily Average Tank Temperatures for Initial Stage 51 Test



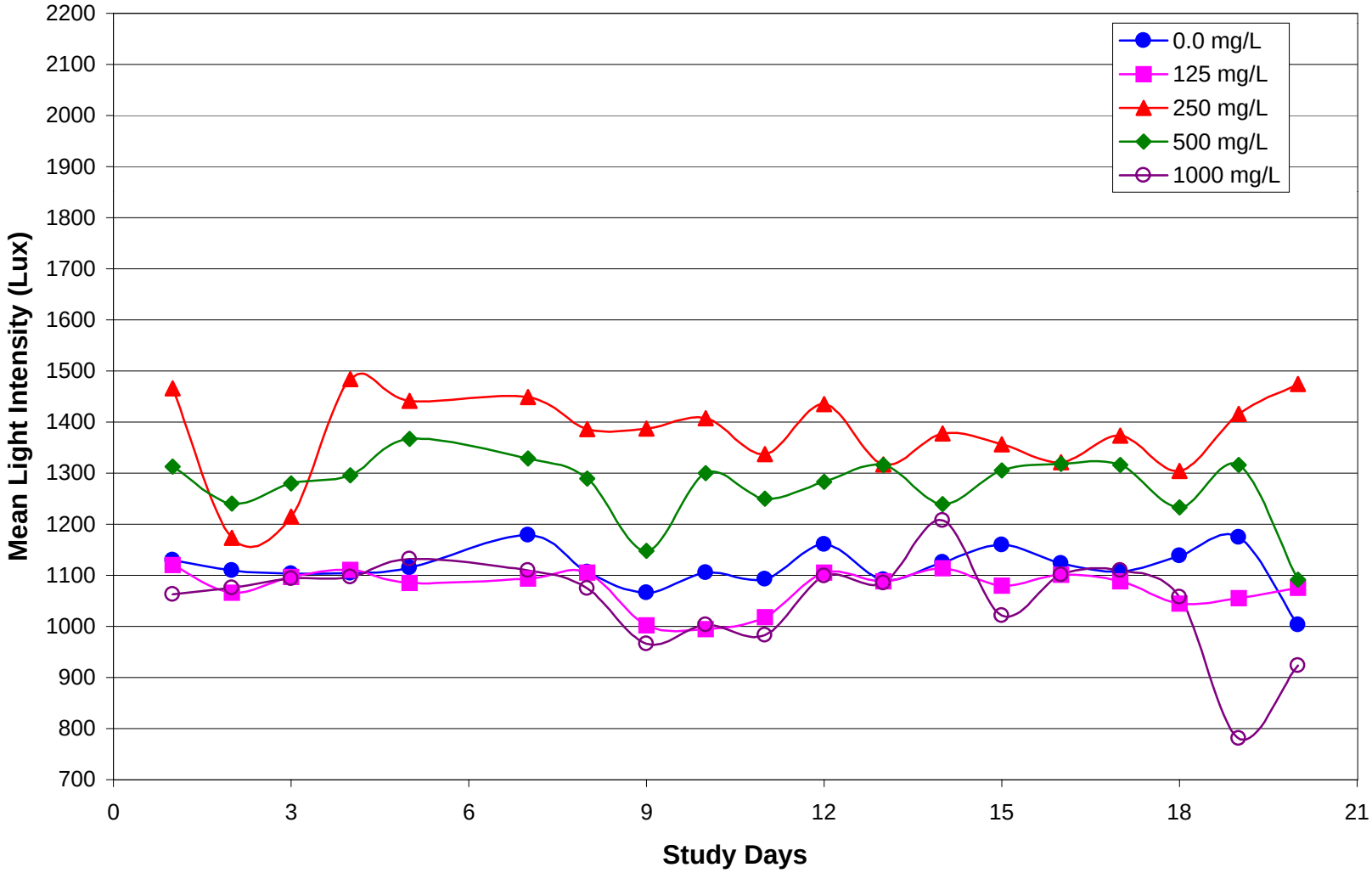
BATT02-00056
Phenobarbital - Spent
Daily Average pH for Initial Stage 51 Test



BATT02-00056
Phenobarbital - Spent
Daily Average Dissolved Oxygen (DO) for Initial Stage 51 Test



BATT02-00056
Phenobarbital - Spent
Daily Average Light Intensity for Initial Stage 51 Test



WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA RENEW

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 0.0 mg/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					Average Measured
		Daily	Cumul.		Tank			Light	Daily	Cumul.		Tank			Light		Tank			Light	Test
Study Day	Study Date	No. Dead	No. Alive	% Survival	Temp (°C)	pH (su)	DO (mg/L)	Intensity (Lux)	No. Dead	No. Alive	% Survival	Temp (°C)	pH (su)	DO (mg/L)	Intensity (Lux)	% Survival	Temp (°C)	pH (su)	DO (mg/L)	Intensity (Lux)	Conc[1] (mg/L)
		Initial No.= 20							**Initial No.= 20**												
0	09/01/04			100.0	21.0	7.0	5.6	-			100.0	21.0	7.0	5.3	-	100.0	21.0	7.0	5.5	-	0.25
1	09/02/04		20	100.0	20.8	7.4	7.3	1149		20	100.0	20.7	7.4	6.0	1254	100.0	20.8	7.4	6.7	1202	
2	09/03/04		20	100.0	20.6	7.3	6.8	1148		20	100.0	20.6	7.3	5.2	1160	100.0	20.6	7.3	6.0	1154	
3	09/04/04		20	100.0	20.5	7.3	5.2	1078		20	100.0	20.6	7.3	5.6	1140	100.0	20.6	7.3	5.4	1109	
4	09/05/04		20	100.0	20.6	7.2	7.7	1248		20	100.0	20.5	7.2	7.8	1164	100.0	20.6	7.2	7.8	1206	
5	09/06/04		20	100.0	20.6	7.1	5.8	1227		20	100.0	20.6	7.1	4.3	1182	100.0	20.6	7.1	5.1	1205	
6	09/07/04		20	100.0	20.4	7.2	7.8	1220		20	100.0	20.4	7.3	7.0	1257	100.0	20.4	7.3	7.4	1239	
7	09/08/04		20	100.0	20.3	7.3	7.6	1152		20	100.0	20.3	7.2	6.4	1356	100.0	20.3	7.3	7.0	1254	
8	09/09/04		20	100.0	20.7	7.5	7.4	1030		20	100.0	20.7	7.5	7.4	1100	100.0	20.7	7.5	7.4	1065	0.24
9	09/10/04		20	100.0	20.4	7.5	7.9	1235		20	100.0	20.4	7.5	7.8	1240	100.0	20.4	7.5	7.9	1238	
10	09/11/04		20	100.0	20.5	7.3	6.6	1047		20	100.0	20.4	7.3	6.5	1255	100.0	20.5	7.3	6.6	1151	
11	09/12/04		20	100.0	20.4	7.2	7.4	1135		20	100.0	20.4	7.2	7.6	1286	100.0	20.4	7.2	7.5	1211	
12	09/13/04		20	100.0	21.5	7.2	7.3	1242		20	100.0	21.5	7.2	6.6	1282	100.0	21.5	7.2	7.0	1262	
13	09/14/04		20	100.0	21.3	7.5	7.8	1334		20	100.0	21.3	7.5	7.5	1364	100.0	21.3	7.5	7.7	1349	
14	09/15/04		20	100.0						20	100.0					100.0					0.06
	Median:				**20.6**	**7.3**	**7.4**	**1152**				**20.6**	**7.3**	**6.6**	**1254**		**20.6**	**7.3**	**7.0**	**1206**	
	Mode:				**20.6**	**7.3**	**7.3**	**#N/A**				**20.4**	**7.3**	**7.8**	**#N/A**		**20.4**	**7.3**	**7.4**	**#N/A**	
	Mean:			**100.0**	**20.7**	**7.3**	**7.0**	**1173**			**100.0**	**20.7**	**7.3**	**6.5**	**1234**	**100.0**	**20.7**	**7.3**	**6.8**	**1203**	**0.182**
	SEM:			**0.00**	**0.09**	**0.04**	**0.24**	**24.51**			**0.00**	**0.10**	**0.04**	**0.29**	**22.37**	**0.00**	**0.10**	**0.04**	**0.25**	**20.08**	**0.06**
	% CV:			**0.00**	**7.80**	**5.60**	**33.57**	**258.05**			**0.00**	**7.88**	**5.60**	**43.10**	**229.63**	**0.00**	**7.82**	**5.55**	**36.25**	**208.72**	**25.75**

[1]All measured test concentrations reported by Battelle as less than method detection limit (MDL) [0.1112 mg/L] or none detected (ND) are expressed as 0.06 mg/L (1/2 of MDL).

DAILY DATA RENEW

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 250 mg/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc (mg/L)
		Initial No.= 20							**Initial No.= 20**												
0	09/01/04			100.0	21.0	7.9	6.3	-			100.0	21.0	8.0	6.4	-	100.0	21.0	8.0	6.4	-	243.00
1	09/02/04		20	100.0	20.5	7.9	7.5	920		20	100.0	20.5	7.9	7.7	1020	100.0	20.5	7.9	7.6	970	
2	09/03/04		20	100.0	20.7	7.8	6.8	760		20	100.0	20.7	7.9	7.2	974	100.0	20.7	7.9	7.0	867	
3	09/04/04		20	100.0	20.6	8.0	7.2	1039		20	100.0	20.7	8.0	7.6	1140	100.0	20.7	8.0	7.4	1090	
4	09/05/04		20	100.0	20.6	7.8	7.3	1035		20	100.0	20.7	7.7	7.5	1155	100.0	20.7	7.8	7.4	1095	
5	09/06/04		20	100.0	20.5	7.7	7.4	1095		20	100.0	20.5	7.7	7.8	1169	100.0	20.5	7.7	7.6	1132	
6	09/07/04		20	100.0	20.6	7.8	6.2	957		20	100.0	20.9	7.7	6.8	1031	100.0	20.8	7.8	6.5	994	
7	09/08/04		20	100.0	20.4	7.7	6.5	948		20	100.0	20.3	7.7	7.3	967	100.0	20.4	7.7	6.9	958	
8	09/09/04		20	100.0	20.5	7.7	6.6	974		20	100.0	20.8	7.8	7.2	1128	100.0	20.7	7.8	6.9	1051	197.90
9	09/10/04		20	100.0	20.6	7.7	6.4	923		20	100.0	20.7	7.7	6.9	1151	100.0	20.7	7.7	6.7	1037	
10	09/11/04		20	100.0	20.7	7.8	6.8	951		20	100.0	20.6	7.7	7.3	984	100.0	20.7	7.8	7.1	968	
11	09/12/04		20	100.0	20.6	7.8	7.2	938		20	100.0	20.7	7.7	7.3	1047	100.0	20.7	7.8	7.3	993	
12	09/13/04		20	100.0	20.8	7.7	7.6	913		20	100.0	20.8	7.6	7.2	1011	100.0	20.8	7.7	7.4	962	
13	09/14/04		20	100.0	20.9	7.7	7.2	989		20	100.0	21.2	7.7	7.3	1187	100.0	21.1	7.7	7.3	1088	
14	09/15/04		20	100.0						20	100.0					100.0					203.60
	Median:				**20.6**	**7.8**	**7.0**	**951**				**20.7**	**7.7**	**7.3**	**1047**		**20.7**	**7.8**	**7.2**	**994**	
	Mode:				**20.6**	**7.7**	**7.2**	**#N/A**				**20.7**	**7.7**	**7.3**	**#N/A**		**20.7**	**7.8**	**7.4**	**#N/A**	
	Mean:			**100.0**	**20.6**	**7.8**	**6.9**	**957**			**100.0**	**20.7**	**7.8**	**7.3**	**1074**	**100.0**	**20.7**	**7.8**	**7.1**	**1016**	**214.833**
	SEM:			**0.00**	**0.04**	**0.03**	**0.13**	**22.20**			**0.00**	**0.06**	**0.03**	**0.10**	**22.73**	**0.00**	**0.05**	**0.03**	**0.11**	**20.57**	**14.18**
	% CV:			**0.00**	**3.63**	**3.40**	**17.91**	**258.78**			**0.00**	**4.89**	**4.54**	**13.65**	**250.01**	**0.00**	**4.04**	**3.77**	**14.84**	**232.70**	**167.56**

DAILY DATA RENEW

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 500 mg/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D								INITIAL STAGE 54 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)		% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**													
0	09/01/04			100.0	20.9	8.2	6.1	-				100.0	20.9	8.2	6.3	-		100.0	20.9	8.2	6.2	-	496.80
1	09/02/04		20	100.0	20.6	8.0	7.6	1261			20	100.0	20.6	8.1	7.7	1301		100.0	20.6	8.1	7.7	1281	
2	09/03/04		20	100.0	20.8	8.0	7.2	1230			20	100.0	20.8	8.0	7.3	1288		100.0	20.8	8.0	7.3	1259	
3	09/04/04		20	100.0	20.7	8.0	7.5	1220			20	100.0	20.7	8.1	7.8	1266		100.0	20.7	8.1	7.7	1243	
4	09/05/04		20	100.0	20.8	7.9	7.8	1222			20	100.0	20.7	8.0	7.7	1225		100.0	20.8	8.0	7.8	1224	
5	09/06/04		20	100.0	20.5	7.8	7.8	1226			20	100.0	20.5	7.9	8.1	1214		100.0	20.5	7.9	8.0	1220	
6	09/07/04		20	100.0	20.9	7.8	6.5	1275			20	100.0	20.9	7.9	7.6	1308		100.0	20.9	7.9	7.1	1292	
7	09/08/04		20	100.0	20.4	7.8	7.5	1245			20	100.0	20.3	7.9	7.9	1294		100.0	20.4	7.9	7.7	1270	
8	09/09/04		20	100.0	20.8	7.9	7.0	1215			20	100.0	20.8	7.9	7.5	1278		100.0	20.8	7.9	7.3	1247	404.75
9	09/10/04		20	100.0	20.7	7.9	6.5	1176			20	100.0	20.7	7.9	7.4	1145		100.0	20.7	7.9	7.0	1161	
10	09/11/04		20	100.0	20.8	7.9	7.2	1223			20	100.0	20.9	7.9	6.9	1287		100.0	20.9	7.9	7.1	1255	
11	09/12/04		20	100.0	20.7	7.9	6.9	1242			20	100.0	20.8	7.8	7.5	1293		100.0	20.8	7.9	7.2	1268	
12	09/13/04		20	100.0	20.9	7.8	6.8	1328			20	100.0	20.9	7.8	7.3	1250		100.0	20.9	7.8	7.1	1289	
13	09/14/04		20	100.0	21.0	7.8	6.8	1321			20	100.0	21.1	7.9	7.2	1207		100.0	21.1	7.9	7.0	1264	
14	09/15/04		20	100.0							20	100.0						100.0					407.50
	Median:				**20.8**	**7.9**	**7.1**	**1230**					**20.8**	**7.9**	**7.5**	**1278**			**20.8**	**7.9**	**7.2**	**1259**	
	Mode:				**20.8**	**7.9**	**7.2**	**#N/A**					**20.9**	**7.9**	**7.7**	**#N/A**			**20.9**	**7.9**	**7.7**	**#N/A**	
	Mean:			**100.0**	**20.8**	**7.9**	**7.1**	**1245**				**100.0**	**20.8**	**8.0**	**7.4**	**1258**		**100.0**	**20.8**	**7.9**	**7.3**	**1252**	**436.350**
	SEM:			**0.00**	**0.04**	**0.03**	**0.14**	**11.80**				**0.00**	**0.05**	**0.03**	**0.12**	**13.29**		**0.00**	**0.05**	**0.03**	**0.12**	**9.75**	**30.24**
	% CV:			**0.00**	**3.63**	**4.06**	**19.54**	**120.56**				**0.00**	**4.37**	**4.11**	**16.50**	**135.04**		**0.00**	**3.96**	**3.92**	**16.71**	**99.39**	**250.70**

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA RENEW

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 1000 mg/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc (mg/L)
		Initial No.= 20								**Initial No.= 20**												
0	09/01/04			100.0	21.0	8.4	6.3	-				100.0	21.0	8.4	6.5	-	100.0	21.0	8.4	6.4	-	1047.10
1	09/02/04		20	100.0	20.6	8.3	7.5	1150			20	100.0	20.4	8.2	7.7	1040	100.0	20.5	8.3	7.6	1095	
2	09/03/04		20	100.0	20.8	8.2	7.3	1166			20	100.0	20.9	8.2	7.7	1236	100.0	20.9	8.2	7.5	1201	
3	09/04/04		20	100.0	20.6	8.2	7.9	1159			20	100.0	20.5	8.2	6.8	1127	100.0	20.6	8.2	7.4	1143	
4	09/05/04		20	100.0	20.6	8.2	7.2	1105			20	100.0	20.6	8.3	7.6	1115	100.0	20.6	8.3	7.4	1110	
5	09/06/04		20	100.0	20.5	8.0	7.4	1045			20	100.0	20.5	8.0	8.0	1034	100.0	20.5	8.0	7.7	1040	
6	09/07/04		20	100.0	21.7	8.0	6.5	1165			20	100.0	21.7	8.0	6.4	1115	100.0	21.7	8.0	6.5	1140	
7	09/08/04		20	100.0	20.5	8.0	7.3	1140			20	100.0	20.5	8.0	7.1	1085	100.0	20.5	8.0	7.2	1113	
8	09/09/04		20	100.0	20.6	8.1	7.1	1131			20	100.0	20.8	8.0	7.1	1086	100.0	20.7	8.1	7.1	1109	
9	09/10/04		20	100.0	20.8	8.0	6.7	1129			20	100.0	20.8	8.0	6.8	1127	100.0	20.8	8.0	6.8	1128	
10	09/11/04		20	100.0	20.8	8.0	6.9	1137			20	100.0	20.7	8.1	6.8	1079	100.0	20.8	8.1	6.9	1108	
11	09/12/04		20	100.0	20.8	8.0	7.6	1128			20	100.0	20.9	8.1	7.1	1087	100.0	20.9	8.1	7.4	1108	
12	09/13/04		20	100.0	20.9	8.0	6.6	1150			20	100.0	20.9	8.0	6.8	1113	100.0	20.9	8.0	6.7	1132	
13	09/14/04		20	100.0	21.4	8.0	7.0	1275			20	100.0	21.4	8.0	7.6	1235	100.0	21.4	8.0	7.3	1255	
14	09/15/04		20	100.0							20	100.0					100.0					814.10
	Median:				**20.8**	**8.0**	**7.2**	**1140**					**20.8**	**8.1**	**7.1**	**1113**		**20.8**	**8.1**	**7.3**	**1113**	
	Mode:				**20.6**	**8.0**	**7.3**	**1150**					**20.9**	**8.0**	**6.8**	**1127**		**20.5**	**8.0**	**7.4**	**#N/A**	
	Mean:			**100.0**	**20.8**	**8.1**	**7.1**	**1145**				**100.0**	**20.8**	**8.1**	**7.1**	**1114**	**100.0**	**20.8**	**8.1**	**7.1**	**1129**	**930.600**
	SEM:			**0.00**	**0.09**	**0.04**	**0.12**	**13.96**				**0.00**	**0.10**	**0.04**	**0.13**	**17.04**	**0.00**	**0.09**	**0.03**	**0.11**	**14.44**	**116.50**
	% CV:			**0.00**	**7.56**	**4.77**	**17.08**	**148.81**				**0.00**	**7.94**	**4.66**	**18.61**	**184.14**	**0.00**	**7.68**	**4.59**	**15.72**	**154.89**	**540.08**

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

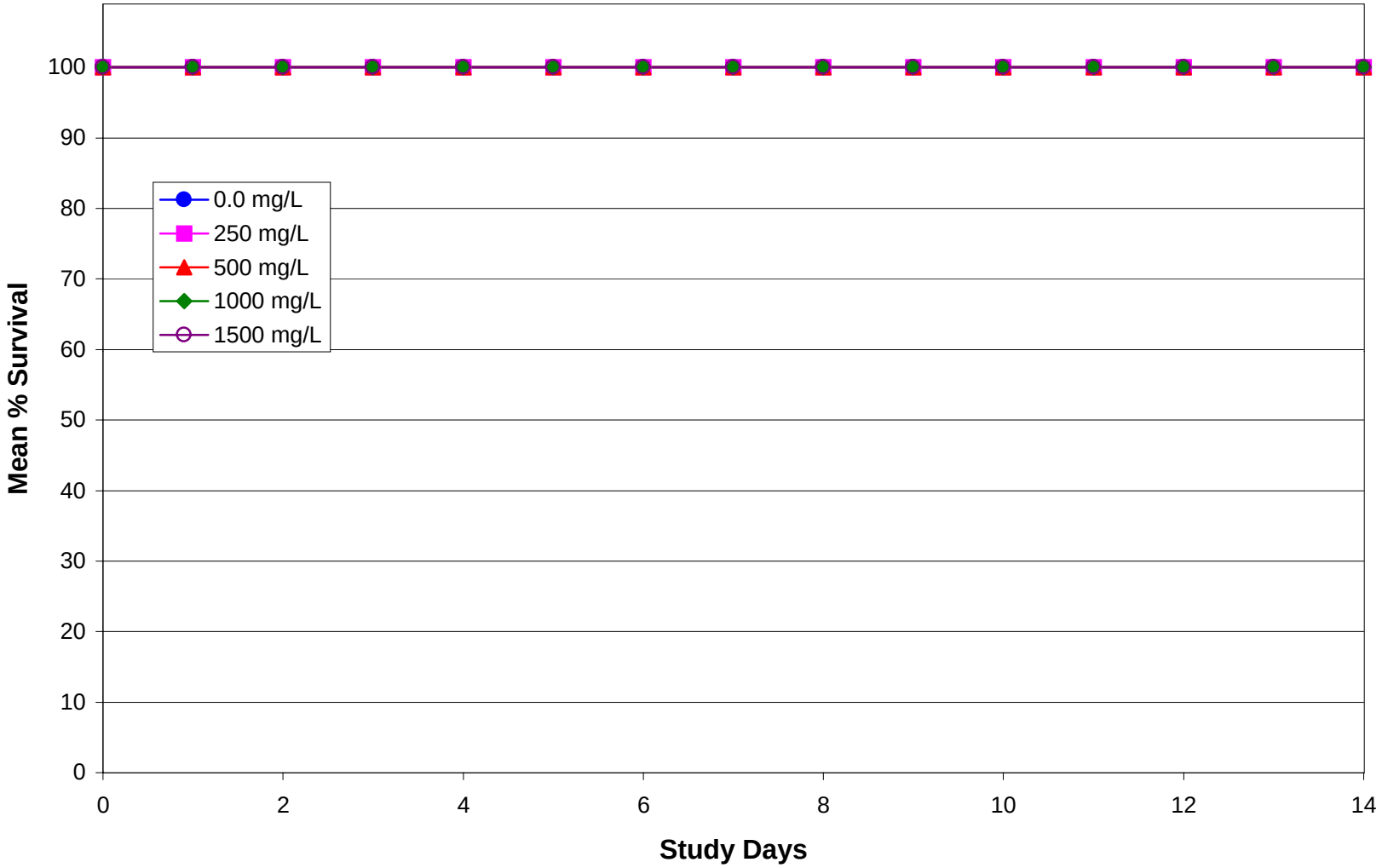
FEL

DAILY DATA RENEW

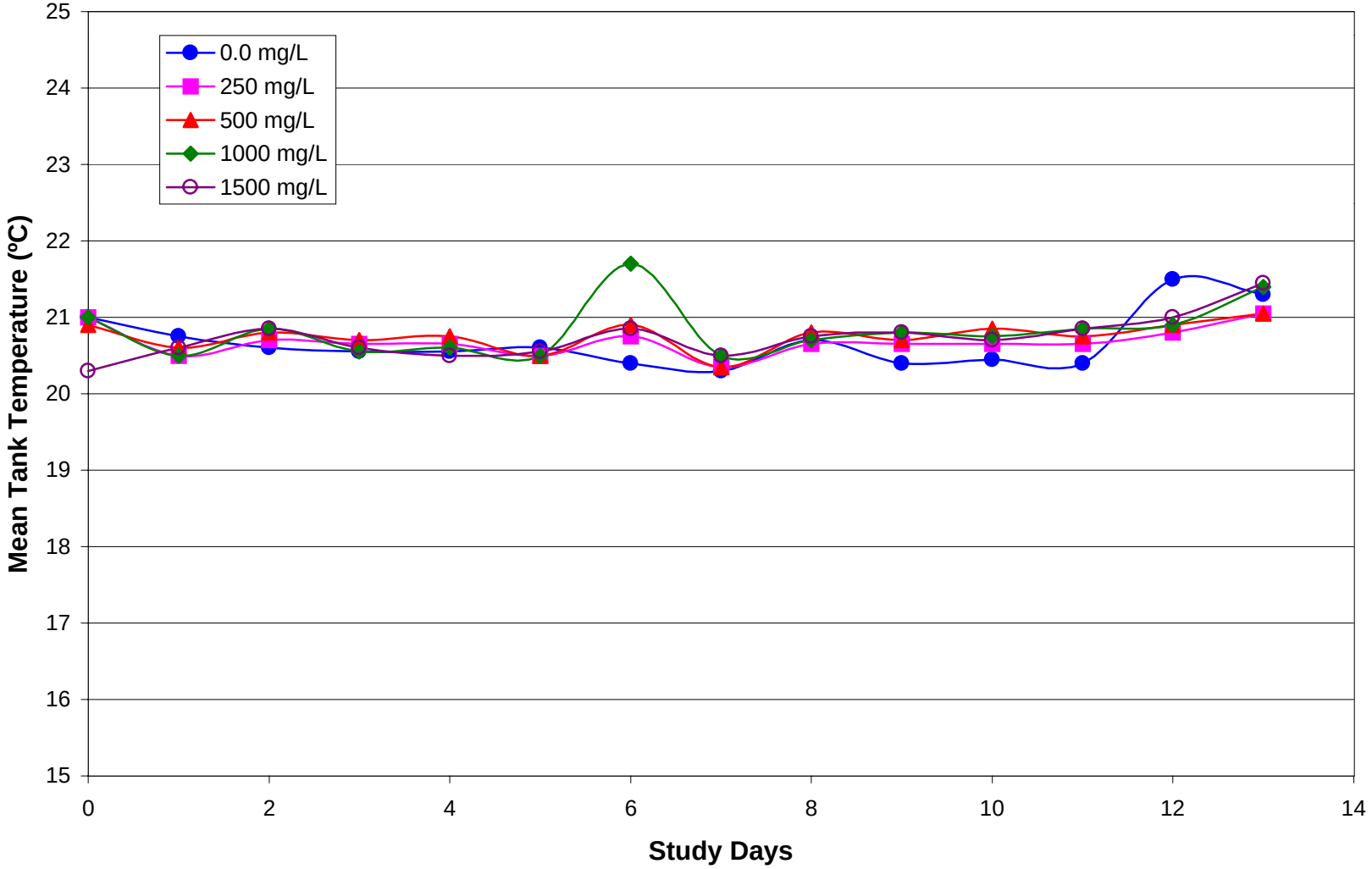
Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 1500 mg/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc (mg/L)
		Initial No.= 20							**Initial No.= 20**												
0	09/01/04			100.0	20.3	8.5	7.3	-			100.0	20.3	8.5	7.4	-	100.0	20.3	8.5	7.4	-	1518.90
1	09/02/04		20	100.0	20.6	8.4	6.9	1001		20	100.0	20.6	8.4	7.1	855	100.0	20.6	8.4	7.0	928	
2	09/03/04		20	100.0	20.9	8.3	7.1	1046		20	100.0	20.8	8.3	7.6	879	100.0	20.9	8.3	7.4	963	
3	09/04/04		20	100.0	20.6	8.3	7.7	937		20	100.0	20.6	8.1	7.8	936	100.0	20.6	8.2	7.8	937	
4	09/05/04		20	100.0	20.5	8.2	7.9	965		20	100.0	20.5	8.3	8.0	925	100.0	20.5	8.3	8.0	945	
5	09/06/04		20	100.0	20.5	8.2	7.4	868		20	100.0	20.6	8.3	8.1	848	100.0	20.6	8.3	7.8	858	
6	09/07/04		20	100.0	20.9	8.1	6.5	1070		20	100.0	20.8	8.2	6.5	980	100.0	20.9	8.2	6.5	1025	
7	09/08/04		20	100.0	20.5	8.1	6.8	885		20	100.0	20.5	8.1	6.7	820	100.0	20.5	8.1	6.8	853	
8	09/09/04		20	100.0	20.7	8.1	6.8	954		20	100.0	20.8	8.1	6.8	927	100.0	20.8	8.1	6.8	941	
9	09/10/04		20	100.0	20.8	8.1	6.1	984		20	100.0	20.8	8.1	6.2	1033	100.0	20.8	8.1	6.2	1009	
10	09/11/04		20	100.0	20.7	8.1	7.1	883		20	100.0	20.7	8.2	7.0	822	100.0	20.7	8.2	7.1	853	
11	09/12/04		20	100.0	20.8	8.1	7.4	864		20	100.0	20.9	8.1	7.4	831	100.0	20.9	8.1	7.4	848	
12	09/13/04		20	100.0	21.0	8.0	6.4	956		20	100.0	21.0	8.1	6.3	890	100.0	21.0	8.1	6.4	923	
13	09/14/04		20	100.0	21.4	8.2	6.3	1090		20	100.0	21.5	8.2	6.0	962	100.0	21.5	8.2	6.2	1026	
14	09/15/04		20	100.0						20	100.0					100.0					1242.70
	Median:				**20.7**	**8.2**	**7.0**	**956**				**20.8**	**8.2**	**7.1**	**890**		**20.7**	**8.2**	**7.0**	**937**	
	Mode:				**20.5**	**8.1**	**7.1**	**#N/A**				**20.8**	**8.1**	**7.4**	**#N/A**		**20.9**	**8.1**	**7.4**	**853**	
	Mean:			**100.0**	**20.7**	**8.2**	**7.0**	**962**			**100.0**	**20.7**	**8.2**	**7.1**	**901**	**100.0**	**20.7**	**8.2**	**7.0**	**931**	**1380.800**
	SEM:			**0.00**	**0.07**	**0.04**	**0.14**	**20.95**			**0.00**	**0.08**	**0.03**	**0.18**	**18.45**	**0.00**	**0.07**	**0.03**	**0.16**	**17.88**	**138.10**
	% CV:			**0.00**	**6.00**	**4.84**	**20.26**	**243.54**			**0.00**	**6.25**	**4.51**	**25.54**	**221.65**	**0.00**	**6.08**	**4.46**	**22.57**	**211.27**	**525.59**

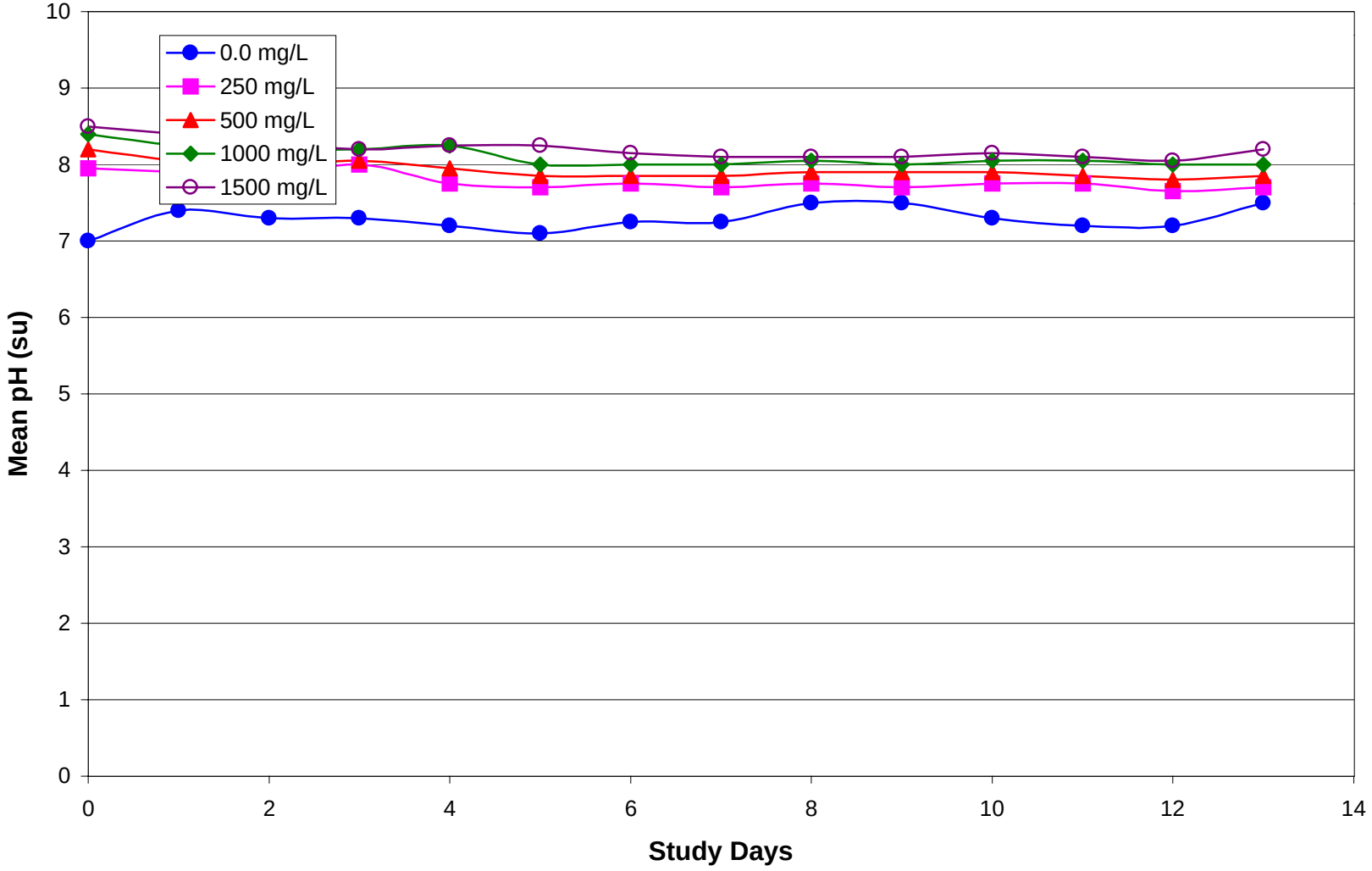
BATT02-00056
Phenobarbital - Renewal
Daily Average of Larval Survival for Initial Stage 54 Test



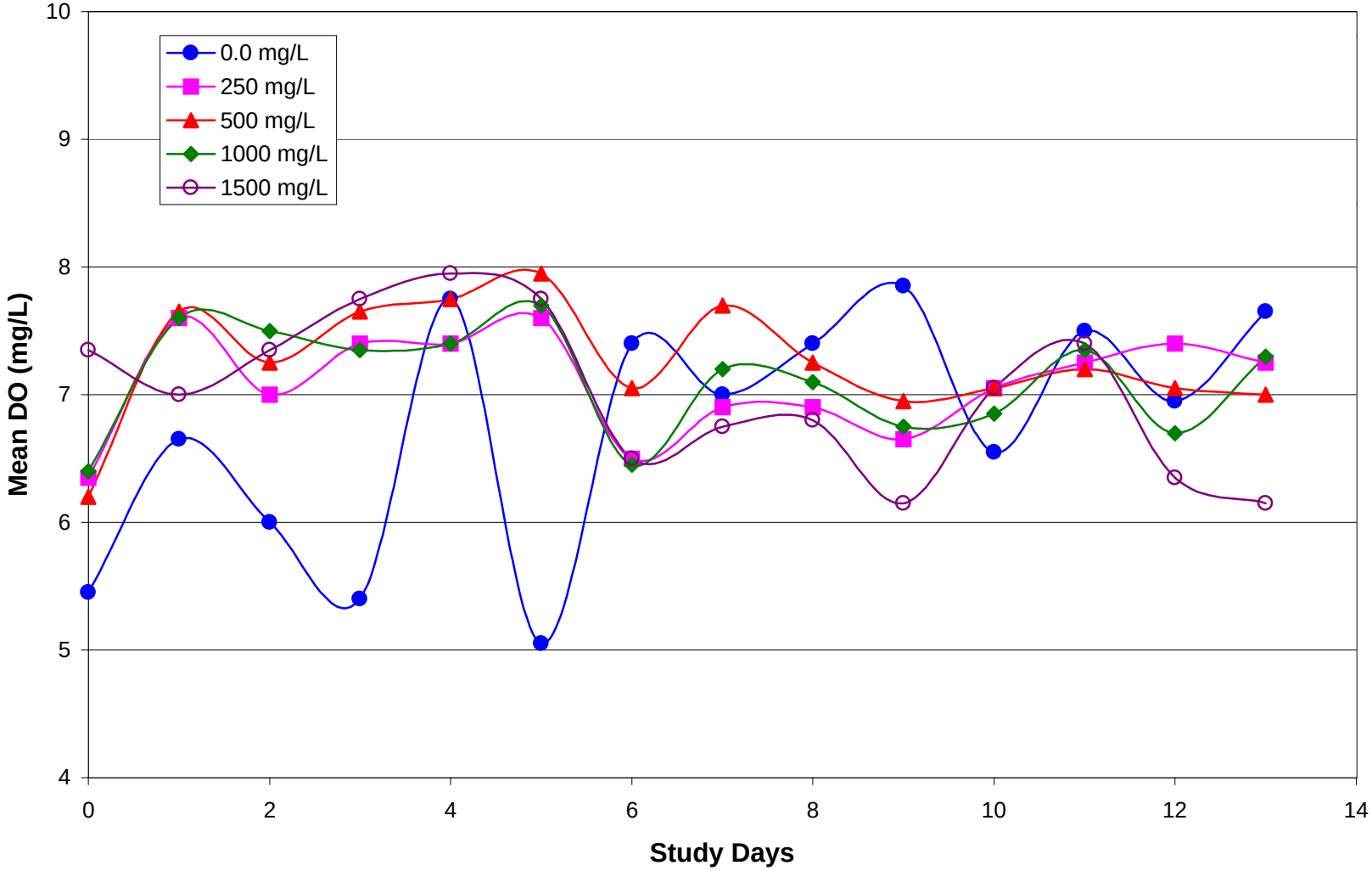
BATT02-00056
Phenobarbital - Renewal
Daily Average Tank Temperatures for Initial Stage 54 Test



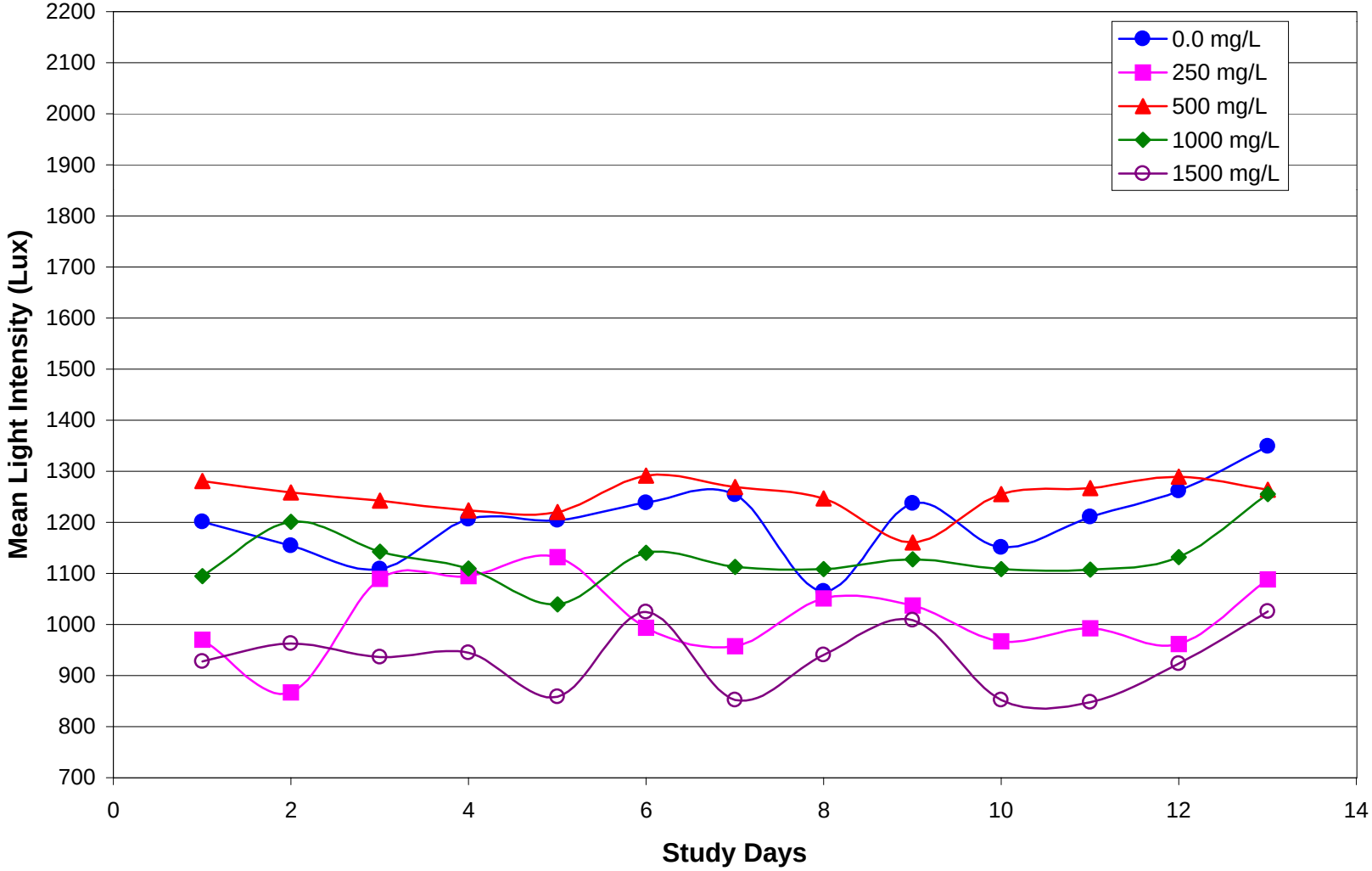
BATT02-00056
Phenobarbital - Renewal
Daily Average pH for Initial Stage 54 Test



BATT02-00056
Phenobarbital - Renewal
Daily Average Dissolved Oxygen (DO) for Initial Stage 54 Test



BATT02-00056
Phenobarbital - Renewal
Daily Average Light Intensity for Initial Stage 54 Test



WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA SPENT

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 0.0 mg/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	09/02/04		20	100.0	20.0	7.5	8.2	1149		20	100.0	20.0	7.5	8.0	1254	100.0	20.0	7.5	8.1	1202	
2	09/03/04		20	100.0	20.0	7.4	8.2	1148		20	100.0	20.0	7.5	7.5	1160	100.0	20.0	7.5	7.9	1154	
3	09/04/04		20	100.0	20.0	7.4	7.5	1078		20	100.0	20.3	7.5	7.3	1140	100.0	20.2	7.5	7.4	1109	
4	09/05/04		20	100.0	20.2	7.4	7.1	1248		20	100.0	20.1	7.3	7.4	1164	100.0	20.2	7.4	7.3	1206	
5	09/06/04		20	100.0	20.2	7.6	8.0	1227		20	100.0	20.1	7.6	7.1	1182	100.0	20.2	7.6	7.6	1205	
6	09/07/04		20	100.0	20.1	7.5	8.1	1220		20	100.0	20.1	7.5	7.6	1257	100.0	20.1	7.5	7.9	1239	
7	09/08/04		20	100.0	20.0	7.5	8.1	1152		20	100.0	20.0	7.5	7.5	1356	100.0	20.0	7.5	7.8	1254	
8	09/09/04		20	100.0	20.0	7.5	8.0	1030		20	100.0	20.0	7.5	6.1	1100	100.0	20.0	7.5	7.1	1065	
9	09/10/04		20	100.0	20.0	7.6	8.1	1235		20	100.0	20.0	7.6	8.1	1240	100.0	20.0	7.6	8.1	1238	
10	09/11/04		20	100.0	20.0	7.3	8.0	1047		20	100.0	20.1	7.4	7.9	1255	100.0	20.1	7.4	8.0	1151	
11	09/12/04		20	100.0	20.1	7.5	8.2	1135		20	100.0	20.0	7.4	7.9	1286	100.0	20.1	7.5	8.1	1211	
12	09/13/04		20	100.0	20.6	7.5	8.1	1242		20	100.0	20.3	7.6	7.9	1282	100.0	20.5	7.6	8.0	1262	
13	09/14/04		20	100.0	20.7	7.6	8.0	1334		20	100.0	20.6	7.6	7.8	1364	100.0	20.7	7.6	7.9	1349	
14	09/15/04		20	100.0	20.9	7.6	8.0	-		20	100.0	20.8	7.6	7.8	-	100.0	20.9	7.6	7.9	-	
	Median:				**20.1**	**7.5**	**8.1**	**1152**				**20.1**	**7.5**	**7.7**	**1254**		**20.1**	**7.5**	**7.9**	**1206**	
	Mode:				**20.0**	**7.5**	**8.0**	**#N/A**				**20.0**	**7.5**	**7.9**	**#N/A**		**20.0**	**7.5**	**8.1**	**#N/A**	
	Mean:			**100.0**	**20.2**	**7.5**	**8.0**	**1173**			**100.0**	**20.2**	**7.5**	**7.6**	**1234**	**100.0**	**20.2**	**7.5**	**7.8**	**1203**	
	SEM:			**0.00**	**0.08**	**0.02**	**0.08**	**24.51**			**0.00**	**0.07**	**0.02**	**0.14**	**22.37**	**0.00**	**0.07**	**0.02**	**0.09**	**20.08**	
	% CV:			**0.00**	**6.76**	**3.35**	**10.80**	**258.05**			**0.00**	**5.55**	**3.35**	**18.57**	**229.63**	**0.00**	**6.01**	**3.12**	**11.79**	**208.72**	

DAILY DATA SPENT

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 250 mg/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	09/02/04		20	100.0	20.0	7.8	7.3	920		20	100.0	20.0	7.8	8.2	1020	100.0	20.0	7.8	7.8	970	
2	09/03/04		20	100.0	20.2	7.7	7.6	760		20	100.0	20.2	7.7	8.0	974	100.0	20.2	7.7	7.8	867	
3	09/04/04		20	100.0	20.4	7.9	7.1	1039		20	100.0	20.2	8.0	7.8	1140	100.0	20.3	8.0	7.5	1090	
4	09/05/04		20	100.0	20.1	7.8	7.8	1035		20	100.0	20.2	7.7	7.6	1155	100.0	20.2	7.8	7.7	1095	
5	09/06/04		20	100.0	20.2	7.6	7.5	1095		20	100.0	20.2	7.7	8.0	1169	100.0	20.2	7.7	7.8	1132	
6	09/07/04		20	100.0	20.1	7.6	7.1	957		20	100.0	20.1	7.6	7.8	1031	100.0	20.1	7.6	7.5	994	
7	09/08/04		20	100.0	20.0	7.6	7.4	948		20	100.0	20.0	7.6	8.0	967	100.0	20.0	7.6	7.7	958	
8	09/09/04		20	100.0	20.0	7.6	7.3	974		20	100.0	20.0	7.7	7.9	1128	100.0	20.0	7.7	7.6	1051	
9	09/10/04		20	100.0	20.0	7.6	7.4	923		20	100.0	20.0	7.7	7.8	1151	100.0	20.0	7.7	7.6	1037	
10	09/11/04		20	100.0	20.1	7.7	7.7	951		20	100.0	20.0	7.6	7.4	984	100.0	20.1	7.7	7.6	968	
11	09/12/04		20	100.0	20.0	7.6	7.7	938		20	100.0	20.0	7.7	7.7	1047	100.0	20.0	7.7	7.7	993	
12	09/13/04		20	100.0	20.6	7.5	7.4	913		20	100.0	20.7	7.5	7.4	1011	100.0	20.7	7.5	7.4	962	
13	09/14/04		20	100.0	20.6	7.7	7.9	989		20	100.0	20.6	7.6	7.5	1187	100.0	20.6	7.7	7.7	1088	
14	09/15/04		20	100.0	20.8	7.6	7.6	-		20	100.0	20.8	7.7	7.7	-	100.0	20.8	7.7	7.7	-	
	Median:				**20.1**	**7.6**	**7.5**	**951**				**20.2**	**7.7**	**7.8**	**1047**		**20.1**	**7.7**	**7.7**	**994**	
	Mode:				**20.0**	**7.6**	**7.4**	**#N/A**				**20.0**	**7.7**	**8.0**	**#N/A**		**20.0**	**7.7**	**7.7**	**#N/A**	
	Mean:			**100.0**	**20.2**	**7.7**	**7.5**	**957**			**100.0**	**20.2**	**7.7**	**7.8**	**1074**	**100.0**	**20.2**	**7.7**	**7.6**	**1016**	
	SEM:			**0.00**	**0.07**	**0.03**	**0.07**	**22.20**			**0.00**	**0.07**	**0.03**	**0.06**	**22.73**	**0.00**	**0.07**	**0.03**	**0.03**	**20.57**	
	% CV:			**0.00**	**5.99**	**3.91**	**8.94**	**258.78**			**0.00**	**6.22**	**4.21**	**8.61**	**250.01**	**0.00**	**6.05**	**3.80**	**4.53**	**232.70**	

DAILY DATA SPENT

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 500 mg/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	09/02/04		20	100.0	20.0	8.0	8.2	1261		20	100.0	20.0	8.0	8.3	1301	100.0	20.0	8.0	8.3	1281	
2	09/03/04		20	100.0	20.2	7.9	7.9	1230		20	100.0	20.1	7.9	8.0	1288	100.0	20.2	7.9	8.0	1259	
3	09/04/04		20	100.0	20.1	7.9	6.4	1220		20	100.0	20.2	7.9	7.9	1266	100.0	20.2	7.9	7.2	1243	
4	09/05/04		20	100.0	20.1	7.8	7.5	1222		20	100.0	20.2	7.9	6.7	1225	100.0	20.2	7.9	7.1	1224	
5	09/06/04		20	100.0	20.2	7.7	7.9	1226		20	100.0	20.2	7.8	8.4	1214	100.0	20.2	7.8	8.2	1220	
6	09/07/04		20	100.0	20.1	7.7	7.0	1275		20	100.0	20.1	7.8	7.9	1308	100.0	20.1	7.8	7.5	1292	
7	09/08/04		20	100.0	20.0	7.7	7.5	1245		20	100.0	20.1	7.8	8.2	1294	100.0	20.1	7.8	7.9	1270	
8	09/09/04		20	100.0	20.0	7.8	7.7	1215		20	100.0	20.0	7.8	8.2	1278	100.0	20.0	7.8	8.0	1247	
9	09/10/04		20	100.0	20.0	7.8	7.7	1176		20	100.0	20.0	7.8	8.2	1145	100.0	20.0	7.8	8.0	1161	
10	09/11/04		20	100.0	20.0	7.7	8.1	1223		20	100.0	20.0	7.8	7.8	1287	100.0	20.0	7.8	8.0	1255	
11	09/12/04		20	100.0	20.0	7.8	7.9	1242		20	100.0	20.0	7.8	7.6	1293	100.0	20.0	7.8	7.8	1268	
12	09/13/04		20	100.0	20.7	7.7	7.2	1328		20	100.0	20.7	7.7	7.8	1250	100.0	20.7	7.7	7.5	1289	
13	09/14/04		20	100.0	20.7	7.6	7.3	1321		20	100.0	20.8	7.7	7.7	1207	100.0	20.8	7.7	7.5	1264	
14	09/15/04		20	100.0	20.8	7.7	7.2	-		20	100.0	20.9	7.7	7.7	-	100.0	20.9	7.7	7.5	-	
	Median:				**20.1**	**7.8**	**7.6**	**1230**				**20.1**	**7.8**	**7.9**	**1278**		**20.1**	**7.8**	**7.8**	**1259**	
	Mode:				**20.0**	**7.7**	**7.9**	**#N/A**				**20.0**	**7.8**	**8.2**	**#N/A**		**20.0**	**7.8**	**8.0**	**#N/A**	
	Mean:			**100.0**	**20.2**	**7.8**	**7.5**	**1245**			**100.0**	**20.2**	**7.8**	**7.9**	**1258**	**100.0**	**20.2**	**7.8**	**7.7**	**1252**	
	SEM:			**0.00**	**0.08**	**0.03**	**0.13**	**11.80**			**0.00**	**0.08**	**0.02**	**0.11**	**13.29**	**0.00**	**0.08**	**0.03**	**0.10**	**9.75**	
	% CV:			**0.00**	**6.56**	**3.83**	**17.71**	**120.56**			**0.00**	**7.06**	**3.09**	**15.03**	**135.04**	**0.00**	**6.78**	**3.36**	**12.81**	**99.39**	

DAILY DATA SPENT

Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 1000 mg/L**

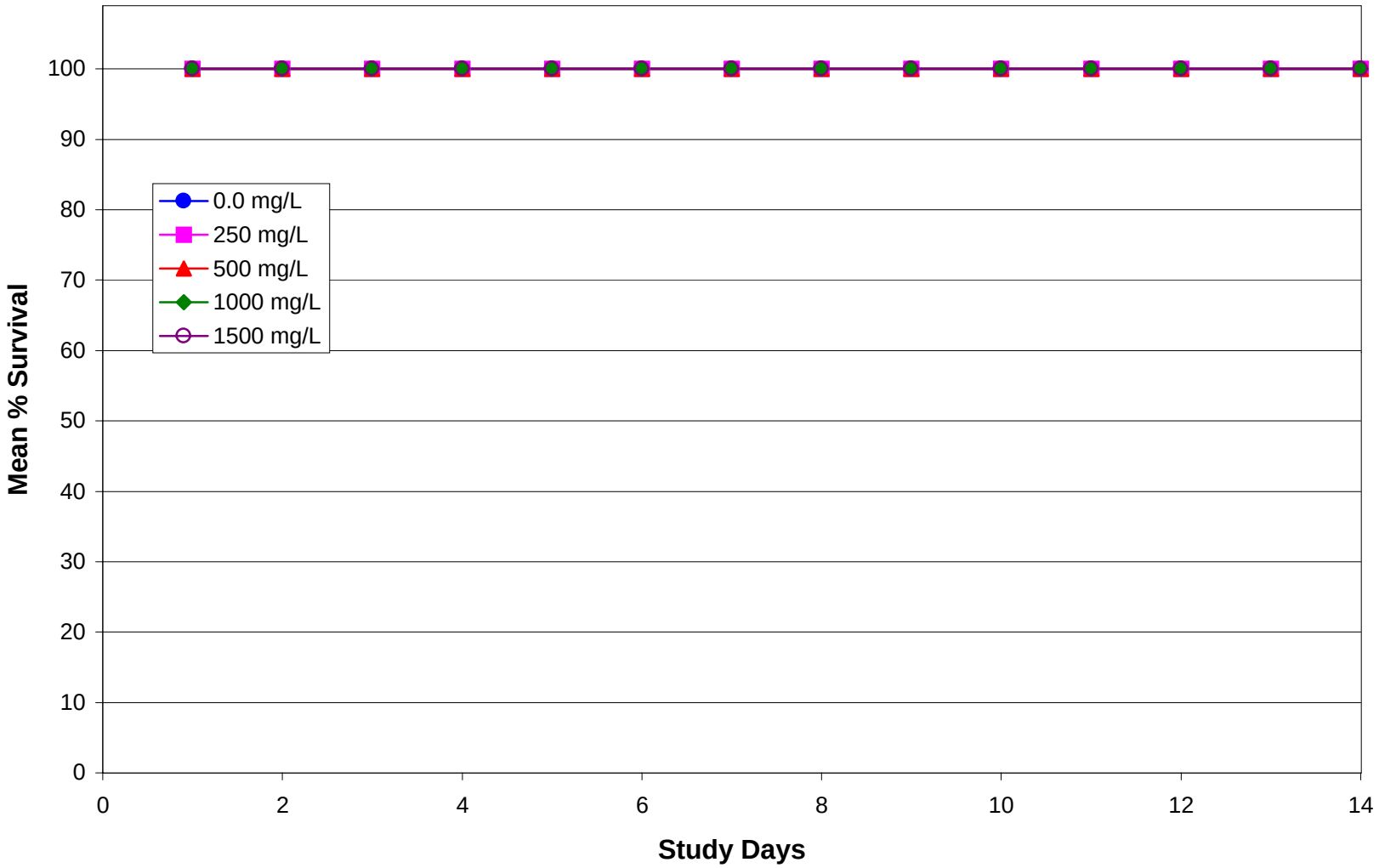
		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	09/02/04		20	100.0	20.1	8.2	7.7	1150		20	100.0	20.1	8.2	8.1	1040	100.0	20.1	8.2	7.9	1095	
2	09/03/04		20	100.0	20.2	8.1	7.3	1166		20	100.0	20.3	8.1	8.0	1236	100.0	20.3	8.1	7.7	1201	
3	09/04/04		20	100.0	20.3	8.2	8.2	1159		20	100.0	20.1	8.2	7.7	1127	100.0	20.2	8.2	8.0	1143	
4	09/05/04		20	100.0	20.2	8.1	7.3	1105		20	100.0	20.3	8.2	7.6	1115	100.0	20.3	8.2	7.5	1110	
5	09/06/04		20	100.0	20.3	7.9	7.0	1045		20	100.0	20.3	7.9	8.1	1034	100.0	20.3	7.9	7.6	1040	
6	09/07/04		20	100.0	20.1	7.9	7.3	1165		20	100.0	20.1	7.9	7.3	1115	100.0	20.1	7.9	7.3	1140	
7	09/08/04		20	100.0	20.0	7.9	7.6	1140		20	100.0	20.0	7.9	7.7	1085	100.0	20.0	7.9	7.7	1113	
8	09/09/04		20	100.0	20.0	7.9	7.8	1131		20	100.0	20.0	7.9	7.9	1086	100.0	20.0	7.9	7.9	1109	
9	09/10/04		20	100.0	20.0	7.9	7.8	1129		20	100.0	20.0	7.9	7.6	1127	100.0	20.0	7.9	7.7	1128	
10	09/11/04		20	100.0	20.0	7.8	7.8	1137		20	100.0	20.1	7.9	7.7	1079	100.0	20.1	7.9	7.8	1108	
11	09/12/04		20	100.0	20.0	7.9	8.0	1128		20	100.0	20.0	7.9	7.4	1087	100.0	20.0	7.9	7.7	1108	
12	09/13/04		20	100.0	20.6	7.9	7.4	1150		20	100.0	20.6	7.9	7.3	1113	100.0	20.6	7.9	7.4	1132	
13	09/14/04		20	100.0	20.8	7.8	6.9	1275		20	100.0	20.8	7.8	7.2	1235	100.0	20.8	7.8	7.1	1255	
14	09/15/04		20	100.0	20.9	7.9	7.2	-		20	100.0	20.9	7.9	7.6	-	100.0	20.9	7.9	7.4	-	
	Median:				**20.2**	**7.9**	**7.5**	**1140**				**20.1**	**7.9**	**7.7**	**1113**		**20.2**	**7.9**	**7.7**	**1113**	
	Mode:				**20.0**	**7.9**	**7.3**	**1150**				**20.1**	**7.9**	**7.7**	**1127**		**20.0**	**7.9**	**7.7**	**#N/A**	
	Mean:			**100.0**	**20.3**	**8.0**	**7.5**	**1145**			**100.0**	**20.3**	**8.0**	**7.7**	**1114**	**100.0**	**20.3**	**8.0**	**7.6**	**1129**	
	SEM:			**0.00**	**0.08**	**0.04**	**0.10**	**13.96**			**0.00**	**0.08**	**0.04**	**0.08**	**17.04**	**0.00**	**0.08**	**0.04**	**0.07**	**14.44**	
	% CV:			**0.00**	**6.79**	**4.76**	**13.88**	**148.81**			**0.00**	**6.73**	**4.90**	**10.58**	**184.14**	**0.00**	**6.72**	**4.79**	**9.23**	**154.89**	

DAILY DATA SPENT

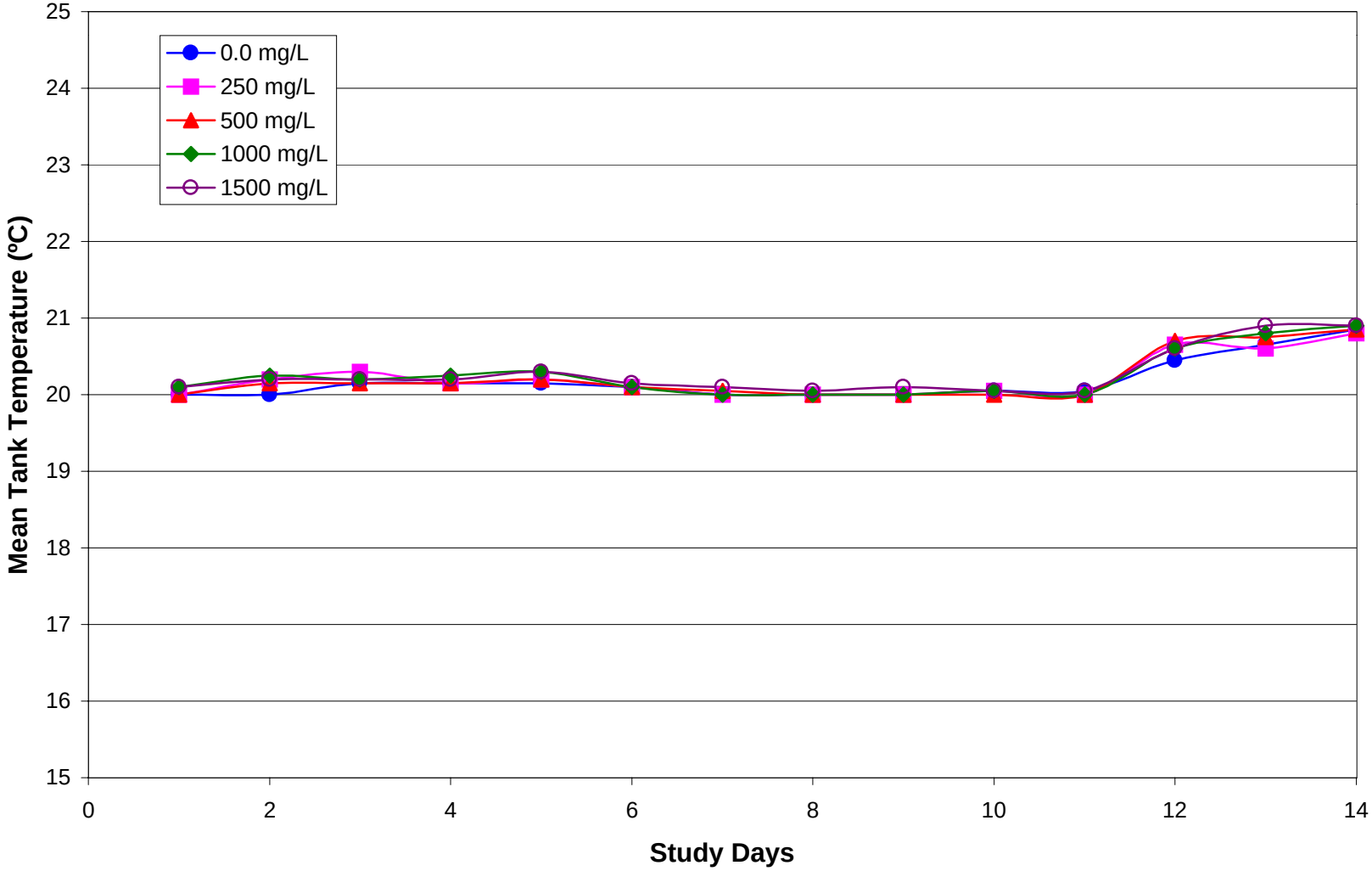
Workorder No: 00056 **Test No: 1**
Test Chemical: Phenobarbital (PBB) **Target Conc: 1500 mg/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	09/02/04		20	100.0	20.1	8.3	7.7	1001		20	100.0	20.1	8.4	8.0	855	100.0	20.1	8.4	7.9	928	
2	09/03/04		20	100.0	20.2	8.2	6.8	1046		20	100.0	20.2	8.2	7.5	879	100.0	20.2	8.2	7.2	963	
3	09/04/04		20	100.0	20.2	8.1	7.8	937		20	100.0	20.2	8.1	8.1	936	100.0	20.2	8.1	8.0	937	
4	09/05/04		20	100.0	20.2	8.2	7.7	965		20	100.0	20.2	8.3	7.9	925	100.0	20.2	8.3	7.8	945	
5	09/06/04		20	100.0	20.3	8.0	6.9	868		20	100.0	20.3	8.0	7.7	848	100.0	20.3	8.0	7.3	858	
6	09/07/04		20	100.0	20.1	8.0	5.8	1070		20	100.0	20.2	8.0	6.2	980	100.0	20.2	8.0	6.0	1025	
7	09/08/04		20	100.0	20.1	8.0	7.0	885		20	100.0	20.1	8.0	7.0	820	100.0	20.1	8.0	7.0	853	
8	09/09/04		20	100.0	20.1	8.0	7.2	954		20	100.0	20.0	8.0	7.3	927	100.0	20.1	8.0	7.3	941	
9	09/10/04		20	100.0	20.1	8.0	7.1	984		20	100.0	20.1	8.0	7.3	1033	100.0	20.1	8.0	7.2	1009	
10	09/11/04		20	100.0	20.0	8.0	7.6	883		20	100.0	20.1	8.0	7.8	822	100.0	20.1	8.0	7.7	853	
11	09/12/04		20	100.0	20.1	7.9	7.5	864		20	100.0	20.0	8.0	7.8	831	100.0	20.1	8.0	7.7	848	
12	09/13/04		20	100.0	20.6	8.0	6.3	956		20	100.0	20.6	8.0	6.6	890	100.0	20.6	8.0	6.5	923	
13	09/14/04		20	100.0	20.9	7.9	6.8	1090		20	100.0	20.9	7.9	6.6	962	100.0	20.9	7.9	6.7	1026	
14	09/15/04		20	100.0	20.9	8.0	5.9	-		20	100.0	20.9	8.0	7.0	-	100.0	20.9	8.0	6.5	-	
	Median:				**20.2**	**8.0**	**7.1**	**956**				**20.2**	**8.0**	**7.4**	**890**		**20.2**	**8.0**	**7.2**	**937**	
	Mode:				**20.1**	**8.0**	**7.7**	**#N/A**				**20.1**	**8.0**	**7.0**	**#N/A**		**20.1**	**8.0**	**#N/A**	**853**	
	Mean:			**100.0**	**20.3**	**8.0**	**7.0**	**962**			**100.0**	**20.3**	**8.1**	**7.3**	**901**	**100.0**	**20.3**	**8.1**	**7.2**	**931**	
	SEM:			**0.00**	**0.08**	**0.03**	**0.17**	**20.95**			**0.00**	**0.08**	**0.04**	**0.16**	**18.45**	**0.00**	**0.08**	**0.03**	**0.16**	**17.88**	
	% CV:			**0.00**	**6.64**	**4.08**	**24.59**	**243.54**			**0.00**	**6.70**	**4.90**	**21.79**	**221.65**	**0.00**	**6.64**	**4.45**	**22.35**	**211.27**	

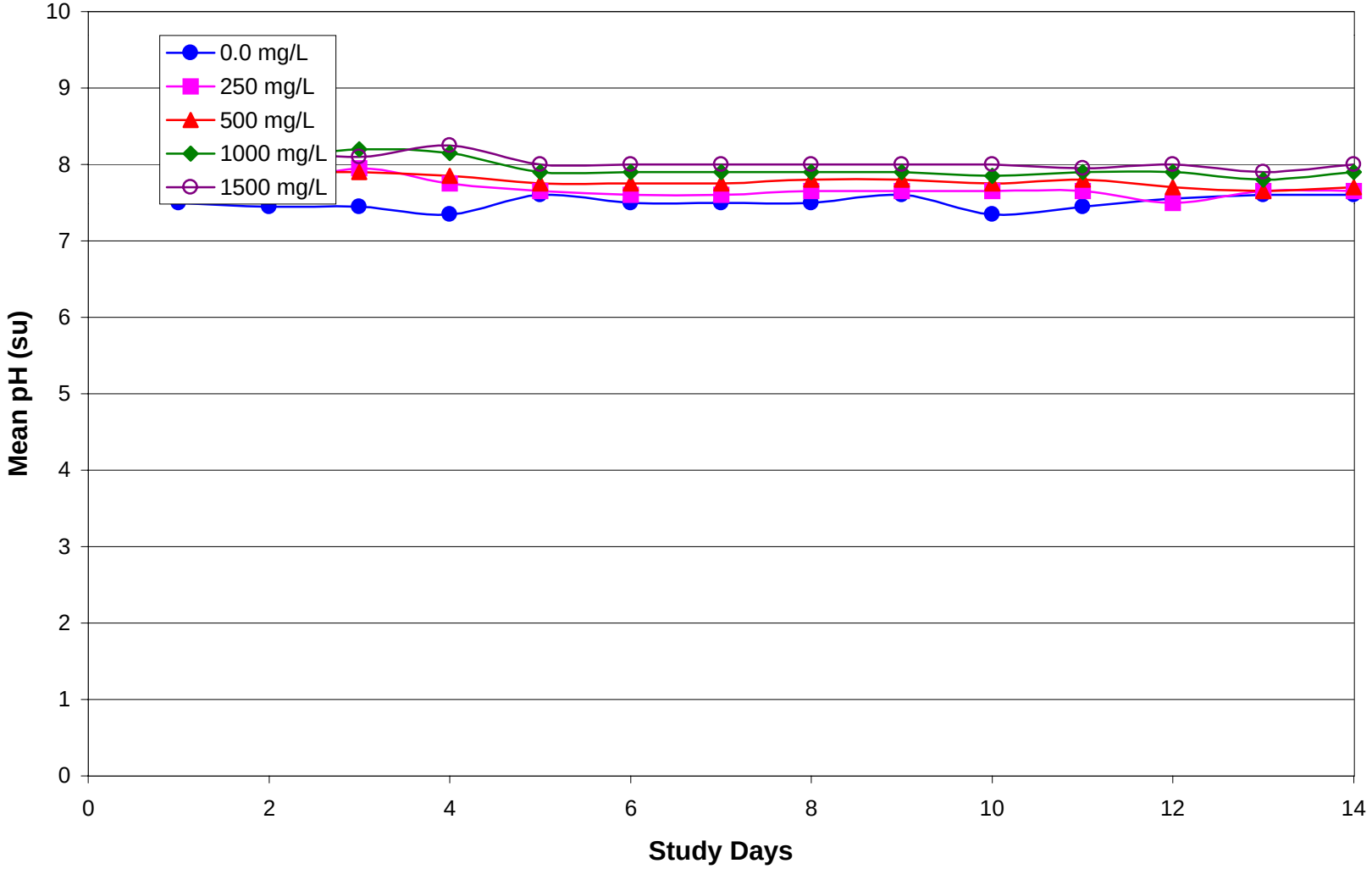
BATT02-00056
Phenobarbital - Spent
Daily Average of Larval Survival for Initial Stage 54 Test



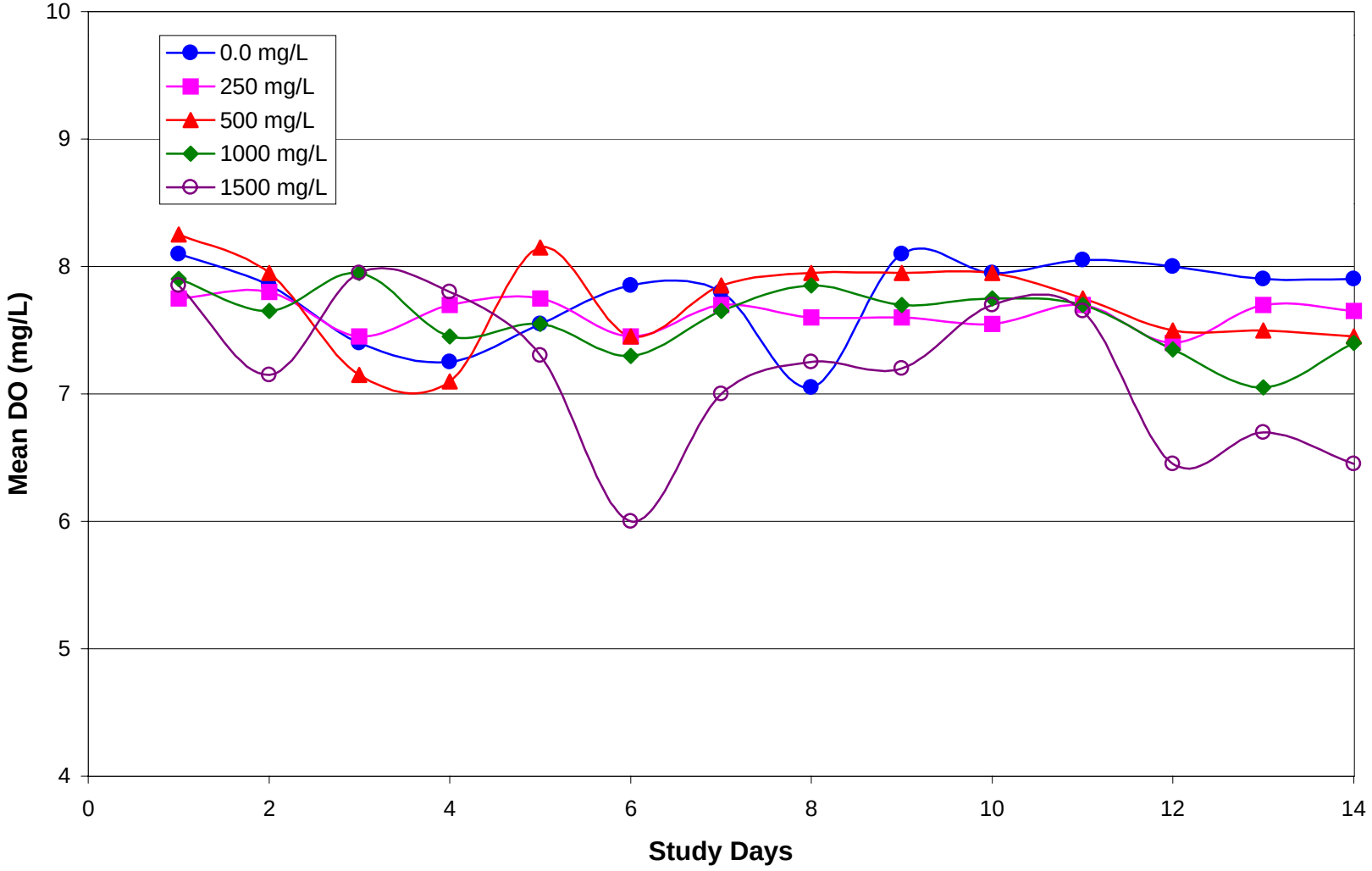
BATT02-00056
Phenobarbital - Spent
Daily Average Tank Temperatures for Initial Stage 54 Test



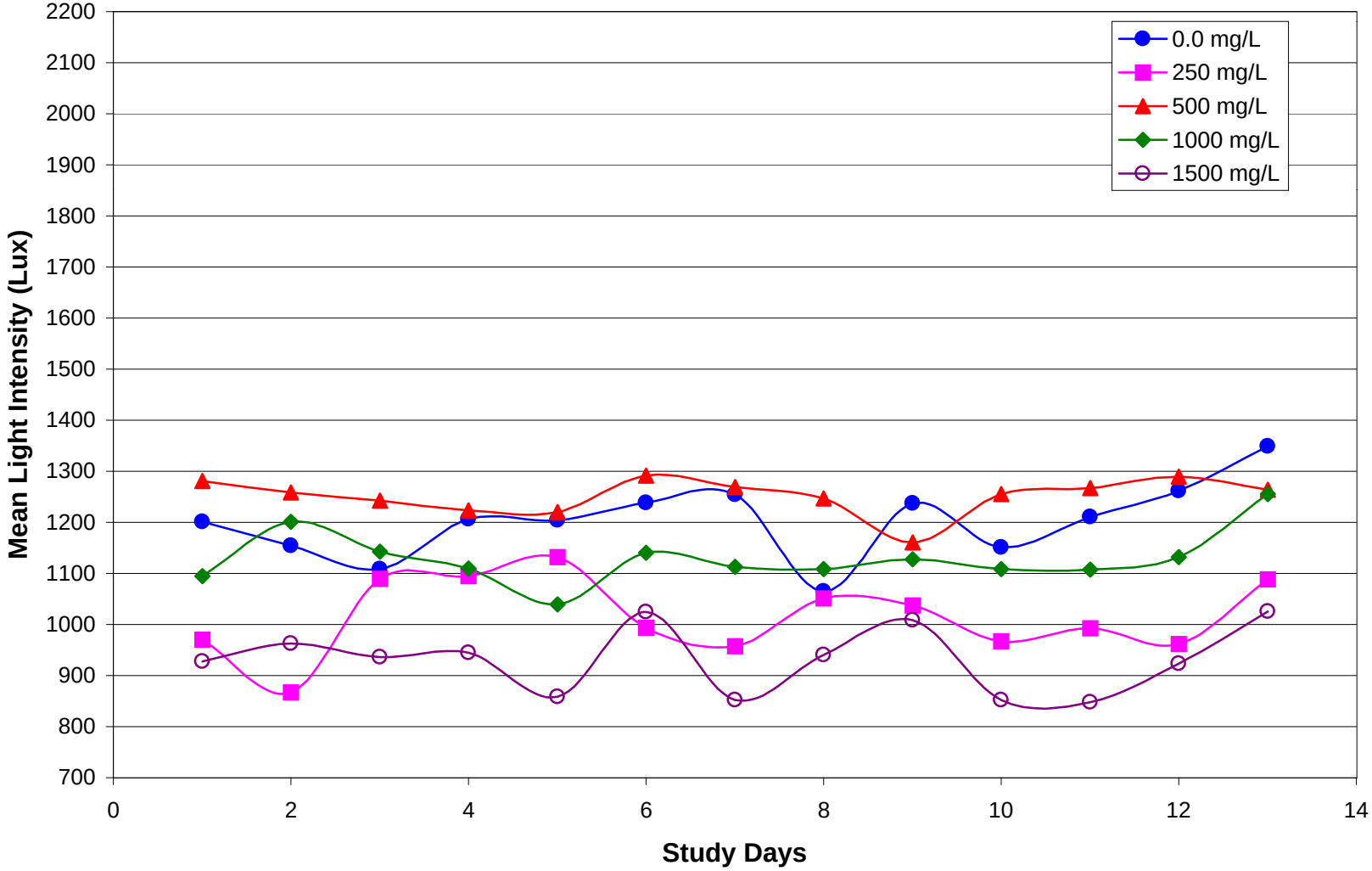
BATT02-00056
Phenobarbital - Spent
Daily Average pH for Initial Stage 54 Test



BATT02-00056
Phenobarbital - Spent
Daily Average Dissolved Oxygen (DO) for Initial Stage 54 Test



BATT02-00056
Phenobarbital - Spent
Daily Average Light Intensity for Initial Stage 54 Test



Phenobarbital
Metamorphosis Assay:
Test Termination Data – Stage and Growth

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00056 **Test No: 1**

Test Chemical: Phenobarbital (PBB) **Target Conc: 0.0 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	201	0.6760	59	
	202	1.0425	59	Kinked Tail
	203	0.7430	59	
	204	1.2365	59	Kinked Tail
	205	1.0078	57	Kinked Tail
	206	1.0984	59	Kinked Tail
	207	0.9664	59	Kinked Tail
	208	0.6780	59	
	209	0.6476	59	
	210	0.7461	57	Kinked Tail
	211	0.6861	57	Kinked Tail
	212	0.6150	59	Kinked Tail
	213	0.9371	59	Kinked Tail
	214	0.9490	57	Kinked Tail
	215	0.9583	57	
	216	0.8680	57	
	217	1.0118	57	Kinked Tail
	218	0.5426	57	
	219	0.7672	59	Kinked Tail
	220	1.0560	57	
	Median:	**0.903**	**59**	
	Mode:		**59**	
	Mean:	**0.862**	**58.100**	
	SEM:	**0.043**	**0.228**	
	%CV:	**20.506**	**13.393**	
B	221	1.3104	59	Kinked Tail
	222	0.9238	57	
	223	1.1856	57	Kinked Tail
	224	0.9114	57	Kinked Tail
	225	1.2596	57	Kinked Tail
	226	0.7690	59	Kinked Tail
	227	0.9819	59	
	228	0.5604	56	
	229	0.8024	57	
	230	0.8433	58	Kinked Tail
	231	0.6826	57	Kinked Tail
	232	0.7732	57	
	233	1.1173	59	Kinked Tail
	234	0.6851	57	Kinked Tail
	235	0.6730	57	
	236	0.6639	57	
	237	0.5798	56	Kinked Tail
	238	0.6005	56	
	239	0.7920	57	Kinked Tail
	240	0.7225	57	
	Median:	**0.783**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.842**	**57.300**	
	SEM:	**0.050**	**0.219**	
	%CV:	**24.547**	**12.929**	
	Median:	**0.797**	**57**	
	Mode:		**57**	
	Total Mean:	**0.852**	**57.700**	
	SEM:	**0.033**	**0.169**	
	%CV:	**22.328**	**14.047**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00056 **Test No: 1**

Test Chemical: Phenobarbital (PBB) **Target Conc: 125 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	241	1.0457	59	
	242	1.2236	59	
	243	1.2878	57	
	244	0.8935	59	
	245	0.9420	59	
	246	0.9180	59	
	247	0.8090	59	
	248	1.0915	59	
	249	1.1299	57	
	250	0.8602	59	
	251	0.9192	59	
	252	1.0825	59	Kinked Tail
	253	0.7089	57	
	254	0.8259	59	
	255	0.8059	57	
	256	0.8996	57	Kinked Tail
	257	0.8325	57	
	258	0.7683	57	
	259	0.7148	57	
	Median:	**0.900**	**59**	
	Mode:		**59**	
	Mean:	**0.935**	**58.158**	
	SEM:	**0.038**	**0.233**	
	%CV:	**17.166**	**13.303**	
B	260	1.0355	59	Kinked Tail
	261	0.9432	57	
	262	0.8948	59	
	263	0.8854	59	
	264	0.5709	59	
	265	0.8751	59	
	266	0.7977	59	
	267	0.7452	59	
	268	1.0668	59	Kinked Tail
	269	1.1809	57	
	270	0.9802	59	
	271	0.6429	58	
	272	0.7874	59	
	273	0.8137	57	
	274	0.8406	57	
	275	0.8123	57	
	276	0.9996	57	
	277	0.9072	57	
	278	1.1374	58	Kinked Tail
	279	0.8961	57	Kinked Tail
	Median:	**0.885**	**58**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.883**	**58.053**	
	SEM:	**0.035**	**0.223**	
	%CV:	**16.196**	**12.735**	
	Median:	**0.894**	**59**	
	Mode:		**59**	
	Total Mean:	**0.912**	**58.128**	
	SEM:	**0.026**	**0.159**	
	%CV:	**16.672**	**12.862**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00056 **Test No: 1**

Test Chemical: Phenobarbital (PBB) **Target Conc: 250 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	280	1.0255	58	Kinked Tail
	281	1.0262	59	
	282	0.8157	59	
	283	1.0874	59	Kinked Tail
	284	1.1360	57	Kinked Tail
	285	0.7288	59	
	286	0.9224	59	
	287	1.1430	59	
	288	1.1237	59	Kinked Tail
	289	1.2999	59	
	290	0.7026	57	
	291	0.9534	59	
	292	0.8283	57	
	293	0.9601	57	Kinked Tail
	294	0.9938	59	
	295	0.8920	59	
	296	1.2015	59	
	297	0.9528	57	
	298	1.0208	58	Kinked Tail
	299	1.0209	59	Kinked Tail
	Median:	**1.007**	**59**	
	Mode:		**59**	
	Mean:	**0.992**	**58.400**	
	SEM:	**0.034**	**0.197**	
	%CV:	**15.323**	**11.549**	
B	300	0.9317	59	Kinked Tail
	301	0.8566	59	
	302	0.8346	59	
	303	0.8138	59	
	304	0.9586	59	
	305	1.1126	59	
	306	0.7950	57	
	307	0.9675	59	Kinked Tail
	308	0.8700	59	
	309	0.7591	58	
	310	0.8804	59	
	311	1.0218	59	Kinked Tail
	312	1.0674	59	
	313	0.7624	57	Kinked Tail
	314	1.2223	59	
	315	1.0493	57	
	316	0.9916	57	
	317	0.6449	57	
	318	1.1068	59	Kinked Tail
	319	0.8470	57	
	Median:	**0.906**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.925**	**58.350**	
	SEM:	**0.032**	**0.209**	
	%CV:	**14.990**	**12.218**	
	Median:	**0.959**	**59**	
	Mode:		**59**	
	Total Mean:	**0.958**	**58.375**	
	SEM:	**0.024**	**0.142**	
	%CV:	**15.365**	**11.739**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00056 **Test No: 1**

Test Chemical: Phenobarbital (PBB) **Target Conc: 500 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	320	0.8876	59	
	321	0.9676	57	
	322	0.7752	57	
	323	0.9241	58	
	324	0.8634	59	
	325	1.1544	59	
	326	0.7439	59	
	327	1.3924	59	
	328	1.2509	59	
	329	0.9098	59	
	330	0.8562	59	
	331	0.9237	59	
	332	1.1131	58	
	333	0.8836	59	
	334	0.7253	59	
	335	0.8317	57	
	336	0.8950	59	
	337	0.6352	60	
	338	0.7828	57	
	339	0.7107	59	
	Median:	**0.886**	**59**	
	Mode:		**59**	
	Mean:	**0.911**	**58.550**	
	SEM:	**0.042**	**0.198**	
	%CV:	**19.780**	**11.593**	
B	340	0.8796	59	
	341	1.0539	59	
	342	0.7863	59	
	343	1.2211	59	
	344	0.9346	59	
	345	0.9495	59	
	346	1.0871	59	
	347	1.0233	59	
	348	0.8948	59	
	349	1.4319	59	
	350	0.9247	57	
	351	0.8736	59	
	352	0.9995	57	
	353	0.7018	57	
	354	1.0896	59	
	355	0.8581	57	
	356	0.7977	57	
	357	0.8456	57	
	358	0.8967	59	
	359	0.8021	57	
	Median:	**0.911**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.953**	**58.300**	
	SEM:	**0.037**	**0.219**	
	%CV:	**17.159**	**12.818**	
	Median:	**0.895**	**59**	
	Mode:		**59**	
	Total Mean:	**0.932**	**58.425**	
	SEM:	**0.028**	**0.147**	
	%CV:	**18.376**	**12.175**	

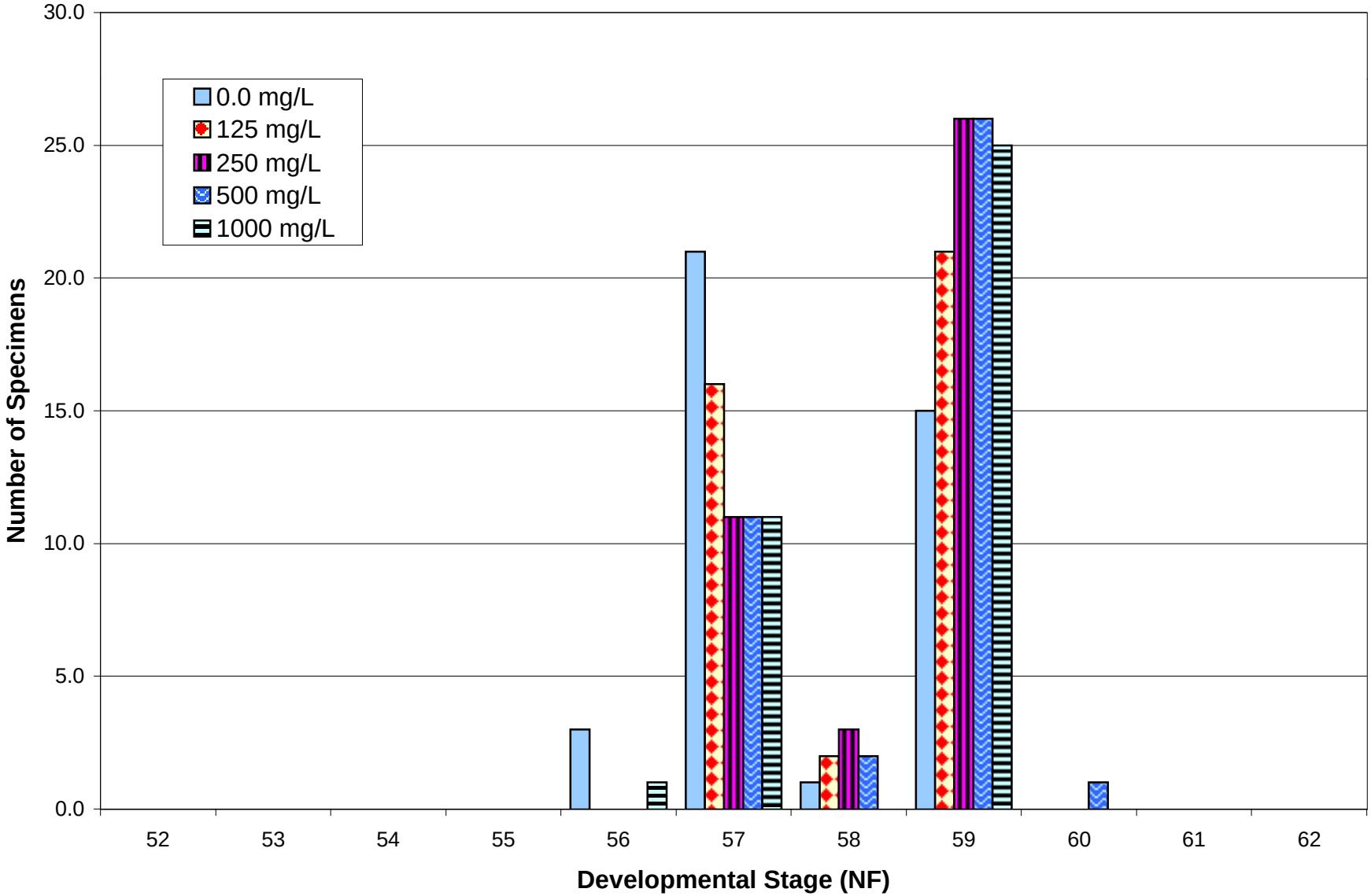
TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00056 **Test No: 1**

Test Chemical: Phenobarbital (PBB) **Target Conc: 1000 mg/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	360	0.9549	59	
	361	1.1909	59	
	362	0.7079	59	
	363	0.9260	57	
	364	0.8236	59	
	365	0.7422	59	
	366	0.9022	59	
	367	1.0852	59	
	368	1.1010	59	
	369	1.0243	59	
	370	1.1905	59	
	371	0.8699	57	
	372	0.8730	57	
	373	0.9923	57	
	374	1.3146	59	
	375	0.9844	59	
	376	0.7898	57	
	377	0.7273	57	Kinked Tail
	378	0.9970	57	
	Median:	**0.955**	**59**	
	Mode:		**59**	
	Mean:	**0.958**	**58.263**	
	SEM:	**0.039**	**0.227**	
	%CV:	**17.161**	**12.986**	
B	379	1.0114	57	
	380	1.0592	59	
	381	1.1191	59	
	382	1.3375	59	
	383	1.0948	59	
	384	0.8940	59	
	385	1.3649	59	
	386	1.0749	59	
	387	0.6639	56	
	388	1.2583	59	
	389	1.0900	59	
	390	0.7690	57	
	391	1.1188	57	
	392	1.2484	57	
	393	1.0126	59	
	394	0.9351	59	
	395	1.2082	59	
	396	1.1463	59	
	Median:	**1.092**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**1.078**	**58.389**	
	SEM:	**0.043**	**0.244**	
	%CV:	**17.587**	**13.570**	
	Median:	**1.013**	**59**	
	Mode:		**59**	
	Total Mean:	**1.016**	**58.324**	
	SEM:	**0.030**	**0.165**	
	%CV:	**18.175**	**13.114**	

BATT02-00056
Phenobarbital
Stage Distribution for Initial Stage 51 Test



TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00056 **Test No: 1**

Test Chemical: Phenobarbital (PBB) **Target Conc: 0.0 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	001	0.8610	57	
	002	1.3063	59	Kinked Tail
	003	0.8890	59	Kinked Tail
	004	0.8870	57	Kinked Tail
	005	0.8777	57	Kinked Tail
	006	1.3188	57	
	007	0.8645	59	
	008	0.8261	59	Kinked Tail
	009	0.8055	58	
	010	1.0443	57	Kinked Tail
	011	1.0459	57	Kinked Tail
	012	1.0142	57	
	013	0.9029	59	Kinked Tail
	014	0.9623	59	
	015	0.6499	56	
	016	0.8215	59	
	017	1.1421	59	Kinked Tail
	018	0.6506	56	Kinked Tail
	019	1.1799	59	
	020	0.8867	59	
	Median:	**0.888**	**59**	
	Mode:		**59**	
	Mean:	**0.947**	**57.950**	
	SEM:	**0.041**	**0.256**	
	%CV:	**18.887**	**15.053**	
D	021	0.6198	60	Kinked Tail
	022	0.8613	57	
	023	0.7607	57	
	024	0.7719	59	
	025	0.9865	59	
	026	0.8438	59	Kinked Tail
	027	0.8007	57	
	028	0.8618	57	
	029	0.7165	59	
	030	0.9992	57	
	031	0.5359	56	
	032	1.1842	58	Kinked Tail
	033	0.7537	59	
	034	1.0475	59	Kinked Tail
	035	1.0888	59	Kinked Tail
	036	0.5476	56	
	037	0.9041	59	Kinked Tail
	038	0.6809	60	
	039	1.2953	58	
	040	0.6883	57	
	Median:	**0.822**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.847**	**58.100**	
	SEM:	**0.046**	**0.280**	
	%CV:	**22.171**	**16.430**	
	Median:	**0.871**	**59**	
	Mode:		**59**	
	Total Mean:	**0.897**	**58.025**	
	SEM:	**0.031**	**0.188**	
	%CV:	**20.925**	**15.586**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00056 **Test No: 1**

Test Chemical: Phenobarbital (PBB) **Target Conc: 250 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	041	0.7966	59	
	042	1.1690	59	
	043	1.6132	59	
	044	1.2351	59	Kinked Tail
	045	1.0388	59	
	046	1.1465	59	
	047	1.4270	59	Kinked Tail
	048	1.0041	57	
	049	1.0719	58	Kinked Tail
	050	0.9095	59	
	051	0.7582	59	
	052	0.9324	59	
	053	1.3944	59	
	054	0.6172	57	
	055	0.7512	57	
	056	0.8543	59	
	057	0.9744	59	
	058	0.6262	59	
	059	1.1938	57	
	060	0.9919	57	
	Median:	**0.998**	**59**	
	Mode:		**59**	
	Mean:	**1.025**	**58.450**	
	SEM:	**0.059**	**0.198**	
	%CV:	**26.162**	**11.602**	
D	061	1.1331	59	
	062	1.3450	59	
	063	1.0460	59	
	064	1.0786	59	
	065	0.9057	59	
	066	0.9907	59	
	067	0.7936	59	
	068	0.9575	59	
	069	0.8110	57	
	070	1.0616	59	
	071	0.9241	59	
	072	1.0406	57	
	073	1.1262	59	
	074	0.6571	57	
	075	0.7390	58	
	076	1.3460	59	
	077	1.3285	59	Kinked Tail
	078	0.8168	57	
	079	0.8705	59	
	080	0.7992	57	Kinked Tail
	Median:	**0.974**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.989**	**58.450**	
	SEM:	**0.045**	**0.198**	
	%CV:	**20.150**	**11.602**	
	Median:	**0.991**	**59**	
	Mode:		**59**	
	Total Mean:	**1.007**	**58.450**	
	SEM:	**0.037**	**0.138**	
	%CV:	**23.177**	**11.453**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00056 **Test No: 1**

Test Chemical: Phenobarbital (PBB) **Target Conc: 500 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	081	1.3631	59	
	082	1.0499	58	
	083	1.1252	59	
	084	0.9718	57	
	085	1.2225	58	
	086	1.6365	58	
	087	1.2690	59	
	088	1.2342	59	
	089	1.0941	59	
	090	0.8406	58	
	091	1.0336	59	
	092	0.7634	57	
	093	1.0173	59	
	094	0.6644	57	
	095	1.1264	58	
	096	0.7346	57	
	097	0.7347	57	
	098	1.0753	57	
	099	1.0062	57	
	100	0.9312	59	
	Median:	**1.042**	**58**	
	Mode:		**59**	
	Mean:	**1.045**	**58.050**	
	SEM:	**0.053**	**0.198**	
	%CV:	**23.155**	**11.642**	
D	101	1.0078	57	
	102	0.8645	57	
	103	1.0841	58	
	104	0.9285	59	
	105	1.4397	57	
	106	0.7343	59	
	107	1.1179	59	
	108	0.7849	57	
	109	1.3095	59	
	110	1.1450	57	
	111	1.3356	59	
	112	1.0423	57	
	113	1.1099	57	
	114	1.1889	57	
	115	0.9200	57	
	116	0.7140	57	
	117	1.3175	59	
	118	1.2130	57	
	119	1.9994	57	
	120	1.6688	57	
	Median:	**1.114**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**1.146**	**57.650**	
	SEM:	**0.070**	**0.209**	
	%CV:	**29.429**	**12.292**	
	Median:	**1.080**	**58**	
	Mode:		**57**	
	Total Mean:	**1.095**	**57.850**	
	SEM:	**0.044**	**0.146**	
	%CV:	**26.734**	**12.112**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00056 **Test No: 1**

Test Chemical: Phenobarbital (PBB) **Target Conc: 1000 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	121	0.9312	59	
	122	1.2113	59	
	123	1.0683	59	
	124	0.9083	59	
	125	1.0201	59	
	126	0.8498	59	
	127	0.9198	59	
	128	0.8088	59	
	129	0.8352	59	
	130	0.9693	59	
	131	0.9983	59	
	132	0.8454	59	
	133	1.0030	59	
	134	0.7442	57	
	135	0.7850	57	
	136	1.0647	57	
	137	1.1782	57	
	138	0.8928	57	
	139	0.7407	57	
	140	0.8464	59	
	Median:	**0.914**	**59**	
	Mode:		**59**	
	Mean:	**0.931**	**58.400**	
	SEM:	**0.030**	**0.210**	
	%CV:	**13.766**	**12.305**	
D	141	1.1278	59	
	142	0.9910	59	
	143	1.2135	59	
	144	1.0769	59	
	145	0.8707	59	
	146	1.0791	59	
	147	1.0408	59	
	148	0.9356	58	
	149	1.0208	57	
	150	1.0527	59	
	151	1.0759	59	
	152	0.7690	59	
	153	0.9410	57	
	154	1.2740	59	
	155	1.1981	59	
	156	0.6445	57	
	157	1.1378	57	
	158	1.2890	57	
	159	0.9228	57	
	160	1.4487	59	
	Median:	**1.064**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**1.055**	**58.350**	
	SEM:	**0.041**	**0.209**	
	%CV:	**18.023**	**12.218**	
	Median:	**0.995**	**59**	
	Mode:		**59**	
	Total Mean:	**0.993**	**58.375**	
	SEM:	**0.027**	**0.146**	
	%CV:	**17.166**	**12.108**	

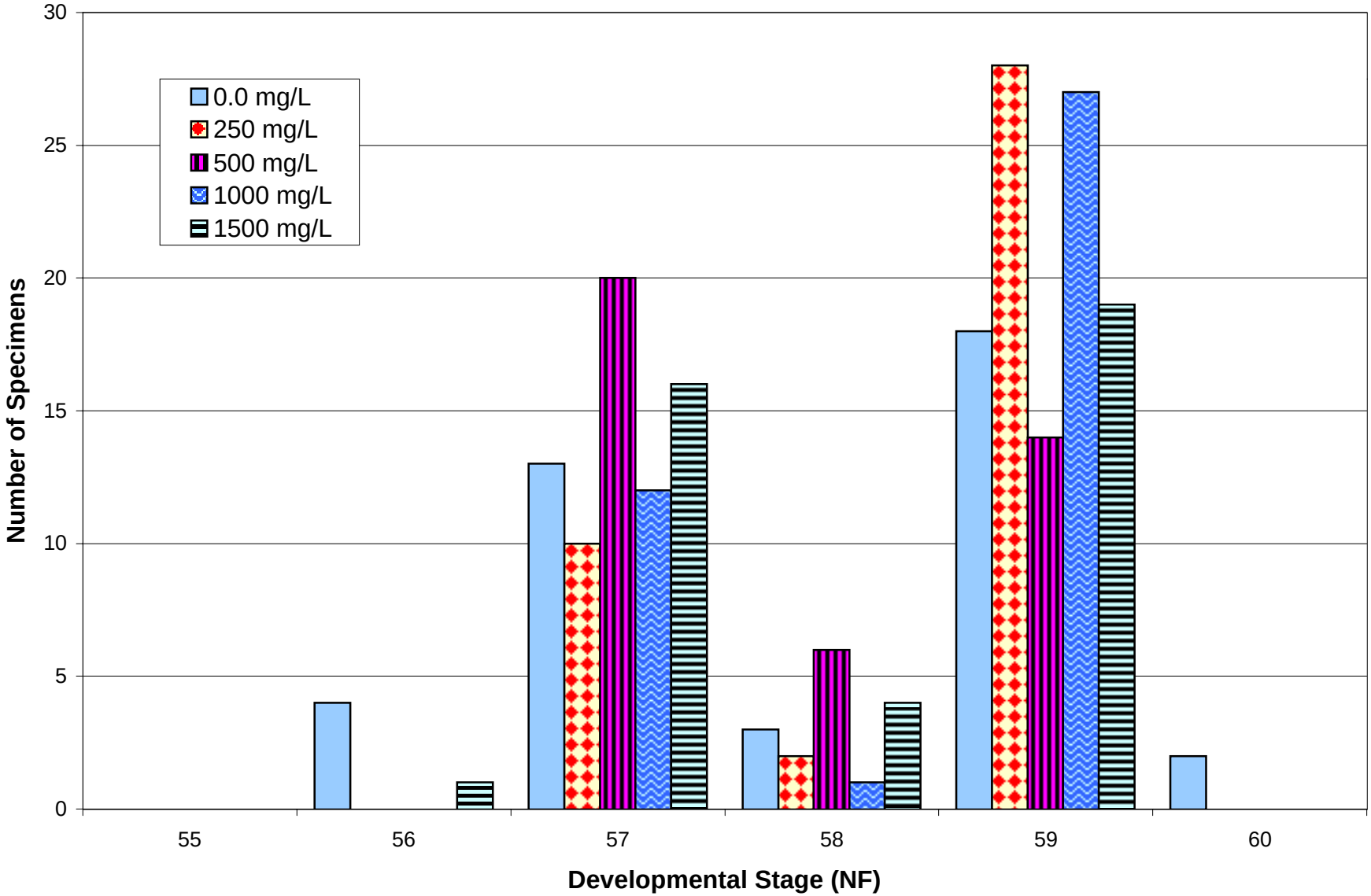
TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00056 **Test No: 1**

Test Chemical: Phenobarbital (PBB) **Target Conc: 1500 mg/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	161	1.2172	59	
	162	1.1304	59	
	163	1.1126	59	
	164	0.9904	59	
	165	0.9434	59	
	166	0.9242	59	
	167	0.9588	57	
	168	1.0608	59	
	169	0.8852	59	
	170	1.0239	59	
	171	1.0492	59	
	172	0.6811	58	
	173	1.5239	59	
	174	0.8457	57	
	175	0.9601	59	
	176	0.9285	59	
	177	0.7245	57	
	178	0.9090	57	
	179	0.9690	57	
	180	0.7783	57	
	Median:	**0.959**	**59**	
	Mode:		**59**	
	Mean:	**0.981**	**58.350**	
	SEM:	**0.041**	**0.209**	
	%CV:	**18.547**	**12.218**	
D	181	1.1813	59	
	182	1.0373	59	
	183	0.8360	59	
	184	1.1219	59	
	185	0.9876	58	
	186	0.6170	56	
	187	1.0972	58	
	188	1.2449	59	
	189	0.7109	57	
	190	0.8972	57	
	191	0.9166	58	
	192	0.7878	57	
	193	0.8679	57	
	194	0.9834	57	
	195	0.8616	57	
	196	0.9026	57	
	197	1.0866	57	
	198	1.0475	59	
	199	0.9330	57	
	200	0.9112	57	
	Median:	**0.925**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.951**	**57.700**	
	SEM:	**0.035**	**0.219**	
	%CV:	**15.948**	**12.885**	
	Median:	**0.951**	**58**	
	Mode:		**59**	
	Total Mean:	**0.966**	**58.025**	
	SEM:	**0.027**	**0.158**	
	%CV:	**17.160**	**13.124**	

BATT02-00056
Phenobarbital
Stage Distribution for Initial Stage 54 Test



Phenobarbital
Metamorphosis Assay:
Statistical Data – Stage and Growth

One Way Analysis of Variance

Data source: BATT02-00056_PBB_Stage 51_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0mg/L	40	0	57	57	59
125mg/L	39	0	59	57	59
250mg/L	40	0	59	57	59
500mg/L	40	0	59	57	59
1000mg/L	37	0	59	57	59

H = 13.340 with 4 degrees of freedom. (P = 0.010)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.010)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
500mg/L vs 0.0mg/L	35.025	2.761	Yes	
250mg/L vs 0.0mg/L	32.087	2.53	Yes	**LOEC**
1000mg/L vs 0.0mg/L	30.717	2.374	No	
125mg/L vs 0.0mg/L	20.064	1.572	Do Not Test	**NOEC**

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00056_PBB_Stage 51_Growth

Normality Test: Passed (P = 0.018)

Equal Variance Test: Passed (P = 0.175)

Group Name	N	Missing	Mean	Std Dev	SEM
0.0mg/L	40	0	0.852	0.206	0.0326
125mg/L	39	0	0.912	0.158	0.0254
250mg/L	40	0	0.958	0.15	0.0238
500mg/L	40	0	0.932	0.177	0.028
1000mg/L	37	0	1.016	0.183	0.0301

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.563	0.141	4.536	0.002
Residual	191	5.928	0.031		
Total	195	6.491			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.002).

Power of performed test with alpha = 0.050: 0.869

Multiple Comparisons versus Control Group (Bonferroni t-test):

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050	
0.0mg/L vs. 1000mg/L	0.165	4.094	<0.001	Yes	
0.0mg/L vs. 250mg/L	0.106	2.702	0.03	Yes	**LOEC**
0.0mg/L vs. 500mg/L	0.0802	2.035	0.173	No	
0.0mg/L vs. 125mg/L	0.0603	1.521	0.519	Do Not Test	**NOEC**

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1).
Note that not testing the enclosed means is a procedural rule, and a result of Do Not Tests should be treated as if there is no significant difference between the means, even though one may appear to exist.

One Way Analysis of Variance

Data source: BATT02-00056_PBB_Stage 54_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0mg/L	40	0	58.5	57	59
250mg/L	40	0	59	57.5	59
500mg/L	40	0	57.5	57	59
1000mg/L	40	0	59	57	59
1500mg/L	40	0	58	57	59

H = 10.876 with 4 degrees of freedom. (P = 0.028)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.028)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
250mg/L vs 0.0mg/L	20.913	1.616	No
1000mg/L vs 0.0mg/L	17.213	1.33	Do Not Test
500mg/L vs 0.0mg/L	11.312	0.874	Do Not Test
1500mg/L vs 0.0mg/L	1.375	0.106	Do Not Test

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00056_PBB_Stage 54_Growth

Normality Test: Passed (P = 0.027)

Equal Variance Test: Passed (P = 0.055)

Group Name	N	Missing	Mean	Std Dev	SEM
0.0mg/L	40	0	0.897	0.198	0.0313
250mg/L	40	0	1.007	0.233	0.0368
500mg/L	40	0	1.095	0.28	0.0442
1000mg/L	40	0	0.993	0.171	0.0271
1500mg/L	40	0	0.966	0.169	0.0267

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.824	0.206	4.491	0.002
Residual	195	8.946	0.0459		
Total	199	9.77			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.002).

Power of performed test with alpha = 0.050: 0.864

Multiple Comparisons versus Control Group (Bonferroni t-test):

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0.0mg/L vs. 500mg/L	0.198	4.142	<0.001	Yes
0.0mg/L vs. 250mg/L	0.11	2.292	0.092	No
0.0mg/L vs. 1000mg/L	0.0961	2.007	0.184	Do Not Test
0.0mg/L vs. 1500mg/L	0.069	1.441	0.605	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1).
Note that not testing the enclosed means is a procedural rule, and a result of Do Not Tests should be treated as if there is no significant difference between the means, even though one may appear to exist.

Phenobarbital
Test Chemical Analytical Data from Battelle:
Purity / Stability Report

Battelle
Putting Technology To Work

Chemical Repository Services for the EDSP

EPA Contract No. 68-W-01-023

1.0 TITLE AND APPROVAL

Chemistry Report for WA 3-11 Phenobarbital in well water

October 11, 2004

Prepared By:

Tim Fortman
Chemical Repository Analyst

10-11-04
Date

Approved By:

Richard M. Ecker
Director, Marine Sciences Laboratory

10/12/04
Date

Michael E Cobb
Chemical Repository Study Director

10-11-04
Date

Battelle Marine Sciences Laboratory

1529 West Sequim Bay Road

Sequim, WA 98382

Submitted to:

Doug Fort

Fort Environmental Laboratories

Stillwater, Oklahoma 74074

2.0 QA REVIEWER AND CONTRIBUTOR PAGE

Chemistry Report for WA 3-11
Phenobarbital in Well Water

Reviewed by: Mary E Lynn

Mary Lynn, EDSP Quality Assurance Representative
Windward Company, Gig Harbor

Date: 10-11-09

Personnel participating in this study:

Analyst: Tim Fortman, Rebecca Wood

Chemical Repository Study Director: Michael Cobb

3.0 EXECUTIVE SUMMARY

Chemistry Report for WA 3-11
Phenobarbital in Well Water

Parameter	Chemical
Compound Name	Phenobarbital
CAS #	50-06-6
Central File No.	1837-1
Initial Receipt Date	06/06/2002
Expiration Date	06/01/2006
Manufacturer	Sigma
Lot Number	81K2620
Battelle Study #	WA 3-11
Method	EDSP.H4-004-01

Executive Summary

Work Assignment (WA) 3-11 of the Environmental Protection Agency's Endocrine Disruptor Screening Program (EDSP) describes a reproductive and developmental study on frogs raised in water spiked with one of six study chemicals. The Chemical Repository (Repository) has the responsibility for carrying out the purity, stability, and in-life concentration determinations of the selected study chemicals for EDSP studies.

The chemical of interest for this portion of the study is Phenobarbital. The chemical purity of Phenobarbital, as determined by the manufacturer, was >99%. The analytical method developed in-house for the Phenobarbital stability study and in-life samples utilized High Performance Liquid Chromatography (HPLC) with UV/Vis detection. The Battelle-Sequim purity result by HPLC was 99.96%.

The stability of the water dosed with Phenobarbital was analyzed at 2 concentrations, one representing the stock feed water (8000 mg/L) the other representing one of the amphibian exposure concentrations (125 mg/L). These concentrations were tested over 21 days. Definition of stability for this project is less than 10% degradation. Test concentrations for both Phenobarbital solutions did not fall below this 10% limit. Phenobarbital is considered stable for 8000 mg/L and 125 mg/L for 21 days.

Notes:

Phenobarbital was not soluble enough to reach the stock concentration of 8000 mg/L. Direction from EPA indicated that adjusting the pH to 8.5 or greater would increase the solubility. This adjustment was done with a solution of sodium hydroxide and the target concentration of 8000 mg/L was achieved.

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5.0 INTRODUCTION

The goal of the Battelle-Sequim, Marine Sciences Laboratory (MSL) Repository for the Endocrine Disruptor Screening Program is to provide the participating laboratory with requested chemicals of documented quality at required concentrations and in a matrix appropriate for different toxicological tests. The EDSP Chemical Repository supplies the manufacturer's information regarding purity and stability, the material safety data sheet (MSDS) chemical information, and independent analysis of purity and stability in a matrix specified by the Study Protocol: Purity and Stability of Test Substances for Work Assignment 3-11 [EDSP Study Number: EDSP.311-01], made in collaboration with the requesting Principal Investigator. Under Work Assignment (WA) 3-11, Fort Environmental Laboratories requested purity and stability testing of Phenobarbital (figure 1).

6.0 GENERAL METHODS

Methods of standard operation of the Repository are currently addressed in MSL SOPs numbered R-001 through R-017. These procedures address chemical procurement including procurement of controlled substances, when applicable, which have unique permitting, ordering, handling, inventory, and storage requirements; chemical receipt and chain of custody, chemical log-in and labeling, inventory, chemical storage; stock solution preparation, documentation and archiving; test solution preparation, documentation and shipping; chemical disposal, and repository maintenance over time. The quality assurance (QA) requirements for procurement of chemicals for use in the Repository are addressed in Version 2 of the Quality Assurance Project Plan for EDSP Chemical Repository (currently in circulation for approval signatures).

6.1 CHEMICAL PROCUREMENT

As requested (Figure 1) by Doug Fort, the Principal Investigator for the Study, Phenobarbital, (CAS No. 50-06-6), was purchased by the Repository in June of 2002 for tests being performed at the time, this same material will be used for purity and stability analysis and used as the test chemical, in a one generational study on frogs. The Phenobarbital was purchased from Sigma, Inc. and the first shipment of lot number 81K2620 was received on 6/6/02 with a expiration date of 6/1/06 (Table 1). The chemical was logged in to the Chemical Management System (CMS) and given a CMS barcode and unique log-in (central file) number (CF-1837-1) as per the QA Project Plan (QAPP) for the EDSP Chemical Repository. The chemical was stored in the Repository in a clean, dry location at 4 deg. C, away from direct sunlight.

Table 1. Chemical Procurement Information

Parameter	Chemical
Compound Name	Phenobarbital
CAS #	50-06-6
Central File No.	1837-1
Initial Receipt Date	06/06/2002
Expiration Date	06/01/2006
Manufacturer	Sigma
Lot Number	81K2620
Battelle Study #	WA 3-11
Method	EDSP.H4-004-01

6.1.1

A 2nd lot was required to finish the frog test. This Phenobarbital was purchased from Sigma, Inc. and lot number 103K2618 was received on 9/3/04 with a expiration date of 9/06

(Table 1a). The chemical was logged in to the Chemical Management System (CMS) and given a CMS barcode and unique log-in (central file) number (CF-2260-1) as per the QA Project Plan (QAPP) for the EDSP Chemical Repository. The chemical was stored in the Repository in a clean, dry location at 4Deg. C, away from direct sunlight.

Table 1a. Chemical Procurement Information

Parameter	Chemical
Compound Name	Phenobarbital
CAS #	50-06-6
Central File No.	2260-1
Initial Receipt Date	09/03/2004
Expiration Date	09/2006
Manufacturer	Sigma
Lot Number	103K2618
Battelle Study #	WA 3-11
Method	EDSP.H4-004-01

EDSP Chemical Request Form

For EPA WA: 3-11

Study Director:
Name: Douglas J. Fort, Ph.D.
Affiliation: Fort Environmental Laboratories
Location: Stillwater, Oklahoma 74074
Telephone number: 405-624-6771

Bioassay Information:

Proposed Bioassay: Frog Metamorphosis Assay Demonstration

Test Chemical: Phenobarbital

Carrier(s): NA

Working Stock Concentration: 8000mg/L

Concentrations/Dilution Series: Stage 51 test 1000, 500, 250 mg/L
Stage 54 test 1500, 1000, 500, 250 mg/L

In vitro or *in vivo* tests? *In vivo*

Organism to be tested: *Xenopus laevis* (South African Clawed Frog)

Method of test solution administration: Static Renewal exposure system

Planned/proposed test duration: 21 days for stage 51 organisms and 14 days for stage 54 organisms

Estimated neat chemical required: 1,500 g (based on 8L/aquarium, 2 replicates/conc, 6 L daily renewal/aquarium, and 35 days of testing, plus additional 25% for contingencies.

Chemical Information:

Chemical Name: Phenobarbital

CAS: 50-06-6

Any known purity information: may refer to attached documentation

Any known stability information: Contact Dr. Joe Tietge, EPA, Duluth, MN

Desired purity (%) for test? ≥98%

Is there a known calibration standard available?
Name: P-3643, 1 mg/ml in Methanol
CAS: 50-06-6
Manufacturer: Sigma

Figure 1. EDSP Chemical Request Form for Phenobarbital

6.2 CHEMICAL PURITY

Chemical purity was verified by high performance liquid chromatographic analysis to determine areas under peaks other than the principal peak and compared to the manufacturer's certificate of analysis/purity (Appendix A). No statistical analyses were performed for the verification of chemical purity. Purity verification was conducted by making a solution of Phenobarbital in 50:50 acetonitrile:water of about 100 µg/mL. This matrix was then run on an HPLC with a UV/Vis detector. An acetonitrile:water blank was also analyzed on the system. The purity was determined by first identifying the peaks in the Phenobarbital chromatogram that were the same as the peaks in the analysis of the blank. The areas associated with these common peaks were then eliminated by inhibiting integration and the remaining peaks were reported as a percentage of the total peak area. The percentage associated with the largest peak represented the purity of Phenobarbital. The HPLC was set up with an autosampler, a 250X4.6 mm reversed-phase HPLC column, a 500 ml loop with a 450 ul injection. The HPLC system was run isocratically, with a 50% acetonitrile/50% water eluent at 1 ml per minute, the detector was set to a wavelength of 225 nm. One replicate was analyzed.

6.3 STABILITY TESTING PLAN DESIGN AND DETAIL

A general study plan for stability testing based on the WA 3-11 Chemical Request from Doug Forte (Figure 1) was developed as the *EDSP Stability Analysis and Purity Testing Plan* and is presented in Appendix B. A stock solution and one diluter solution were prepared to arrive at the chemical concentrations requested for stability analysis (Table 2). All samples were analyzed in triplicate for calculation of a mean concentration and relative standard deviation (RSD).

Phenobarbital stock matrices were prepared on 8/3/04 for testing as described in Table 2. Briefly, for the 8000 mg/L Phenobarbital 1.6 g was weighed into a 250 ml amber bottle and 200 ml of water from Fort Environmental was added using a 250 ml class A graduated cylinder. 5 ml of a 10% NaOH was added to adjust the pH to greater than 8.5 (this pH was over shot and was readjusted with 1 ml of a 50% HCL solution). The solution was agitated by hand shaking for approximately five minutes until all of the Phenobarbital was dissolved. For the 125 mg/L 1.6 mL of the above 8000 mg/L stock was measured into another 250 ml amber bottle containing 246 mL water from Fort Environmental. All measurements were done with Class A graduated cylinders. The solution was agitated to insure uniformity. Solutions were stored on the counter at room temperature for the duration of the test.

Table 2. Stock Matrix Composition for Stability Testing

Study, Duration	Test Chemical	Target Conc.	Nominal Conc.	Sample ID	Stock Matrix
WA 3-11, 21 days	Phenobarbital	8000 mg/L	7767 mg/L	Stock	1.6 g in 206 ml Water
		125 ug/L	124.3 mg/L	Diluter	4 ml of 8000 mg/L in 250 ml water

6.4 ANALYTICAL CHEMISTRY FOR STABILITY TESTING

Chemical stability was evaluated under storage conditions and matrix specifications as requested by the participating laboratory. At initiation and at each time period throughout the duration of the test, the chemical concentration was determined by chromatographic analysis. Triplicate aliquots were tested. The frequency of determinations and the duration of testing

were determined by the requesting principal investigator and the chemists based on *a priori* knowledge about chemical stability.

Phenobarbital stock solution was sampled by taking 0.025 mL of the stock using a positive displacement pipette and diluting it with 2.5 mL of mobile phase, using a 5 ml graduated pipette into a 4 mL vial. A transfer pipette was used to mix this solution and transfer it to an autosampler vial compatible with the HPLC autosampler.

Phenobarbital diluter solution was sampled by taking 0.1 mL of the diluter sample using a syringe and diluting it to 1 mL with 0.9 mL of mobile phase, using a 1 mL syringe into a 1.8 mL vial. A transfer pipette was used to mix this solution and transfer it to an autosampler vial compatible with the HPLC autosampler. Water blanks were prepared the same way. Spike solution, prepared similarly to the stability solutions, but stored at 4 deg. C. was used as a QA/QC sample, this sample was prepared for analysis in the same way as the stability solutions. These solutions was then analyzed on the HPLC/UV/Vis for quantification. Initial calibration verification and continuing calibration verification (ICV/CCV) samples were analyzed to demonstrate on-going calibration accuracy.

Table 3. HPLC Conditions

Detector	UV/Vis, Wavelength, 225 nm
Flow Rate	1 ml/minute
Injection Volume	450 ul
Eluent; flow pattern	50% Acetonitrile:50% water, Isocratic
Column	Reverse Phase, Phenominex Hydro RP

7.0 RESULTS

7.1 CHEMICAL PURITY

Battelle-Sequim conducted an HPLC-UV/Vis purity scan on both lots of Phenobarbital. The chromatograms, after blank correction, showed only one large peak that had the appropriate retention time for Phenobarbital with several smaller peaks. The area of the Phenobarbital peak was 99.96% of the total area of all peaks in the chromatogram for lot 081K2620 and 100.67% of the total area of all peaks in the chromatogram. Chemical purity of Phenobarbital determined by the manufacturer via TLC was >99% (Appendix A).

7.2 ANALYTICAL CHEMISTRY FOR STABILITY TESTING

Chemical stability testing was initiated on 08/04/04. Chemical concentrations were determined 7 times over a period of 21 days. Concentrations measured in the stock Stability test ranged from 94% to 109% of the target concentration. Concentrations measured in the diluter Stability test ranged from 99% to 111% of the target concentration. The analytical and quality control (QC) results are presented in Appendix C. 5 point calibration curves all met the R^2 criteria of 0.995, see table 3. Blanks and matrix spikes were analyzed with every batch for QC purposes. All blanks were less than 3 times the detection limit of Phenobarbital of 0.111 mg/L for diluter samples and 1.112 mg/ml stock solutions. ICV/CCV results ranged from 96% to 107%. All of the QC data were inside acceptance criteria. The MDL was determined to be 0.111 mg/L.

Table 4. Calibration Acceptance

Calibration Date	R2 Value (≥ .995)	Curve Fit
8/3/04	0.999709	Quadratic

8/4/04	0.999733	Quadratic
8/4/04	0.999803	Quadratic
8/6/04	0.999792	Quadratic
8/10/04	0.999809	Quadratic
8/13/04	0.999779	Quadratic
8/17/04	0.999852	Quadratic
8/20/04	0.999888	Quadratic
8/25/04	0.999898	Quadratic

8.0 CONCLUSIONS

Stability of the stock solution of Phenobarbital remained within 90% of the day zero concentration on all 21 days of the study. Samples will be collected and shipped to the lab in Sequim for analysis twice during the in-life study. All analyses will be carried out within 21 days of day of sampling.

APPENDIX A

MANUFACTURER'S CERTIFICATE OF ANALYSIS/PURITY

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Phenobarbital
Product Number	P1636
Product Brand	SIGMA
CAS Number	50-06-6
Molecular Formula	$C_{12}H_{12}N_2O_3$
Molecular Weight	232.24

TEST	SPECIFICATION	LOT 081K2620 RESULTS
APPEARANCE	WHITE POWDER	CONFORMS
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 50 MG/ML IN ETHANOL	CONFORMS
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONFORMS
PURITY BY SODIUM HYDROXIDE TITRATION	NLT 99%	100.8%
PURITY BY THIN LAYER CHROMATOGRAPHY	NLT 99%	>99%
SHELF LIFE SOP QC-12-006	5 YEARS	SEPTEMBER 2006
QC ACCEPTANCE DATE		SEPTEMBER 2001

Lori Schulz, Manager
Analytical Services

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Phenobarbital
Product Number	P1636
Product Brand	SIGMA
CAS Number	50-06-6
Molecular Formula	$C_{12}H_{12}N_2O_3$
Molecular Weight	232.24

TEST	SPECIFICATION	LOT 103K2618 RESULTS
APPEARANCE	WHITE POWDER	CONFORMS
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 50MG/ML IN ETHANOL	CONFORMS
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONFORMS
PURITY BY SODIUM HYDROXIDE TITRATION	NLT 99%	100.8%
PURITY BY THIN LAYER CHROMATOGRAPHY	NLT 99%	>99%
SHELF LIFE SOP QC-12-006	5 YEARS	SEPTEMBER 2006
QC ACCEPTANCE DATE		OCTOBER 2003

Lori Schulz, Manager
Analytical Services

APPENDIX B

PURITY AND STABILITY TESTING PROTOCOL

Study Protocol:
Purity and Stability of Test Substances for Work Assignment 3-11
EDSP Study Number: EDSP.311-01

Study Objective: •

To verify the purity of 6 test substances (listed in Table 1), the solubility, (at the concentrations specified in Table 2), and to determine the stability of same dissolved in well water over a 21 day period. This study is in support of EPA contract number 68-W-01-023, MSL Work Assignment Number 3-11, Task 3, Amphibian Metamorphosis Assay Demonstration Study.

Address of Testing Facility and Sponsor's Representative:

Battelle – Marine Science Laboratory
1529 West Sequim Bay Road
Sequim, Washington

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propyl-2-thiouracil	PTU
L-Thyroxine	T4
Phenobarbital	ΦBarb
Pregnenolone-16-Alpha-carbonitrile	P16αc
Dexamethosone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	SOP MSL-O-	TBA	TBA	TBA	TBA	TBA

TABLE 3
Reference Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	TCI America	Fluka	TBA	TBA	TBA	Aldrich
CAS #	51-52-5	51-48-9	TBA	TBA	TBA	60-56-0
Lot #	0630144	411874/1	TBA	TBA	TBA	06109DP
Purity req.	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%	≥ 98%
Mfg. Purity claim	> 99%	99.6%	TBA	TBA	TBA	99.7%

Proposed experimental start and termination dates:

Start Date – November 1, 2003
Termination Date – June 22, 2004

Experimental Design:

- Solubility of the substances will be determined as per SOP EDSP.C-0XX-01 (EDSP Chemical Characterization-TBA) in the diluent (well water) at a concentration approximately 10% above the stock solution concentration (see Table 2).
- If the solubility of the test substance is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP EDSP.C-0XX-01. Note: exposure concentrations won't change, so stability of an exposure solution will be carried out at the target concentration.
- Substance purity will be verified using HPLC, GC/MS or IC/ICP/MS as appropriate, and should be 98% or greater and be within ±2% of the value provided on the Certificate of Analysis by the manufacturer
- Stock solution – made up to concentrations delineated in Table 2 (approximate levels) in well water (obtained from MSL).
- Stability test solutions – Stability testing will be carried out on stock solutions and at a second level near or within the in-life exposure dose range. Nominal concentrations to be tested are delineated in Table 2 in well water (obtained from MSL).
- Storage and Labeling Requirements of Test Solution – stored in the dark (i.e., same storage conditions of solutions employed in the in-life studies of WA 3-11) at room temperature (20-25˚C). Minimally, containers will be labeled with the name of the test substance, the date of preparation, the solution concentration, and the study number.
- Test Schedule – Samples will be analyzed the day of collection from the test solution
- Replicates – 3 per sample tested at each analysis time point
- Sampling Timing – Sampling will be carried out on the first day and then as appropriate for statistical analysis for duration of the 21 day study
- For details of the analytical methods see the appropriate SOP associated with the analytical method employed for the specific substance: MSL-O-018-00 (HPLC), MSL-X-XXX-XX (GC/MS)-TBA, MSL-X-XXX-XX (ICP/MS)-TBA.

Statistical Analysis:

Log linear degradation curves will be fit to the stability data to describe the trend of the chemical concentration over time. Lack of fit and residual plots will be evaluated to determine the form of

the regression. Power calculations based on the observed variability will be used to determine the sensitivity of the test to detect degraded concentrations.

Regulatory requirements:

This study will be conducted in compliance with EPA FIFRA Good Laboratory Practices (40CFR, Part 160).

Records to be maintained:

All records and the final report generated as a result of this study will be transported to and maintained in the EDSP Data Coordination Center for archival purposes at the following address:

EDSP Data Coordination Center
Battelle Columbus Operations (PNL)
505 King Ave
Columbus, OH 43201

Sponsor Representative_____ _____
Michael Blanton **Date**

Study Director_____ _____
Michael Cobb **Date**

PROTOCOL AMENDMENT
STUDY NUMBER: EDSP.311-01
AMENDMENT NUMBER: A-1
DATE: FEBRUARY 4, 2004
Page 1 of 2

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01			DATE: February 4, 2004		
AMENDMENT NUMBER: A-1			WAL/STUDY DIRECTOR:		
NOTEBOOK NUMBER: N/A			Michael Blanton/Michael Cobb		
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

1. Solubility of the substances will be determined as per SOP EDSP.C-0XX-01 (EDSP Chemical Characterization-TBA)…
   If the solubility of the test substance is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP EDSP.C-0XX-01.
2. For details of the analytical methods see the appropriate SOP associated with the analytical method employed for the specific substance: MSL-O-018-00 (HPLC), MSL-X-XXX-XX (GC/MS)-TBA, MSL-X-XXX-XX (ICP/MS)-TBA.
3. All records and the final report generated as a result of this study will be transported to and maintained in the EDSP Data Coordination Center for archival purposes at the following address:
   EDSP Data Coordination Center
   Battelle Columbus Operations (PNL)
   505 King Ave
   Columbus, OH 43201

4. Sponsor Representative_____ _____
   Michael Blanton Date

AMENDMENT:

1. Sentences amended to read: “Solubility of the substances will be determined as per SOP MSL-R-009 (Chemical Characterization)”…
   “If the solubility of the test substances is low, tests will be carried out at about 10% less than the solubility limit as specified by SOP MSL-R-009.”
   Last several SOPs identified in #2 below.
   Sentence amended to read: “For details of the analytical methods, see the method identified in Table 2 and provided in each specific chemical characterization notebook. These methods are supported by specific operation and maintenance SOPs appropriate to the analytical method employed for the particular substance under study: MSL-R-012 (Operation and Maintenance of HPLC Systems), MSL-O-008-02 (Operation and Maintenance of Gas Chromatographs (GC) and Gas Chromatograph/Mass
2. Spectrometer (GC/MS) Systems), MSL-R-014 (Operation & Maintenance of IC ICP/MS Systems – currently being drafted).

PROTOCOL AMENDMENT
STUDY NUMBER: EDSP.311-01
AMENDMENT NUMBER: A-1
DATE: FEBRUARY 4, 2004
Page 2 of 2

3. Sentence amended to read: "All records and the final report generated as a result of this study will be maintained and archived per SOP MSL-D-003 (Archiving of Records, Data and Retired SOPs). In addition, the documentation will be transported in electronic form to the EDSP Data Coordination Center for archival purposes at the following address:

   EDSP Data Coordination Center
   Battelle Columbus Operations
   505 King Ave
   Columbus, OH 43201

4. Sponsor Representative_____ _____
   David Houchens Date

REASON FOR CHANGE:

1. The SOP numbers were not available (in the "Experimental Design" section) when the protocol was written. These SOP numbers are now available and are provided in this amendment.
2. Our new documentation approach involves generation of a specific "Analytical Method" document developed for the chemical characterization and provided in the final report. Each specific "Method" will correspond to a generic SOP.
3. Original document section in "Records to be maintained" section requires clarification. Battelle Columbus Operations incorrectly labeled as PNL.
4. Correct Sponsor Representative is EDSP Program Manager.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01				DATE: February 24, 2004	
AMENDMENT NUMBER: A-2				WAL/STUDY DIRECTOR:	
NOTEBOOK NUMBER: N/A				Michael Blanton/Michael Cobb	
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

Table entries that are earmarked for change in this amendment are highlighted in Yellow

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	SOP MSL-0-018-00 (HPLC)	TBA	TBA	TBA	TBA	TBA

AMENDMENT:
New table entries are highlighted in **Orange**

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	0, 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50, 100 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

REASON FOR CHANGE:

Information was not available when protocol was originally generated.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.311-01			DATE: March 30, 2004		
AMENDMENT NUMBER: A-3			WAL/STUDY DIRECTOR:		
NOTEBOOK NUMBER: N/A			Michael Blanton/Michael Cobb		
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: Work Assignment 3-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

Table entries that are earmarked for change in this amendment are presented underlined and italicized in ***Georgia Font***

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propyl-*2*-thiouracil	PTU
L-Thyroxine	T4
Phenobarbital	ΦBarb
Pregnenolone-16-Alpha-carbonitrile	P16αc
Dexameth*o*sone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	*0,* 2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50*, 100* mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

TBA = To Be Amended

AMENDMENT:

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
6-n-Propylthiouracil	PTU
L-Thyroxine	T4
Phenobarbital	ΦBarb
Pregnenolone-16-Alpha-carbonitrile	P16αc
Dexamethasone	DxM
Methimazole	Mtmz

TABLE 2
Test Substance Specifications:

TBA = To Be Amended

Chemical Name	PTU	T4	ΦBarb	P16αc	DxM	Mtmz
Manufacturer	Sigma	Sigma	TBA	TBA	TBA	Sigma
CAS #	51-52-5	51-48-9	50-06-6	1434-54-4	50-02-2	60-56-0
Lot #	0630144	092K1061	TBA	TBA	TBA	073K3519
Purity req.	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%	≥ 97%
Mfg. Purity Claim	100%	99.4%	TBA	TBA	TBA	99.9%
Solubility Requirement	≥ 770 mg/L	≥ 4.44 mg/L	TBA	TBA	TBA	2750 mg/L
Concentration Stock Solution	700 mg/L	4.04 mg/L	TBA	TBA	TBA	2500 mg/L
Duration Stability Study	21 days	21 days	21 days	21 days	21 days	21 days
Concentrations For Stability Study	1.5 mg/L and 700 mg/L	1.0 µg/L & 4.04 mg/L	TBA	TBA	TBA	2500 mg/L & 25 mg/L
In-life Exposure Concentrations	2.5, 10, 20 mg/L	0, 0.5, 1.0, 2.0, 4.0 µg/L	TBA	TBA	TBA	6.25, 12.5, 25, 50 mg/L
Carrier	Well Water	Well Water	Well Water	Well Water	Well Water	Well Water
Analytical Method	EDSP.H4-001-00	EDSP.I8-003-00	EDSP.H4-004-00	EDSP.H4-005-00	EDSP.H4-006-00	EDSP.H4-002-00

REASON FOR CHANGE:

Information was not available when protocol was originally generated.

Approval:

Work Assignment Leader _____ Date _____

Study Director _____ Date _____

EDSP QA Representative _____ Date _____

MSL Laboratory Director _____ Date _____

EDSP Program Management _____ Date _____

EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

ENDOCRINE DISRUPTOR SCREENING PROGRAM DEVIATION FORM

STUDY NUMBER: EDSP.311-01				WAL/STUDY DIRECTOR: Michael Blanton/Michael Cobb for WA 3-11/Eric Crecelius for EDSP Chemical Repository	
DEVIATION NUMBER: EDSP.311-01-D3				Date: 10/11/2004	
TITLE OF STUDY: Purity and Stability of Test Substances for Work Assignment 3-11					
QAPP/PROTOCOL ID: See above					
DEVIATION RELATING TO:					
[ ]	QAPP	[ ]	QMP	[X]	Protocol
[ ]	SOP	[ ]	Method	[ ]	Miscellaneous Documentation

ORIGINAL DOCUMENT SPECIFICATIONS
Table 2 of Amendment A-4 of Chemical Repository protocol EDSP.311-01 shows the lot number for Phenobarbital as 081K2620.
DEVIATION
An additional order of Phenobarbital, lot # 62K8804, was required to carry out the in-life studies.
REASON/IMPACT
The new lot of test substance, # 62K8804, was not listed on the original protocol. The new material was purchased, purity was verified, and it was shipped to Fort Laboratories for use in the in-life studies.
There is no impact to the project or data.
PROPOSED CORRECTIVE ACTION AND SCHEDULE FOR COMPLETION:
None, beyond this documentation.
ACTIONS TO PREVENT RECURRENCE:
Not applicable.

Approval:

Michael Blanton
Work Assignment Leader _____ Date _____

Michael Cobb,
Study Director _____ Date _____
Mary Lynn,
EDSP QAU Representative _____ Date _____
Richard Ecker
MSL Laboratory Director _____ Date _____
David Houchens
EDSP Program Management _____ Date _____
Terri Pollock
EDSP Battelle QAM _____ Date _____

cc: Send final approved copies to:
Michael Cobb
Chemical Repository Study Director

NOTE: A signed copy of this protocol and all relevant Amendments and deviations are available in the files of the Chemical Repository Study Director in MSL 5.

APPENDIX C
ANALYTICAL RESULTS OF STABILITY TESTING

Table C1. Phenobarbital concentration in Fort Environmental Water (mg/L)

Target Conc. (mg/L)	Sample ID	Date	Phenobarbital (mg/L)	Average (mg/L)	Recovery	RSD
7767	stock-1r R-1	08/04/04	7847			
7767	stock-r1R-2	08/04/04	7798	7735	99.58%	1.99%
7767	stock-1r R-3	08/04/04	7559			
7767	stock-2 R-1	08/06/04	7647			
7767	stock-2 R-2	08/06/04	7673	7748	99.75%	1.98%
7767	stock-2 R-3	08/06/04	7924			
7767	stock-3r R-1	08/10/04	8600			
7767	stock-3rR-2	08/10/04	8116	8501	109.45%	4.08%
7767	stock-3r R-3	08/10/04	8787			
7767	stock-4r R-1	08/13/04	8085			
7767	stock-4rR-2	08/13/04	8182	7922	102.00%	4.67%
7767	stock-4r R-3	08/13/04	7499			
7767	stock-5r R-1	08/17/04	7438			
7767	stock-5rR-2	08/17/04	7726	7723	99.43%	3.66%
7767	stock-5r R-3	08/17/04	8004			
7767	stock-6r R-1	08/20/04	7229			
7767	stock-6rR-2	08/20/04	6958	7263	93.52%	4.46%
7767	stock-6r R-3	08/20/04	7603			
7767	stock-7 R-1	08/25/04	7280			
7767	stock-7 R-2	08/25/04	7426	7352	94.65%	0.99%
7767	stock-7 R-3	08/25/04	7349			
124.3	Dil-1r R-1	08/04/04	131.6			
124.3	Dil-1r R-2	08/04/04	130.8	129.5	104.22%	2.22%
124.3	Dil-1r R-3	08/04/04	126.2			
124.3	Dil-2 R-1	08/06/04	124.0			
124.3	Dil-2 R-2	08/06/04	124.4	123.5	99.39%	0.94%
124.3	Dil-2 R-3	08/06/04	122.2			
124.3	Dil-3r R-1	08/10/04	133.9			
124.3	Dil-3r R-2	08/10/04	133.0	132.5	106.62%	1.21%
124.3	Dil-3r R-3	08/10/04	130.7			
124.3	Dil-4r R-1	08/13/04	136.9			
124.3	Dil-4r R-2	08/13/04	129.6	130.9	105.31%	4.19%
124.3	Dil-4r R-3	08/13/04	126.2			
124.3	Dil-5r R-1	08/17/04	126.5			
124.3	Dil-5r R-2	08/17/04	122.1	123.6	99.41%	2.07%
124.3	Dil-5r R-3	08/17/04	122.1			
124.3	Dil-6r R-1	08/20/04	135.9			
124.3	Dil-6r R-2	08/20/04	139.8	138.0	111.01%	1.43%
124.3	Dil-6r R-3	08/20/04	138.2			
124.3	Dil-7 R-1	08/25/04	122.8			
124.3	Dil-7 R-2	08/25/04	126.0	125.9	101.27%	2.41%
124.3	Dil-7 R-3	08/25/04	128.8			

Table C2. ICV/CCV Data for Phenobarbital in Fort Environmental Water

Sample Name	Date	Expected Phenobarbital (mg/L)	Measured Phenobarbital (mg/L)	Recovery
Phen4 20 ug-ml ICV	08/04/04	20	20.3924	101.96%
Phen2C 20 ug-ml CCV	08/04/04	20.16	20.8770	103.56%
Phen2C 20 ug-ml CCV	08/04/04	20.16	21.1033	104.68%
Phen2C 20 ug-ml CCV	08/04/04	20.16	20.7259	102.81%
Phen4 20 ug-ml ICV	08/06/04	20	20.6769	103.38%
Phen2C 20 ug-ml CCV	08/06/04	20.16	20.9436	103.89%
Phen2C 20 ug-ml CCV	08/06/04	20.16	20.8940	103.64%
Phen2C 20 ug-ml CCV	08/06/04	20.16	20.4105	101.24%
Phen4 20 ug-ml ICV	08/10/04	20	21.2341	106.17%
Phen2C 20 ug-ml CCV	08/10/04	20.16	21.3954	106.13%
Phen2C 20ug-ml CCV	08/10/04	20.16	21.3538	105.92%
Phen2C 20 ug-ml CCV	08/10/04	20.16	20.5565	101.97%
Phen4 20 ug-ml ICV	08/13/04	20	20.5459	102.73%
Phen2C 20 ug-ml CCV	08/13/04	20.16	20.7950	103.15%
Phen2C 20ug-ml CCV	08/13/04	20.16	20.7061	102.71%
Phen2C 20 ug-ml CCV	08/13/04	20.16	20.3014	100.70%
Phen4 20 ug-ml ICV	08/17/04	20	19.6424	98.21%
Phen2C 20 ug-ml CCV	08/17/04	20.16	20.1210	99.81%
Phen2C 20ug-ml CCV	08/17/04	20.16	19.5725	97.09%
Phen2C 20 ug-ml CCV	08/17/04	20.16	19.3046	95.76%
Phen4 20 ug-ml ICV	08/20/04	20	20.2991	101.50%
Phen2C 20 ug-ml CCV	08/20/04	20.16	20.9681	104.01%
Phen2C 20ug-ml CCV	08/20/04	20.16	20.8303	103.33%
Phen2C 20 ug-ml CCV	08/20/04	20.16	21.4887	106.59%
Phen4 20 ug-ml ICV	08/25/04	20	20.5075	102.54%
Phen7 20 cal check	08/25/04	20.08	20.1495	100.35%
Phen2C 20 ug-ml CCV	08/25/04	20.16	20.8747	103.55%
Phen2C 20ug-ml CCV	08/25/04	20.16	21.0802	104.56%
Phen2C 20 ug-ml CCV	08/25/04	21.16	21.3754	101.02%

Table C3. Spike Recovery Data for Phenobarbital in Fort Environmental Water

Target Conc. (mg/L)	Sample ID	Date	Phenobarbital (mg/L)	Average (mg/L)	Recovery	RSD
201.6	Spike 1r R-1	08/04/04	206.1			
201.6	Spike 1r R-2	08/04/04	210.2	207.9	103.1%	1.00%
201.6	Spike 1r R-3	08/04/04	207.4			
201.6	Spike 2 R-1	08/06/04	204.0			
201.6	Spike 2 R-2	08/06/04	202.1	203.1	100.7%	0.47%
201.6	Spike 2 R-3	08/06/04	203.0			
201.6	Spike 3r R-1	08/10/04	213.7			
201.6	Spike 3r R-2	08/10/04	220.9	214.8	106.6%	2.61%
201.6	Spike 3r R-3	08/10/04	209.9			
201.6	Spike 4r R-1	08/13/04	217.4			
201.6	Spike 4r R-2	08/13/04	215.5	217.5	107.9%	0.94%
201.6	Spike 4r R-3	08/13/04	219.6			
201.6	Spike 5r R-1	08/17/04	201.5			
201.6	Spike 5r R-2	08/17/04	210.0	206.2	102.3%	2.11%
201.6	Spike 5r R-3	08/17/04	207.2			
201.6	Spike 6r R-1	08/20/04	204.5			
201.6	Spike 6r R-2	08/20/04	218.6	213.6	106.0%	3.71%
201.6	Spike 6r R-3	08/20/04	217.8			
201.6	Spike 7 R-1	08/25/04	209.0			
201.6	Spike 7 R-2	08/25/04	220.9	215.1	106.7%	2.76%
201.6	Spike 7 R-3	08/25/04	215.3			

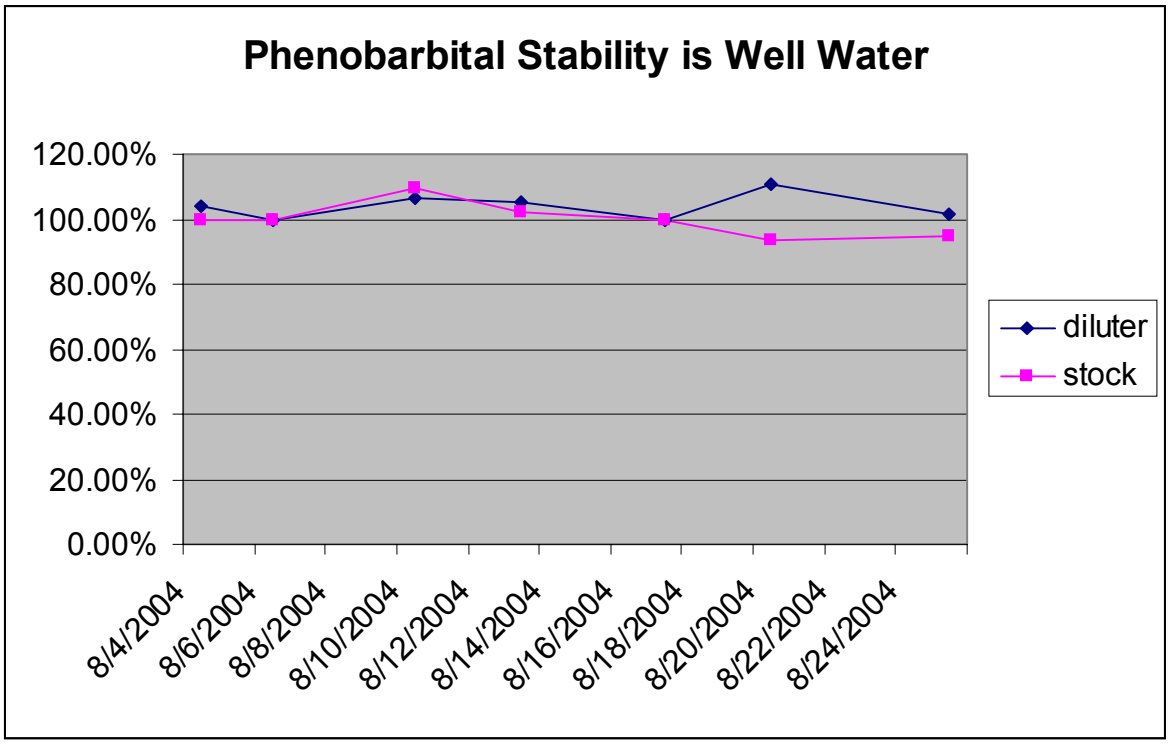


Figure 2. Observed concentration of Phenobarbital with a target concentration of 8000 mg/L (Stock) and 125 mg/L (Diluter) plotted against time

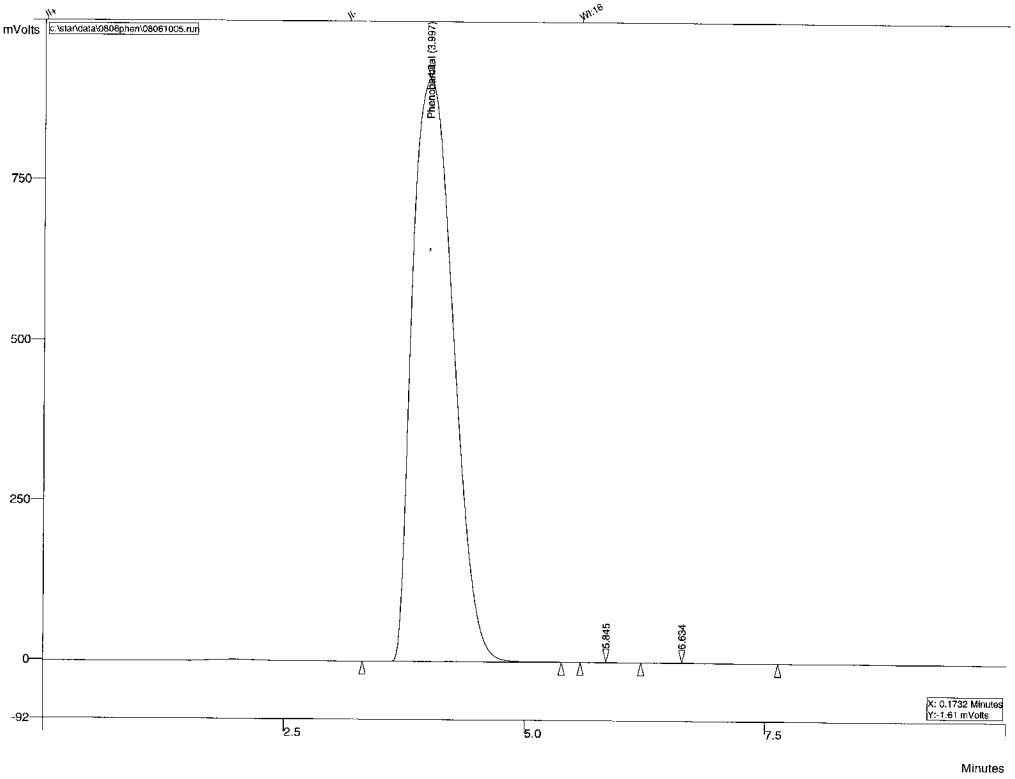


Figure 3. Typical Chromatogram, 50 ug/ml Phenobarbital

Phenobarbital
Test Chemical Analytical Data from Battelle:
In-Life Test Chemical Analysis Report

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE. USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client: Battelle
Submit Report To: Doug Fort
Sampler's Signature: Andrea Walker
P.O. #:

BATT02-00056

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
002	8000 mg/L Phenobarbital Stock	8/25/04 1600	2	G	Blue Ice	Phenobarbital
003	$DeCl_2$ Tap H_2O	8/26/04 1600	1			
004	125 mg/L Phenobarbital		1			
005	250 mg/L Phenobarbital		1			
006	500 mg/L Phenobarbital		1			
007	1000 mg/L Phenobarbital		2			
008	8000 mg/L Phenobarbital Stock	8/31/04 1000	1			
009	$DeCl_2$ Tap H_2O	9/1/04 1600	1			
010	250 mg/L Phenobarbital		1			
011	500 mg/L Phenobarbital		1			
012	1000 mg/L Phenobarbital		1			
013	1500 mg/L Phenobarbital		1			
014	8000 mg/L Phenobarbital Stock		2			

SPECIAL INSTRUCTIONS:
002 (NEW STOCK)
003-007 (STAGE 5T, REP A)
008 (NEW STOCK)
009-013 (STAGE 5T, REP C)
014 (1d OLD STOCK)

DATE:
INITIALS:

Andrea Walker	9/2/04 1700	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE. USING BLACK INK

COPY

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Walker
P.O. #:	

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road

Stillwater, OK 74074

405-824-6771 Fax 405-377-3388

mail@fortlabs.com

BATID2-000516

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
015	8000 mg/L Phenobarbital Stock	9/3/04 1600	1	G	Wet Ice	Phenobarbital
017	8000 mg/L Phenobarbital Stock	9/8/04 1430	1	"	"	"
019	8000 mg/L Phenobarbital Stock	9/7/04 0930	1	"	"	"
019	Dech. Tap H_2O B	9/9/04 1030	1	"	"	"
020	Dech. Tap H_2O D	" "	1	"	"	"
021	250 mg/L Phenobarbital	" "	1	"	"	"
022	500 mg/L Phenobarbital	" "	2	"	"	"

SPECIAL INSTRUCTIONS:

015 - (New Stock)

017 - (New Stock)

019 - (4 d old Stock)

019 & 021 - (Stage 51, Rep. B)

020 & 022 - (Stage 54, Rep. D)

Please return ice chest.

DATE.

INITIALS:

Andrea Walker	9/9/04 1700	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

Mistake. AW 9/9/04

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

COPY

Client: Battelle
Submit Report To: Doug Fort
Sampler's Signature: Andria Walker
P.O. #:

Fort Lab Use Only
BATT02-00056

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only		
024	8000 mg/L Phenobarbital Stock	9/10/04 1130	1	G	Blue ice	Phenobarbital			
025	DeCl$_2$ Tap H_2O	9/15/04 1600	1						
026	250 mg/L Phenobarbital		1						
027	500 mg/L Phenobarbital		1						
028	1000 mg/L Phenobarbital		1						
029	1500 mg/L Phenobarbital		2						
030	DeCl$_2$ Tap H_2O	9/16/04 1600	1						
031	125 mg/L Phenobarbital		1						
032	250 mg/L Phenobarbital		1						
033	500 mg/L Phenobarbital		1						
034	1000 mg/L Phenobarbital		2						

SPECIAL INSTRUCTIONS:
024 - (New Stock)
025 - 029 (Stage 54, Rep. D)
030-034 (Stage 51, Rep. B)
Please return ice chest.

DATE:
INITIALS:

Andria Walker	9/16/04 1700	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

Phenobarbital
Thyroid Histology Data from EPL:
Sample Identification Key
Thyroid Pathology Report

EPL Sample Key for Phenobarbital

FEL Sample Number	Individual Specimen Number[1]	Conc. Replicate Number	Initial Specimen Stage	Chemical Conc. (mg/L)
035	205, 211, 214, 216, 218 222, 223, 229, 231, 235	A B	51	0
036	243, 249, 255, 257, 258 261, 269, 273, 275, 277	A B	51	125
037	303, 305, 310, 314, 318 286, 288, 291, 294, 296	A B	51	250
038	320, 324, 326, 330, 339 341, 344, 349, 351, 358	A B	51	500
039	360, 362, 368, 374, 375 381, 384, 386, 393, 395	A B	51	1000
040	002, 003, 007, 016, 019 024, 026, 033, 035, 037	C D	54	0
041	042, 047, 050, 052, 058 063, 066, 071, 073, 079	C D	54	250
042	081, 083, 088, 091, 100 104, 107, 109, 111, 117	C D	54	500
043	124, 126, 129, 131, 140 144, 147, 150, 152, 155	C D	54	1000
044	161, 164, 168, 173, 176 182, 183, 184, 188, 198	C D	54	1500

[1] Individual specimens for thyroid histology were randomly selected using *Principles and Procedures of Statistics, A Biometrical Approach*, 2nd Ed., by Robert G.D. Steel and James H. Torrie, McGraw-Hill Book Co., USA, 1980.

EPL®

EXPERIMENTAL PATHOLOGY LABORATORIES, INC.

BATTELLE
STUDY NUMBER BATT02-00056
EPL PROJECT NUMBER 237-012

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

DRAFT PATHOLOGY REPORT

Submitted by:

Experimental Pathology Laboratories, Inc.
P.O. Box 474
Herndon, VA 20172-0474
(703) 471-7060

Submitted to:

Battelle
Columbus, OH 43201

March 3, 2005

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DRAFT PATHOLOGY SUMMARY

BATTELLE
STUDY NUMBER BATT02-00056
EPL PROJECT NUMBER 237-012

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

DRAFT PATHOLOGY SUMMARY

Histomorphological evaluation was performed on thyroid glands of *Xenopus laevis* (South African clawed frogs) that were exposed to various concentrations of phenobarbital to determine the differential sensitivity of Stage 51 and Stage 54 larvae and to establish the necessary duration of exposure. Stage 51 larvae were exposed to phenobarbital for 21 days and Stage 54 larvae were exposed to phenobarbital for 14 days. The experimental designs were as follows:

Stage 51 Frog Larvae Exposed for 21 Days

Group/ Replicate	Phenobarbital (mg/L)	No. of Frogs/Group	Individual Frog Numbers
035A	0	5	205, 211, 214, 216, 218
035B	0	5	222, 223, 229, 231, 235
036A	125	5	243, 249, 255, 257, 258
036B	125	5	261, 269, 273, 275, 277
037A	250	5	303, 305, 310, 314, 318
037B	250	5	286, 288, 291, 294, 296
038A	500	5	320, 324, 326, 330, 339
038B	500	5	341, 344, 349, 351, 358
039A	1000	5	360, 362, 368, 374, 375
039B	1000	5	381, 384, 386, 393, 395

Stage 54 Frog Larvae Exposed for 14 Days

Group/ Replicate	Phenobarbital (mg/L)	No. of Frogs/Group	Individual Frog Numbers
040C	0	5	002, 003, 007, 016, 019
040D	0	5	024, 026, 033, 035, 037
041C	250	5	042, 047, 050, 052, 058
041D	250	5	063, 066, 071, 073, 079
042C	500	5	081, 083, 088, 091, 100
042D	500	5	104, 107, 109, 111, 117
043C	1000	5	124, 126, 129, 131, 140
043D	1000	5	144, 147, 150, 152, 155
044C	1500	5	161, 164, 168, 173, 176
044D	1500	5	182, 183, 184, 188, 198

MATERIALS AND METHODS

Slides were prepared by Experimental Pathology Laboratories, Inc. (EPL®). Head samples were trimmed from the frog larvae and placed in cassettes with the cut side down so that the heads were sectioned in a transverse plane from caudal to rostral. Five step sections, approximately 30 microns apart, were taken from each block at the level of the thyroid glands and were mounted on one slide. The slides were examined by a technician to assure that a sufficient amount of thyroid tissue was present bilaterally in the sections. Three duplicate slides with the five step sections were cut. One slide was stained with hematoxylin and eosin (H&E), one slide was stained with periodic acid-Schiff (PAS), and the third slide was not stained.

Microscopic findings for thyroid glands from each frog are listed in the attached Histopathology Incidence Tables. The changes were graded from 1 to 5 depending upon severity (1 = minimal, 2- slight/mild, 3 =moderate,

4=moderately severe, 5-severe/high). All lesions are summarized by stage and treatment group in the Summary Incidence Tables.

RESULTS

Stage 51 *Xenopus laevis* exposed to phenobarbital for 21 days

There were minimal to mild exposure-related changes in the thyroid glands of several animals exposed to the highest dose (1000 mg/L); these included follicular cell hyperplasia and diffuse enlargement of the thyroid gland. Minimal hyperplasia of follicular cells was present in six of ten animals exposed at 1000 mg/L; this change was not evident in any animals exposed at lower doses. Follicular cell hyperplasia was characterized by minimally increased follicular cell height and piling up of cells.

Diffuse enlargement of thyroid glands was present in two of ten animals exposed at 1000 mg/L, but was not evident in any animals exposed at lower doses. This enlargement was graded minimal to slight/mild in the two affected animals.

In the most of the control and treated animals, the colloid stained homogeneous and eosinophilic with H&E and homogeneous and magenta with PAS. Pale colloid was present in occasional follicles from two animals exposed at 125 mg/L. This change was graded as minimal and was evident on both the sections stained with H&E and the sections stained with PAS. This change was not considered related to treatment with phenobarbital because it was not evident in any of the animals exposed to the three high doses. Staining intensity and amount of colloid were not remarkable on either the H&E stained sections or on the PAS-stained sections from any of the other Stage 51 larvae in this study.

Interfollicular pigment was present in scattered thyroid glands from all exposure concentrations and varied in severity from minimal to moderate. There was no relationship between either the incidence or severity of this finding with exposure concentration of phenobarbital. See Table 1 for a tabulation of the prevalence of selected changes in the thyroid glands described above in Stage 51 frogs.

Table 1: Prevalence With Replicates Combined of Selected Changes in Thyroid Glands of Stage 51 Frogs After Exposure to Various Concentrations of Phenobarbital for 21 Days

Group	35 A & B	36 A & B	37 A & B	38 A & B	39 A & B
Exposure Conc.	0 mg/L	125 mg/L	250 mg/L	500 mg/L	1000 mg/L
Thyroid (No. Examined)	10	10	10	10	10
Hyperplasia, Follicular Cells	0	0	0	0	6
Enlargement, Diffuse	0	0	0	0	2
Interfollicular Pigment	3	4	2	2	3
Colloid, Pale	0	2	0	0	0

See Appendix A for photographs of thyroid glands from the Stage 51 frogs. The photographs are annotated to illustrate various features of thyroid glands and changes that resulted from exposure to phenobarbital.

Stage 54 *Xenopus laevis* exposed to phenobarbital for 14 days

There were rare, exposure-related changes in the thyroid glands of frogs exposed to the highest concentration of phenobarbital (1500 mg/L). Minimal hyperplasia of thyroid follicular cells was present in two of ten animals exposed at 1500 mg/L. Follicular cell hyperplasia was characterized by minimally increased follicular cell height and piling up of cells.

The control and treated animals, the colloid stained homogeneous and eosinophilic with H&E and homogeneous and magenta with PAS. Staining intensity and amount of colloid was not remarkable on either the H&E-stained sections or on the PAS-stained sections for any of the Stage 54 larvae in this study.

Interfollicular pigment was present in scattered thyroid glands from all exposure concentrations and varied in severity from minimal to moderate. There was no relationship between either the incidence or severity of this finding with exposure concentration of phenobarbital. See Table 2 for a tabulation of the prevalence of selected changes in the thyroid glands described above in Stage 54 frogs.

Table 2: Prevalence With Replicates Combined of Selected Changes in Thyroid Glands of Stage 54 Frogs After Exposure to Various Concentrations of Phenobarbital for 14 Days

Group	40 C & D	41 C & D	42 C & D	43 C & D	44 C & D
Exposure Conc.	0 mg/L	250 mg/L	500 mg/L	1000 mg/L	1500 mg/L
Thyroid (No. Examined)	9	10	10	10	10
Hyperplasia, Follicular Cells	0	0	0	0	2
Interfollicular Pigment	2	4	3	2	4

DISCUSSION

In rats, phenobarbital causes thyroid follicular cell hypertrophy and hyperplasia and increases the risk of development of benign thyroid tumors. Phenobarbital is a potent inducer of hepatic microsomal enzymes in mammals; these enzymes are the rate-limiting step in excretion of thyroid hormones. The increased excretion of T3 and T4 that occurs following sustained phenobarbital

administration causes stimulation of the hypothalamic-pituitary-thyroid axis and increases thyroid stimulating hormone (TSH) excretion. Increased TSH causes thyroid follicular cell hypertrophy and hyperplasia.[1]

The effect of phenobarbital and related compounds on hepatic microsomal enzymes in amphibians, particularly aquatic species, is different than in mammals. In one study, pentobarbital caused no induction of hepatic microsomal cytochrome P450 in adult *Xenopus laevis*.[2] Administration of phenobarbital in separate studies to leopard frogs (*Rana pipiens*) and to Italian water frogs similarly caused no increase in hepatic microsomal enzymes.[3,4]

Differences between mammals and aquatic amphibians in the induction of hepatic microsomal enzymes by phenobarbital likely played a role in the results of this study. Lesions of diffuse gland enlargement and follicular cell hyperplasia in this study were limited to frogs exposed to only the highest concentrations of phenobarbital; severity of these lesions was usually graded as minimal and rarely as slight/mild.

No changes in colloid amount or staining characteristics were associated with administration of phenobarbital to frogs in this study. The only colloid changes were in two Stage 51 frogs exposed at the lowest concentration (125 mg/L) and were considered unrelated to phenobarbital administration. In rats, phenobarbital caused colloid depletion in animals exposed to phenobarbital for time periods of two to four weeks; PAS has been shown to be a sensitive stain to detect colloid changes.[5]

CONCLUSIONS

Exposure of *Xenopus laevis* larvae to phenobarbital resulted in minimal to mild changes in thyroid glands at exposure concentrations of 1000 mg/L in Stage 51 frogs and 1500 mg/L in Stage 54 frogs. Changes were limited to minimal hyperplasia of thyroid follicular cells in both Stage 51 and 54 frogs and minimal to mild diffuse gland enlargement in low numbers of Stage 51 frogs. Stage 51 frogs had a higher incidence of treatment-related changes at a lower exposure concentration and so appeared more sensitive to phenobarbital exposure than did Stage 54 frogs. Unlike in rats, no treatment-related changes in colloid were visible on either H&E or PAS-stained sections.

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Veterinary Pathologist

SLH/skh

REFERENCES

[1] Capen, C.C., DeLellis, R.A., and Yarrington, J.T. (1991). Endocrine System. In "Handbook of Toxicologic Pathology". (Haschek, W.M. and Rousseaux, C.G. (eds.). Academic Press, San Diego. pp. 723-724.

[2] Khan, M.A., Qadri, S.Y., Tomar, S., Fish, D., Gururajan, L., and Poria, M.S. (1998). Induction of hepatic cytochrome P-450 by phenobarbital in semiaquatic frog (Rana pipiens). *Biochem Biophys Res Commun.* **244**(3):737-744.

[3] Schwen, R.J. and Mannering, G.J. (1982). Hepatic cytochrome P-450-dependent monooxygenase systems of the trout, frog and snake—III. Induction. *Comp Biochem Physiol B.* **71**(3):445-453.

[4] Longo, V., Marini, S., Salvetti, A., Angelucci, S., Bucci, S., and Gervasi, P.G. (2004). Effects of beta-naphthoflavone, phenobarbital and dichlobenil on the drug-metabolizing system of liver and nasal mucosa of Italian water frogs. *Aquat Toxicol.* **69**(3):259-270.

[5] O'Connor, J.C., Frame, S.R., Davis, L.G., and Cook, J.C. (1999). Detection of thyroid toxicants in a tier I screening battery and alterations in thyroid endpoints over 28 days of exposure. *Toxicol Sci.* **51**:54-70.

COMPLIANCE STATEMENT

Client Name	Battelle	EPL Project Coordinator	Dr. Marilyn J. Wolfe
Client Study	BATT02-00056	EPL Pathologist	Dr. Sarah L. Hale
Species	*Xenopus laevis*	EPL Project Number	237-012
Study Title	Amphibian Metamorphosis Assay Demonstration Study		
Test Article	Phenobarbital		

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 160.

EPL Project Coordinator

Date

3/03/05 DRAFT

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Amphibian Metamorphosis Assay Demonstration Study

Client Study: BATT02-00056 EPL Project Coordinator: Dr. Marilyn J. Wolfe

EPL Project Number: 237-012 EPL Pathologist: Dr. Sarah L. Hale

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 Project Coordinator and Management are indicated below.

	Dates	
Area Inspected	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management XXX

Date of last quarterly facility inspection 1/05

EPL Quality Assurance Unit Date

SUMMARY INCIDENCE TABLES – Stage 51 Frogs

SUMMARY INCIDENCE TABLE

BATT02-00056
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 035A	GROUP 035B	GROUP 036A	GROUP 036B	GROUP 037A	GROUP 037B
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Colloid, Pale, H&E			1	1		
Colloid, Pale, PAS			1	1		
Enlargement, Diffuse						
Hyperplasia, Follicular Cell						
Interfollicular Pigment	1	2	1	3	1	1

SUMMARY INCIDENCE TABLE

BATT02-00056
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 038A	GROUP 038B	GROUP 039A	GROUP 039B		
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)		
Colloid, Pale, H&E						
Colloid, Pale, PAS						
Enlargement, Diffuse				2		
Hyperplasia, Follicular Cell			2	4		
Interfollicular Pigment	1	1	2	1		

HISTOPATHOLOGY INCIDENCE TABLES – Stage 51 Frogs

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00056
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 035A						GROUP 035B						GROUP 036A							
ANIMAL	51-205	51-211	51-214	51-216	51-218		51-222	51-223	51-229	51-231	51-235		51-243	51-249	51-255	51-257	51-258			
THYROID		X	X	X	X			X		X	X		X		X	X				
Colloid, Pale, H&E														1						
Colloid, Pale, PAS														1						
Enlargement, Diffuse																				
Hyperplasia, Follicular Cell																				
Interfollicular Pigment	3						2		2								3			

EPL
Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00056
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 036B						GROUP 037A						GROUP 037B							
ANIMAL	51-261	51-269	51-273	51-275	51-277		51-303	51-305	51-310	51-314	51-318		51-286	51-288	51-291	51-294	51-296			
THYROID		X		X			X		X	X	X			X	X	X	X			
Colloid, Pale, H&E			1																	
Colloid, Pale, PAS			1																	
Enlargement, Diffuse																				
Hyperplasia, Follicular Cell																				
Interfollicular Pigment	1		2		1			2					2							

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HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00056
Stage 51/21 Day Sacrifice
Xenopus laevis

	GROUP 038A						GROUP 038B						GROUP 039A						
ANIMAL	51-320	51-324	51-326	51-330	51-339		51-341	51-344	51-349	51-351	51-358		51-360	51-362	51-368	51-374	51-375		
THYROID	X	X	X	X				X	X	X	X			X					
Colloid, Pale, H&E																			
Colloid, Pale, PAS																			
Enlargement, Diffuse																			
Hyperplasia, Follicular Cell													1		1				
Interfollicular Pigment					3		1									2	1		

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1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
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HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00056
Stage 51/21 Day Sacrifice
Xenopus laevis

GROUP
039B

ANIMAL	51-381	51-384	51-386	51-393	51-395
THYROID				X	
Colloid, Pale, H&E					
Colloid, Pale, PAS					
Enlargement, Diffuse		2			1
Hyperplasia, Follicular Cell	1	1	1		1
Interfollicular Pigment					2

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

SUMMARY INCIDENCE TABLES – Stage 54 Frogs

SUMMARY INCIDENCE TABLE

BATT02-00056
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 040C	GROUP 040D	GROUP 041C	GROUP 041D	GROUP 042C	GROUP 042D
THYROID (NO. EXAMINED)	(4)	(5)	(5)	(5)	(5)	(5)
Hyperplasia, Follicular Cell						
Interfollicular Pigment	1	1	3	1		3

SUMMARY INCIDENCE TABLE

BATT02-00056
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 043C	GROUP 043D	GROUP 044C	GROUP 044D		
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)		
Hyperplasia, Follicular Cell			1	1		
Interfollicular Pigment	1	1	3	1		

HISTOPATHOLOGY INCIDENCE TABLES – Stage 54 Frogs

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00056
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 040C						GROUP 040D						GROUP 041C								
ANIMAL	54-002	54-003	54-007	54-016	54-019		54-024	54-026	54-033	54-035	54-037		54-042	54-047	54-050	54-052	54-058				
THYROID	X	X		N	X		X		X	X	X		X			X					
Hyperplasia, Follicular Cell																					
Interfollicular Pigment			2					1						1	2		2				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00056
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 041D						GROUP 042C						GROUP 042D							
ANIMAL	54-063	54-066	54-071	54-073	54-079		54-081	54-083	54-088	54-091	54-100		54-104	54-107	54-109	54-111	54-117			
THYROID	X	X		X	X		X	X	X	X	X		X		X					
Hyperplasia, Follicular Cell																				
Interfollicular Pigment			2											2		3	2			

EPL
Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00056
Stage 54/14 Day Sacrifice
Xenopus laevis

	GROUP 043C						GROUP 043D						GROUP 044C							
ANIMAL	54-124	54-126	54-129	54-131	54-140		54-144	54-147	54-150	54-152	54-155		54-161	54-164	54-168	54-173	54-176			
THYROID		X	X	X	X			X	X	X	X					X				
Hyperplasia, Follicular Cell													1							
Interfollicular Pigment	1						1							1	2		1			

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

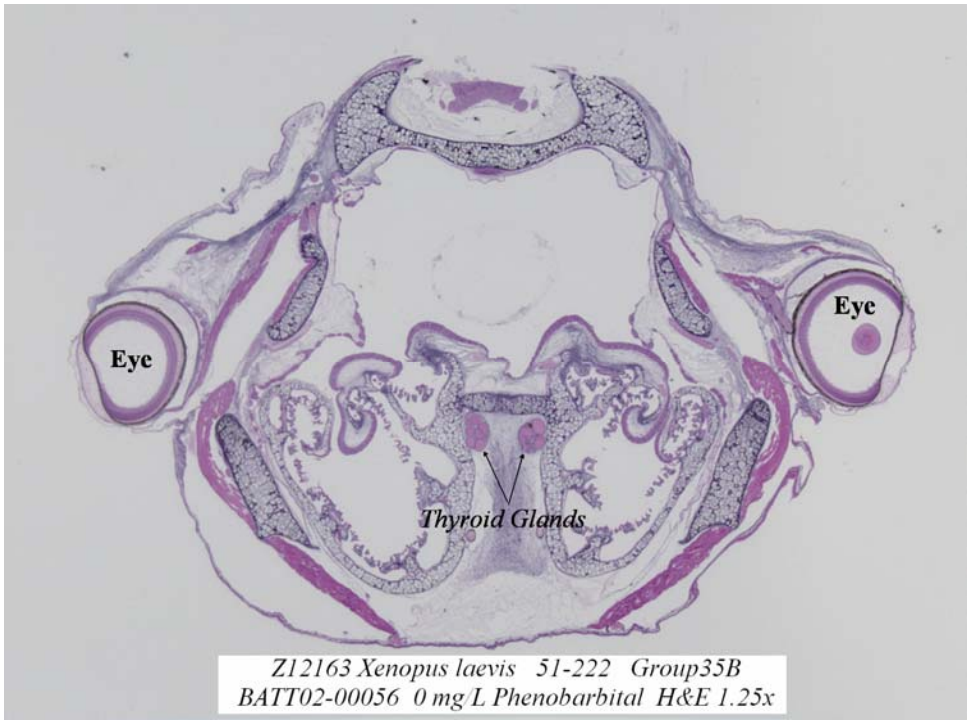
BATT02-00056
Stage 54/14 Day Sacrifice
Xenopus laevis

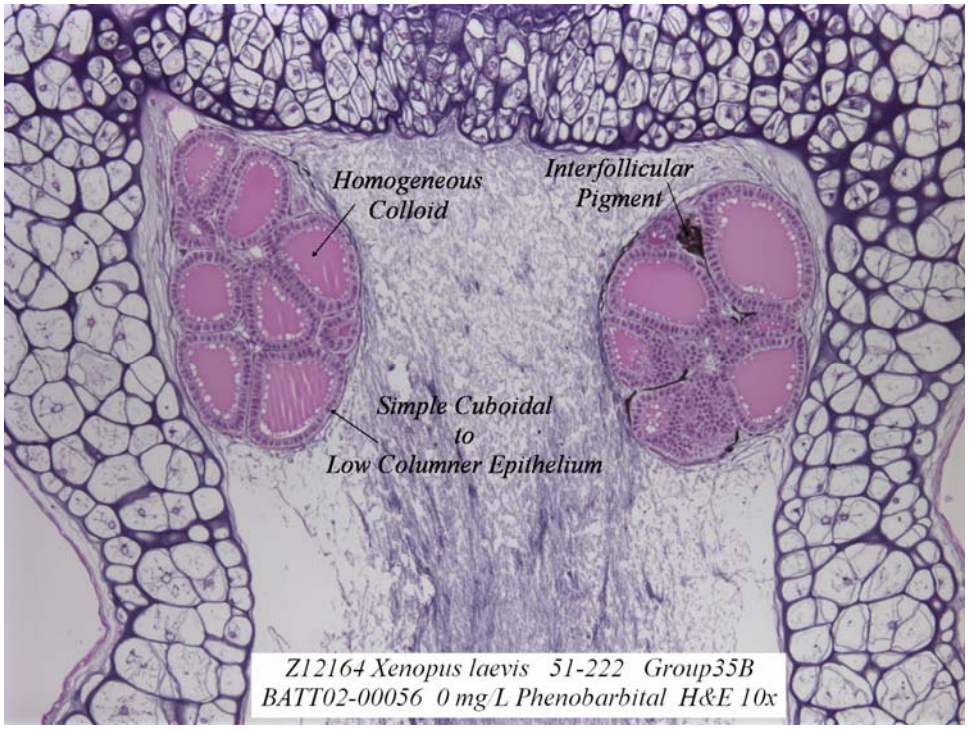
GROUP
044D

ANIMAL	54-182	54-183	54-184	54-188	54-198																
THYROID	X	X		X	X																
Hyperplasia, Follicular Cell			1																		
Interfollicular Pigment			2																		

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

APPENDIX A: PHOTOGRAPHS – STAGE 51 FROGS



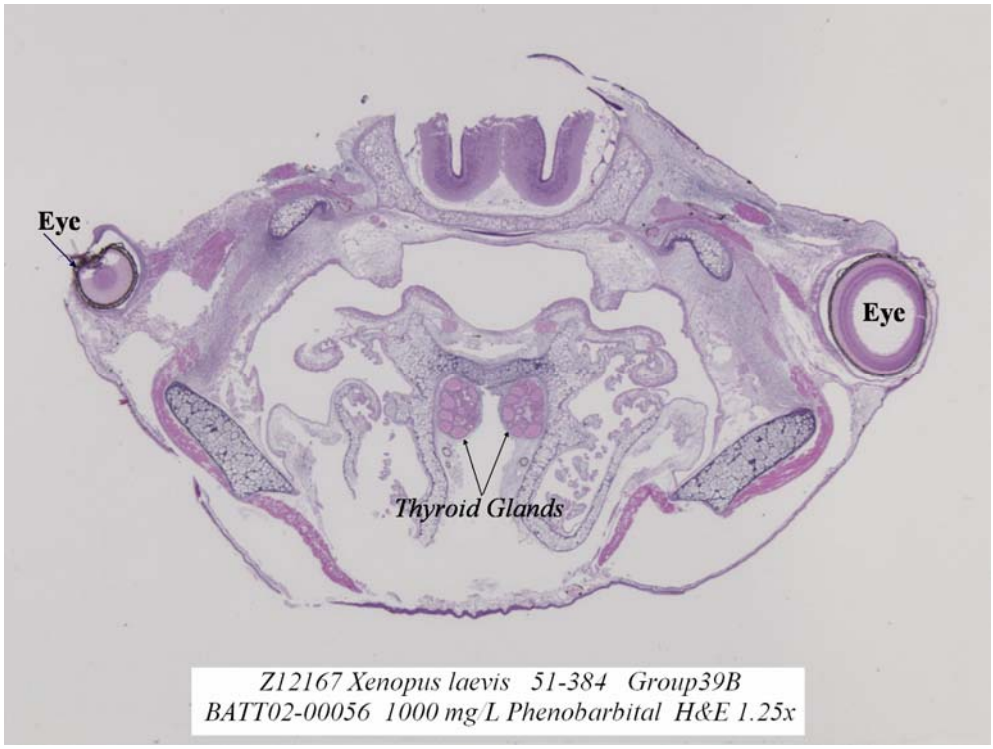


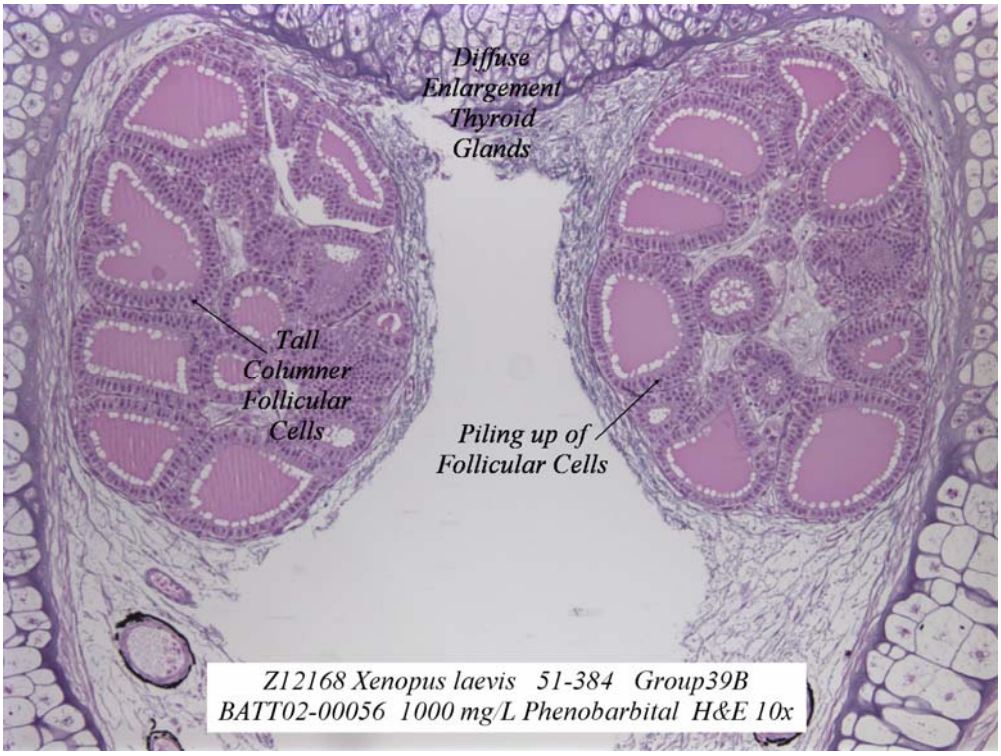
Z12163 and Z12164. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* control. The colloid is homogeneous and eosinophilic, and the follicles are lined by simple cuboidal to low columnar epithelium.





Z12165 and Z12166. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands stained with PAS from Stage 51 *Xenopus laevis* control. The colloid is homogeneous and magenta.





Z12167 and Z12168. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* exposed to 1000 mg/L Phenobarbital for 21 days. There is mild diffuse thyroid gland enlargement and minimal follicular cell hyperplasia.



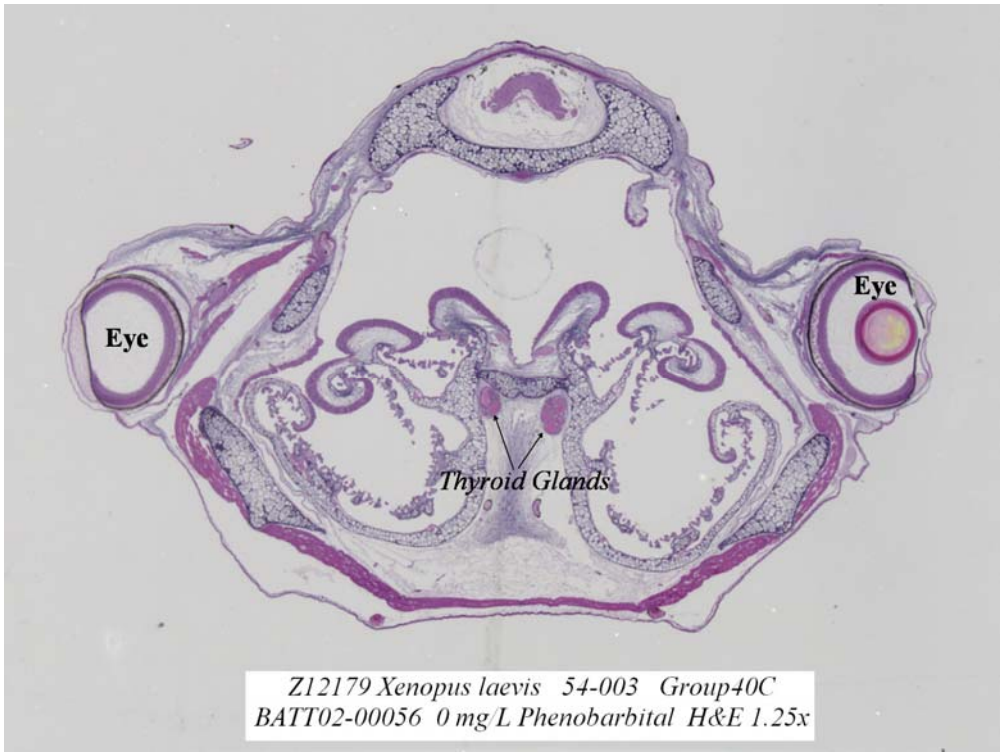
Z12174. High magnification (10x) photomicrograph of thyroid glands stained with PAS from Stage 51 *Xenopus laevis* exposed to 1000 mg/L Phenobarbital for 21 days. The colloid is homogeneous and magenta.

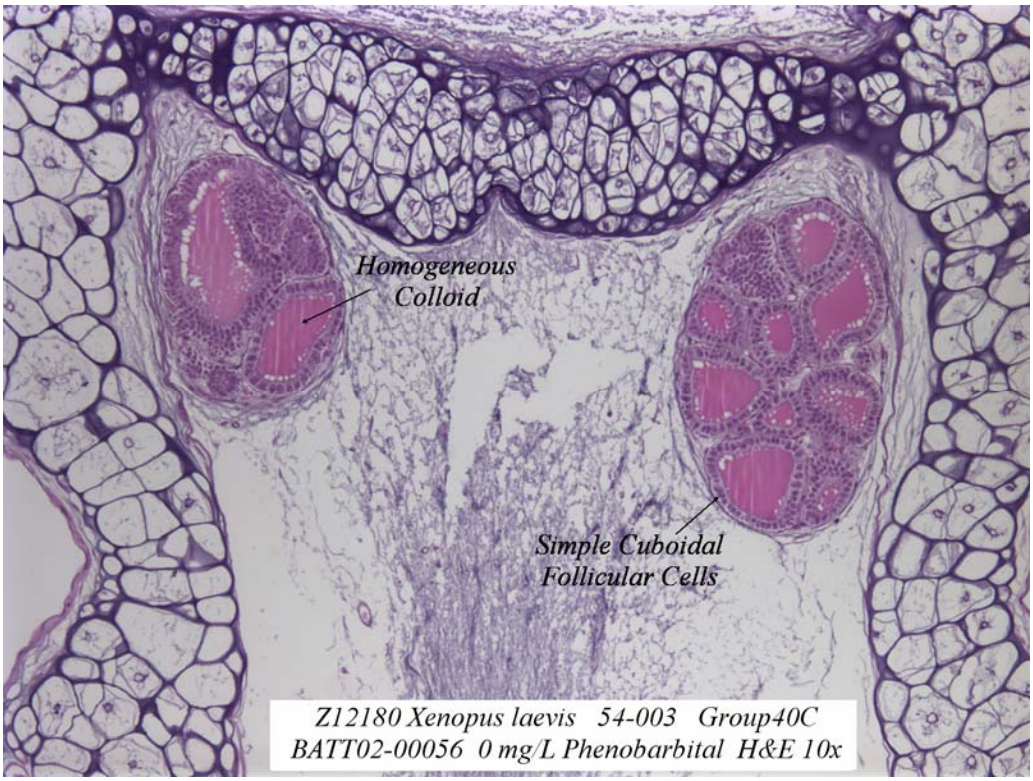




Z12178 and Z12176. High (10x) magnification photomicrographs of thyroid glands stained with H&E and PAS from Stage 51 *Xenopus laevis* exposed to 125 mg/L Phenobarbital for 21 days. Note that the colloid in rare follicle stains pale with both H&E and PAS.

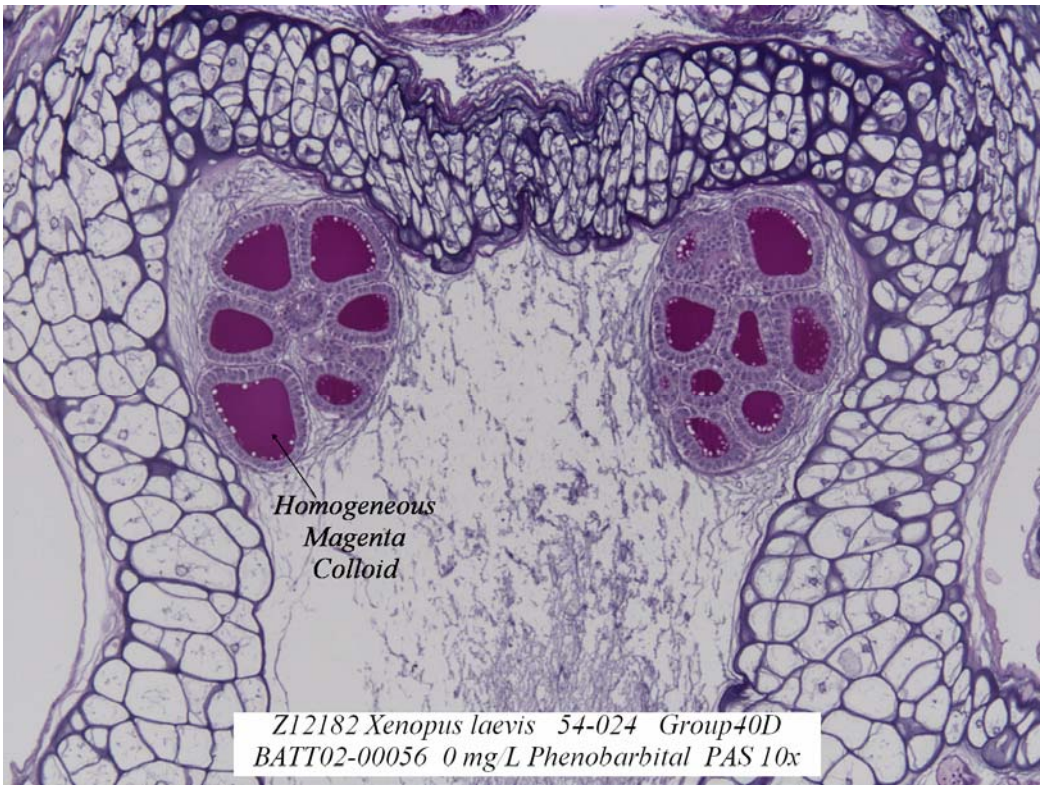
APPENDIX B: PHOTOGRAPHS – STAGE 54 FROGS



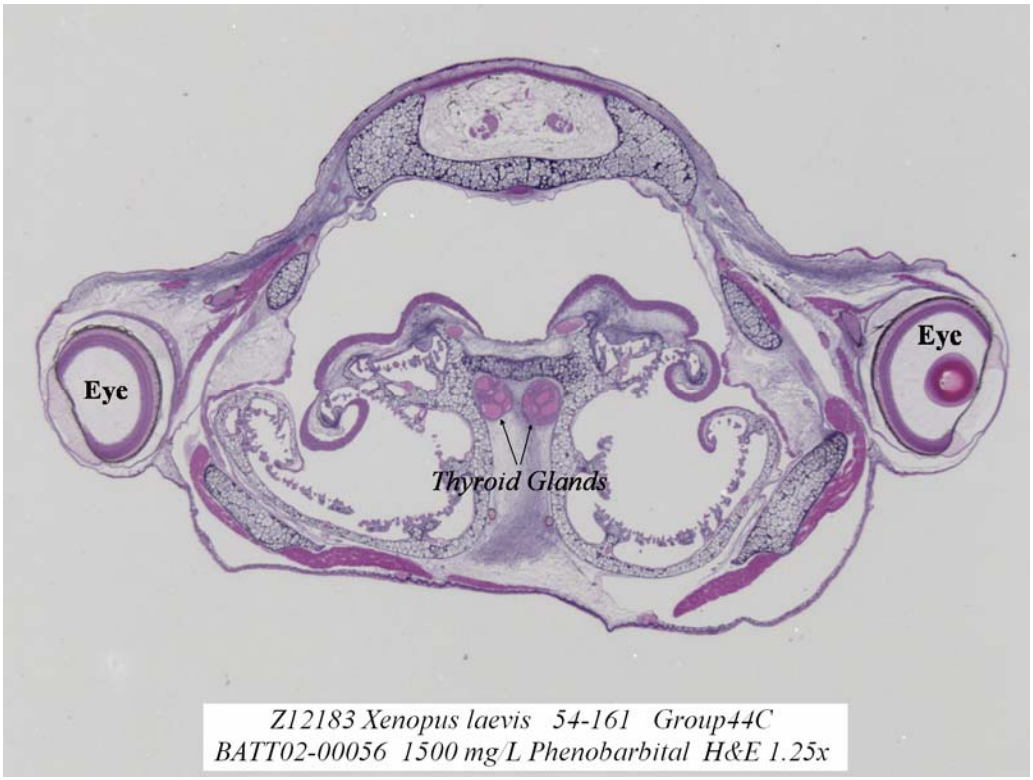


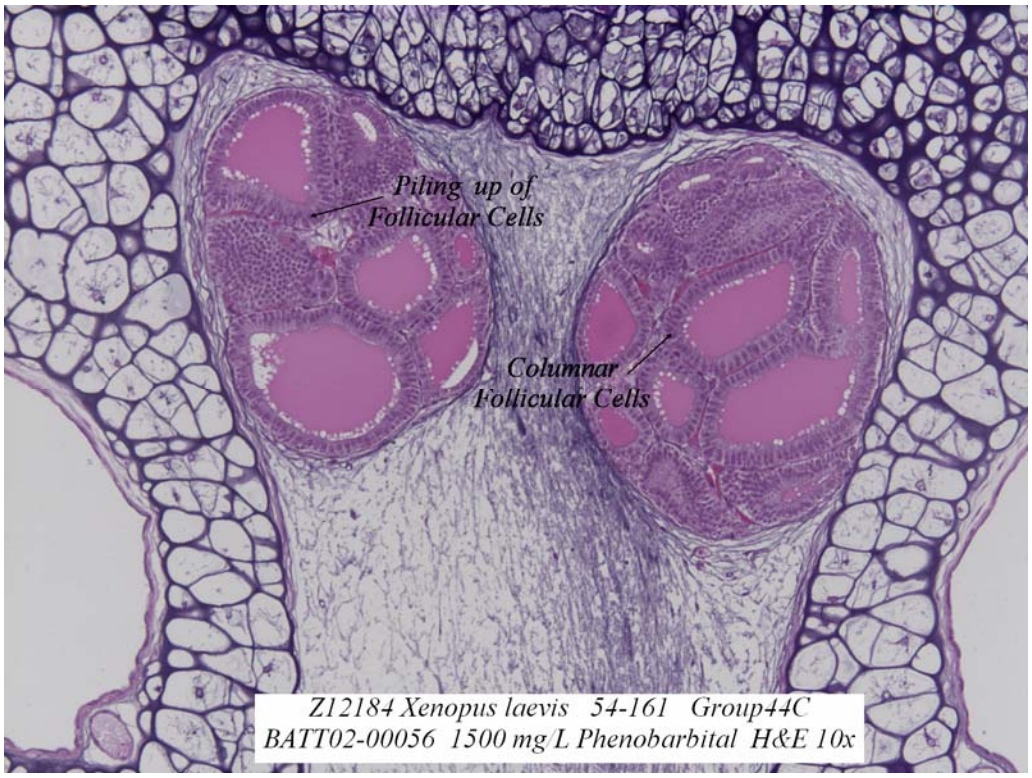
Z12179 and Z12180. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 54 control *Xenopus laevis*. The colloid is homogeneous and eosinophilic, and the follicles are lined by simple cuboidal to low columnar epithelium.





Z12181 and Z12182. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands stained with PAS from Stage 54 *Xenopus laevis* control. The colloid is homogeneous and magenta.





Z12183 and Z12184. Low (1.25x) and high (10x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* exposed to 1500 mg/L Phenobarbital for 14 days. There is minimal follicular cell hyperplasia.



Z12188. High (10x) magnification photomicrograph of thyroid glands stained with PAS from Stage 54 *Xenopus laevis* exposed to 1500 mg/L Phenobarbital for 14 days. The colloid is homogeneous and magenta.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

Client:	Ba Helle
Submit Report To:	Doug Fort
Sampler's Signature:	Jeb H. Howe
P.O. #:	

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

BAT02-00056

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
035	Specimen #s: 205, 211,	11/30/04 1000	10	G	10% Formalin	Histological examination of thyroid gland
↓	214, 216, 218, 222,	↓ ↓			↓	~~as per protocol # BAT02-1~~ ①
↓	223, 229, 231, 235					
036	Specimen #s: 243, 249,		10	G		
↓	255, 257, 258, 261,					
↓	269, 273, 275, 277					
037	Specimen #s: 303, 305,		10	G		
↓	310, 314, 318, 286,					
↓	288, 291, 294, 296					
038	Specimen #s: 320, 324,		10	G		
↓	326, 330, 339, 341,					
↓	344, 349, 351, 358					
039	Specimen #s: 360, 362,		10	G		
↓	368, 374, 375, 381,					
↓	384, 386, 393, 395	↓ ↓			↓	

SPECIAL INSTRUCTIONS: Organisms exposed to Pharbitis l

DATE:
INITIALS:

Andrea Walden	12/2/04 1000		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

① N/A - JT - 12/2/04

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

Client: Ba Helle
Submit Report To: Doug East
Sampler's Signature: [signature]
P.O. #:

FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Lab Use Only
Client Project # / WO #: BATT02-0005

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only ID#	Lab Use Only Temp	Lab Use Only pH
040	Specimen #s: 002, 003,	11/30/04 1000	10	G	10% formalin	Histological examination of Thyroid Gland ~~as per protocol # BATT02~~ + ①			
↓	007, 016, 019, 024,								
↓	026, 033, 035, 037								
041	Specimen #s: 042, 047,		10	G					
↓	050, 052, 058, 063,								
↓	066, 071, 073, 079								
042	Specimen #s: 081, 083,		10	G					
↓	088, 091, 100, 104,								
↓	107, 109, 111, 117								
043	Specimen #s: 124, 126,		10	G					
↓	129, 131, 140, 144,								
↓	147, 150, 152, 155								
044	Specimen #s: 161, 164,		10	G					
↓	168, 173, 176, 182,								
↓	183, 184, 188, 198								

SPECIAL INSTRUCTIONS: Organisms exposed to Phenobarbital

DATE:
INITIALS:

Sample Relinquished By:	Date-Time	Sample Received By:	Date-Time
Andrea Walker	12/2/04 1006		

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

① N/A 12/2/04

Appendix H
Pregnenolone-16-α-Carbonitrile (PCN)

Metamorphosis Assay – Raw Data:

Daily Operational Data
Test Termination Data – Stage and Growth
Statistical Data – Stage and Growth

Test Chemical Analytical Data from Battelle:

Purity / Stability Report
In-Life Test Chemical Analysis Report

Thyroid Histology Data from EPL:

Sample Identification Key
Thyroid Pathology Report

Pregnenolone-16-α-Carbonitrile (PCN) Metamorphosis Assay: Daily Operational Data

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA RENEW

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 0.0 ug/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Conc[1] (ug/L)
		Initial No.= 20							Initial No.= 20												
0	10/14/04			100.0	20.9	7.1	9.9	1189			100.0	20.9	7.1	10.1	1202	100.0	20.9	7.1	10.0	1196	0.51
1	10/15/04		20	100.0	20.5	7.1	5.6	1175		20	100.0	20.5	7.1	5.3	1250	100.0	20.5	7.1	5.5	1213	
2	10/16/04		20	100.0	21.4	7.0	5.0	1204		20	100.0	21.2	7.0	5.1	1251	100.0	21.3	7.0	5.1	1228	
3	10/17/04		20	100.0	21.0	7.0	5.1	1183		20	100.0	20.9	7.0	5.2	1254	100.0	21.0	7.0	5.2	1219	
4	10/18/04		20	100.0	20.5	7.4	7.6	1236		20	100.0	20.5	7.4	7.9	1200	100.0	20.5	7.4	7.8	1218	
5	10/19/04		20	100.0	21.0	7.2	6.1	1273		20	100.0	21.0	7.2	5.4	1259	100.0	21.0	7.2	5.8	1266	
6	10/20/04		20	100.0	21.0	7.2	6.6	1211		20	100.0	21.0	7.2	5.6	1140	100.0	21.0	7.2	6.1	1176	0.51
7	10/21/04		20	100.0	21.3	7.0	6.6	1040		20	100.0	21.3	7.0	6.8	1010	100.0	21.3	7.0	6.7	1025	
8	10/22/04		20	100.0	21.4	7.1	5.1	1180		20	100.0	21.2	7.3	5.9	1150	100.0	21.3	7.2	5.5	1165	
9	10/23/04		20	100.0	21.4	7.1	5.7	1248		20	100.0	21.3	7.0	6.0	1234	100.0	21.4	7.1	5.9	1241	
10	10/24/04		20	100.0	21.4	7.1	5.9	1172		20	100.0	21.3	7.1	6.0	1253	100.0	21.4	7.1	6.0	1213	
11	10/25/04		20	100.0	21.2	7.0	6.6	1088		20	100.0	21.0	7.0	6.7	1074	100.0	21.1	7.0	6.7	1081	
12	10/26/04		20	100.0	21.6	7.1	4.6	1099		20	100.0	21.4	7.1	5.1	1071	100.0	21.5	7.1	4.9	1085	
13	10/27/04		20	100.0	21.2	7.2	6.3	1080		20	100.0	21.2	7.2	7.1	1190	100.0	21.2	7.2	6.7	1135	
14	10/28/04		20	100.0	21.4	7.1	5.0	1260		20	100.0	21.4	7.1	6.1	1249	100.0	21.4	7.1	5.6	1255	0.51
15	10/29/04		20	100.0	21.2	7.1	6.0	1119		20	100.0	21.1	7.1	6.2	1152	100.0	21.2	7.1	6.1	1136	
16	10/30/04		20	100.0	20.6	7.3	6.4	1120		20	100.0	20.6	7.3	6.2	1179	100.0	20.6	7.3	6.3	1150	
17	10/31/04		20	100.0	21.0	7.2	6.5	1123		20	100.0	21.1	7.3	6.5	1164	100.0	21.1	7.3	6.5	1144	
18	11/01/04		20	100.0	20.6	7.2	6.6	1130		20	100.0	20.3	7.4	8.2	1160	100.0	20.5	7.3	7.4	1145	
19	11/02/04		20	100.0	21.4	6.9	4.1	1284		20	100.0	21.4	7.0	5.1	1258	100.0	21.4	7.0	4.6	1271	
20	11/03/04		20	100.0	20.8	7.1	5.6	1100		20	100.0	20.7	7.1	6.8	1120	100.0	20.8	7.1	6.2	1110	
21	11/04/04		20	100.0						20	100.0					100.0					
	Median:				**21.2**	**7.1**	**6.0**	**1175**				**21.1**	**7.1**	**6.1**	**1190**		**21.1**	**7.1**	**6.1**	**1176**	
	Mode:				**21.4**	**7.1**	**6.6**	**#N/A**				**21.2**	**7.1**	**5.1**	**#N/A**		**20.5**	**7.1**	**6.1**	**1213**	
	Mean:			**100.0**	**21.1**	**7.1**	**6.0**	**1167**			**100.0**	**21.0**	**7.1**	**6.3**	**1182**	**100.0**	**21.1**	**7.1**	**6.2**	**1175**	**0.510**
	SEM:			**0.00**	**0.07**	**0.02**	**0.26**	**15.12**			**0.00**	**0.07**	**0.03**	**0.27**	**15.52**	**0.00**	**0.07**	**0.03**	**0.26**	**14.42**	**0.00**
	% CV:			**0.00**	**7.30**	**4.21**	**49.23**	**202.85**			**0.00**	**7.15**	**4.96**	**48.72**	**206.93**	**0.00**	**7.14**	**4.41**	**47.35**	**192.84**	**0.00**

[1]All measured test concentrations reported by Battelle as less than limit of detection (LOD) [1.018 µg/L] or none detected (ND) are expressed as 0.51 µg/L (1/2 of LOD).

DAILY DATA RENEW

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 7.4 ug/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Conc[1] (ug/L)
		Initial No.= 20							**Initial No.= 20**												
0	10/14/04			100.0	20.5	7.1	5.7	1182			100.0	20.3	7.1	5.8	901	100.0	20.4	7.1	5.8	1042	0.51
1	10/15/04		20	100.0	20.2	7.2	6.3	1185		20	100.0	20.2	7.2	6.4	875	100.0	20.2	7.2	6.4	1030	
2	10/16/04		20	100.0	21.1	7.1	5.9	1192		20	100.0	21.2	7.0	6.0	884	100.0	21.2	7.1	6.0	1038	
3	10/17/04		20	100.0	20.9	7.1	6.6	1187		20	100.0	20.9	7.2	7.1	890	100.0	20.9	7.2	6.9	1039	
4	10/18/04		20	100.0	20.3	7.5	7.4	1240		20	100.0	20.4	7.5	7.7	941	100.0	20.4	7.5	7.6	1091	
5	10/19/04		20	100.0	21.0	7.3	7.4	1198		20	100.0	21.0	7.3	7.5	947	100.0	21.0	7.3	7.5	1073	
6	10/20/04		20	100.0	20.8	7.3	7.4	1181		20	100.0	20.9	7.3	7.8	1201	100.0	20.9	7.3	7.6	1191	0.51
7	10/21/04		20	100.0	21.1	7.2	7.9	1212		20	100.0	21.0	7.2	8.2	920	100.0	21.1	7.2	8.1	1066	
8	10/22/04		20	100.0	21.5	7.3	8.0	910		20	100.0	21.5	7.4	8.3	1200	100.0	21.5	7.4	8.2	1055	
9	10/23/04		20	100.0	21.4	7.1	8.1	1197		20	100.0	21.2	7.2	8.3	1029	100.0	21.3	7.2	8.2	1113	
10	10/24/04		20	100.0	21.4	7.1	7.2	1089		20	100.0	21.3	7.1	7.3	902	100.0	21.4	7.1	7.3	996	
11	10/25/04		20	100.0	20.8	7.2	7.7	1123		20	100.0	20.8	7.2	7.7	886	100.0	20.8	7.2	7.7	1005	
12	10/26/04		20	100.0	21.4	7.2	7.8	1022		20	100.0	21.4	7.2	7.8	932	100.0	21.4	7.2	7.8	977	
13	10/27/04		20	100.0	21.0	7.3	7.9	1150		20	100.0	21.0	7.3	7.9	880	100.0	21.0	7.3	7.9	1015	
14	10/28/04		20	100.0	21.4	7.3	6.5	1185		20	100.0	21.4	7.2	6.8	927	100.0	21.4	7.3	6.7	1056	0.51
15	10/29/04		20	100.0	21.4	7.1	6.5	1160		20	100.0	21.4	7.2	6.8	905	100.0	21.4	7.2	6.7	1033	
16	10/30/04		20	100.0	20.4	7.3	8.6	1197		20	100.0	20.6	7.4	8.5	910	100.0	20.5	7.4	8.6	1054	
17	10/31/04		20	100.0	21.4	7.4	8.4	1193		20	100.0	21.3	7.4	8.4	916	100.0	21.4	7.4	8.4	1055	
18	11/01/04		20	100.0	20.6	7.2	8.0	1070		20	100.0	20.7	7.2	8.0	920	100.0	20.7	7.2	8.0	995	
19	11/02/04		20	100.0	21.4	7.1	6.6	1219		20	100.0	21.4	7.1	6.6	910	100.0	21.4	7.1	6.6	1065	
20	11/03/04		20	100.0	20.7	7.2	6.9	1180	1	19	95.0	21.0	7.2	6.9	900	97.5	20.9	7.2	6.9	1040	
21	11/04/04		20	100.0						19	95.0					97.5					
	Median:				**21.0**	**7.2**	**7.4**	**1185**				**21.0**	**7.2**	**7.7**	**910**		**21.0**	**7.2**	**7.6**	**1042**	
	Mode:				**21.4**	**7.1**	**7.4**	**1185**				**21.0**	**7.2**	**7.7**	**920**		**21.4**	**7.2**	**6.7**	**#N/A**	
	Mean:			**100.0**	**21.0**	**7.2**	**7.3**	**1156**			**99.5**	**21.0**	**7.2**	**7.4**	**942**	**99.8**	**21.0**	**7.2**	**7.3**	**1049**	**0.510**
	SEM:			**0.00**	**0.09**	**0.02**	**0.18**	**16.76**			**0.31**	**0.08**	**0.03**	**0.17**	**20.07**	**0.16**	**0.09**	**0.02**	**0.18**	**10.04**	**0.00**
	% CV:			**0.00**	**9.09**	**4.18**	**30.59**	**225.91**			**14.75**	**8.38**	**4.45**	**29.39**	**299.73**	**7.36**	**8.65**	**4.18**	**29.84**	**142.01**	**0.00**

[1]All measured test concentrations reported by Battelle as less than limit of detection (LOD) or none detected (ND) are expressed as 0.51 µg/L (1/2 of LOD).

DAILY DATA RENEW

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 22.2 ug/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Conc[1] (ug/L)
		Initial No.= 20							**Initial No.= 20**												
0	10/14/04			100.0	20.3	7.2	8.4	1431			100.0	20.4	7.2	8.4	1403	100.0	20.4	7.2	8.4	1417	0.77
1	10/15/04		20	100.0	20.3	7.2	7.6	975		20	100.0	20.3	7.2	7.3	1110	100.0	20.3	7.2	7.5	1043	
2	10/16/04		20	100.0	21.2	7.0	6.8	1410		20	100.0	21.2	7.1	6.8	1387	100.0	21.2	7.1	6.8	1399	
3	10/17/04		20	100.0	20.9	7.2	6.8	1221		20	100.0	20.9	7.2	6.8	1406	100.0	20.9	7.2	6.8	1314	
4	10/18/04		20	100.0	20.6	7.5	7.5	1456		20	100.0	20.6	7.5	7.5	1442	100.0	20.6	7.5	7.5	1449	
5	10/19/04		20	100.0	21.0	7.3	8.3	1436		20	100.0	21.0	7.3	8.2	1436	100.0	21.0	7.3	8.3	1436	
6	10/20/04		20	100.0	21.0	7.3	7.9	1450		20	100.0	21.0	7.3	7.8	1446	100.0	21.0	7.3	7.9	1448	0.51
7	10/21/04		20	100.0	21.0	7.2	7.3	1158		20	100.0	21.1	7.2	7.0	1117	100.0	21.1	7.2	7.2	1138	
8	10/22/04		20	100.0	21.6	7.2	7.3	1390		20	100.0	21.5	7.2	7.1	1430	100.0	21.6	7.2	7.2	1410	
9	10/23/04		20	100.0	21.3	7.2	7.9	1441		20	100.0	21.4	7.2	7.4	1230	100.0	21.4	7.2	7.7	1336	
10	10/24/04		20	100.0	21.2	7.2	7.5	1437		20	100.0	21.2	7.1	7.6	1412	100.0	21.2	7.2	7.6	1425	
11	10/25/04		20	100.0	20.9	7.1	7.0	1416		20	100.0	21.0	7.1	6.9	1403	100.0	21.0	7.1	7.0	1410	
12	10/26/04		20	100.0	21.5	7.2	7.0	1220		20	100.0	21.5	7.2	6.9	1248	100.0	21.5	7.2	7.0	1234	
13	10/27/04		20	100.0	21.0	7.3	7.2	1430		20	100.0	21.1	7.3	7.1	1370	100.0	21.1	7.3	7.2	1400	
14	10/28/04		20	100.0	21.3	7.2	7.2	1455		20	100.0	21.3	7.2	7.1	1420	100.0	21.3	7.2	7.2	1438	0.51
15	10/29/04		20	100.0	21.5	7.2	7.1	1305		20	100.0	21.4	7.2	7.1	1350	100.0	21.5	7.2	7.1	1328	
16	10/30/04		20	100.0	20.6	7.4	8.5	1430		20	100.0	20.7	7.3	8.4	1428	100.0	20.7	7.4	8.5	1429	
17	10/31/04		20	100.0	21.5	7.4	8.1	1422		20	100.0	21.5	7.3	8.2	1398	100.0	21.5	7.4	8.2	1410	
18	11/01/04		20	100.0	20.9	7.4	7.8	1240		20	100.0	21.1	7.4	7.8	1320	100.0	21.0	7.4	7.8	1280	
19	11/02/04		20	100.0	21.2	7.1	7.2	1445		20	100.0	21.4	7.1	7.3	1439	100.0	21.3	7.1	7.3	1442	
20	11/03/04		20	100.0	21.1	7.2	7.4	1410		20	100.0	21.1	7.1	7.4	1390	100.0	21.1	7.2	7.4	1400	
21	11/04/04		20	100.0						20	100.0					100.0					
	Median:				**21.0**	**7.2**	**7.4**	**1422**				**21.1**	**7.2**	**7.3**	**1403**		**21.1**	**7.2**	**7.4**	**1410**	
	Mode:				**21.0**	**7.2**	**7.2**	**1410**				**21.1**	**7.2**	**7.1**	**1403**		**21.0**	**7.2**	**7.2**	**1410**	
	Mean:			**100.0**	**21.0**	**7.2**	**7.5**	**1361**			**100.0**	**21.1**	**7.2**	**7.4**	**1361**	**100.0**	**21.1**	**7.2**	**7.5**	**1361**	**0.597**
	SEM:			**0.00**	**0.08**	**0.03**	**0.11**	**27.81**			**0.00**	**0.08**	**0.02**	**0.11**	**22.02**	**0.00**	**0.08**	**0.02**	**0.11**	**23.65**	**0.09**
	% CV:			**0.00**	**8.03**	**4.31**	**18.65**	**345.51**			**0.00**	**7.56**	**3.89**	**19.04**	**273.48**	**0.00**	**7.75**	**4.01**	**18.65**	**293.74**	**19.43**

[1]All measured test concentrations reported by Battelle as less than limit of detection (LOD) or none detected (ND) are expressed as 0.51 µg/L (1/2 of LOD).

WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA RENEW

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 66.7 ug/L**

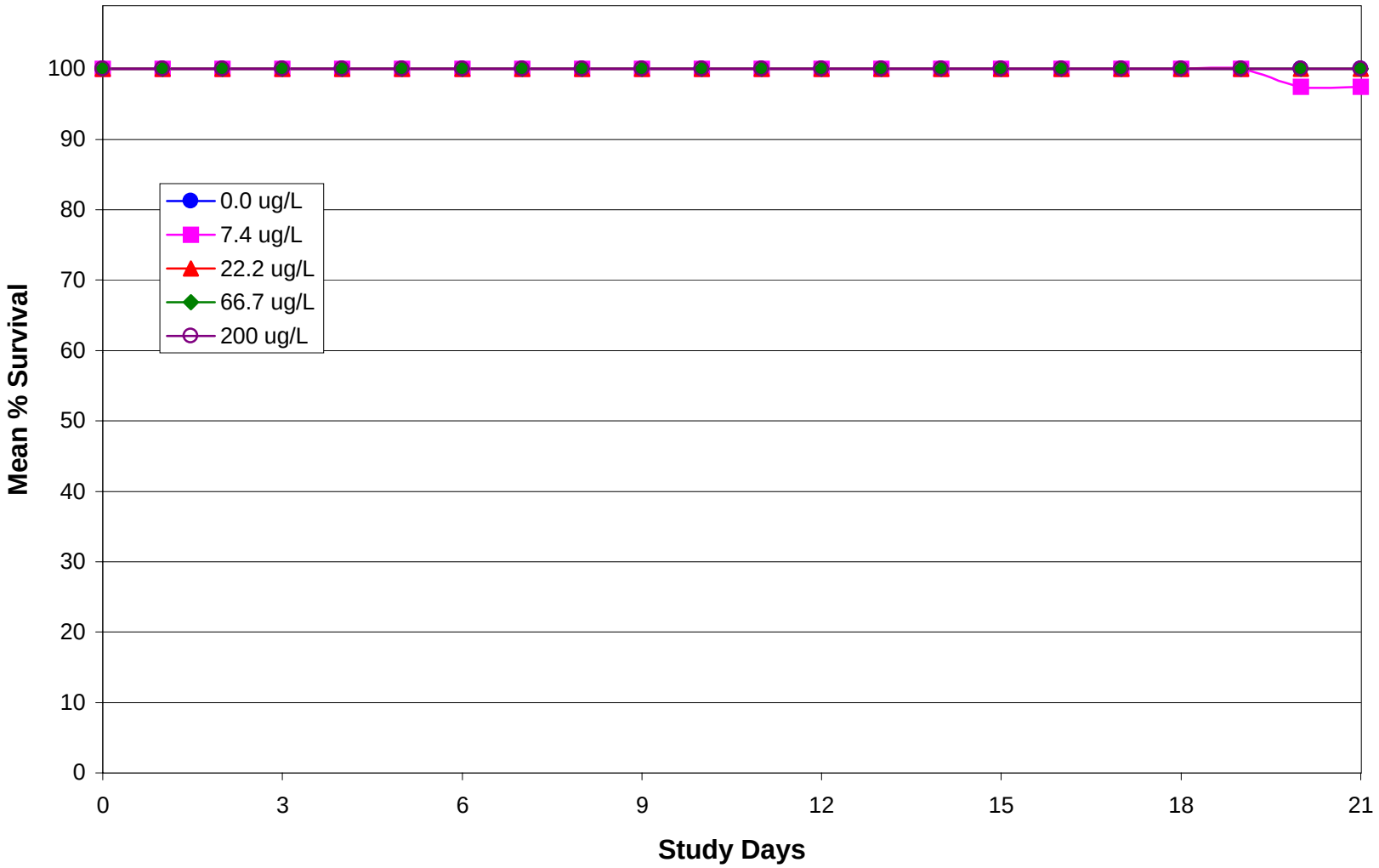
		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc (ug/L)
		Initial No.= 20							**Initial No.= 20**												
0	10/14/04			100.0	20.5	7.2	9.5	1253			100.0	20.6	7.2	9.5	1336	100.0	20.6	7.2	9.5	1295	5.69
1	10/15/04		20	100.0	20.4	7.2	8.1	1220		20	100.0	20.4	7.2	8.3	1200	100.0	20.4	7.2	8.2	1210	
2	10/16/04		20	100.0	21.3	7.1	7.5	1080		20	100.0	21.2	7.1	7.4	1075	100.0	21.3	7.1	7.5	1078	
3	10/17/04		20	100.0	21.0	7.2	7.0	1242		20	100.0	21.0	7.2	7.3	1301	100.0	21.0	7.2	7.2	1272	
4	10/18/04		20	100.0	20.6	7.5	7.4	1323		20	100.0	20.6	7.5	7.8	1339	100.0	20.6	7.5	7.6	1331	
5	10/19/04		20	100.0	21.0	7.3	8.4	1325		20	100.0	21.0	7.3	8.5	1358	100.0	21.0	7.3	8.5	1342	
6	10/20/04		20	100.0	21.1	7.3	8.0	1338		20	100.0	21.1	7.3	7.9	1361	100.0	21.1	7.3	8.0	1350	2.20
7	10/21/04		20	100.0	21.3	7.2	6.4	1325		20	100.0	21.3	7.2	6.3	1336	100.0	21.3	7.2	6.4	1331	
8	10/22/04		20	100.0	21.7	7.3	6.6	1230		20	100.0	21.7	7.2	6.3	1300	100.0	21.7	7.3	6.5	1265	
9	10/23/04		20	100.0	21.5	7.2	6.7	1208		20	100.0	21.5	7.3	7.0	1058	100.0	21.5	7.3	6.9	1133	
10	10/24/04		20	100.0	21.3	7.2	7.6	1272		20	100.0	21.4	7.2	7.3	1093	100.0	21.4	7.2	7.5	1183	
11	10/25/04		20	100.0	21.2	7.1	6.4	1180		20	100.0	21.2	7.1	6.2	1244	100.0	21.2	7.1	6.3	1212	
12	10/26/04		20	100.0	21.4	7.2	6.8	1304		20	100.0	21.4	7.2	6.8	1320	100.0	21.4	7.2	6.8	1312	
13	10/27/04		20	100.0	21.2	7.3	7.1	1300		20	100.0	21.2	7.3	6.9	1330	100.0	21.2	7.3	7.0	1315	
14	10/28/04		20	100.0	21.4	7.3	8.0	1311		20	100.0	21.4	7.3	7.8	1342	100.0	21.4	7.3	7.9	1327	4.74
15	10/29/04		20	100.0	21.5	7.2	7.6	1190		20	100.0	21.5	7.2	7.5	1277	100.0	21.5	7.2	7.6	1234	
16	10/30/04		20	100.0	20.7	7.4	8.3	1327		20	100.0	20.7	7.4	8.3	1345	100.0	20.7	7.4	8.3	1336	
17	10/31/04		20	100.0	21.5	7.4	8.0	1333		20	100.0	21.6	7.4	8.1	1350	100.0	21.6	7.4	8.1	1342	
18	11/01/04		20	100.0	21.2	7.4	7.9	1190		20	100.0	21.2	7.4	7.8	1280	100.0	21.2	7.4	7.9	1235	
19	11/02/04		20	100.0	21.3	7.2	7.9	1336		20	100.0	21.3	7.2	7.7	1375	100.0	21.3	7.2	7.8	1356	
20	11/03/04		20	100.0	21.1	7.2	7.9	1320		20	100.0	21.1	7.2	7.9	1370	100.0	21.1	7.2	7.9	1345	
21	11/04/04		20	100.0						20	100.0					100.0					
	Median:				**21.2**	**7.2**	**7.6**	**1300**				**21.2**	**7.2**	**7.7**	**1330**		**21.2**	**7.2**	**7.6**	**1312**	
	Mode:				**21.3**	**7.2**	**8.0**	**1325**				**21.2**	**7.2**	**7.8**	**1336**		**21.2**	**7.2**	**7.9**	**1342**	
	Mean:			**100.0**	**21.2**	**7.3**	**7.6**	**1267**			**100.0**	**21.2**	**7.3**	**7.6**	**1285**	**100.0**	**21.2**	**7.3**	**7.6**	**1276**	**4.208**
	SEM:			**0.00**	**0.08**	**0.02**	**0.17**	**15.21**			**0.00**	**0.08**	**0.02**	**0.18**	**21.32**	**0.00**	**0.08**	**0.02**	**0.17**	**16.98**	**1.04**
	% CV:			**0.00**	**7.58**	**3.82**	**27.81**	**195.78**			**0.00**	**7.55**	**3.82**	**29.31**	**272.48**	**0.00**	**7.55**	**3.77**	**28.34**	**217.78**	**87.92**

DAILY DATA RENEW

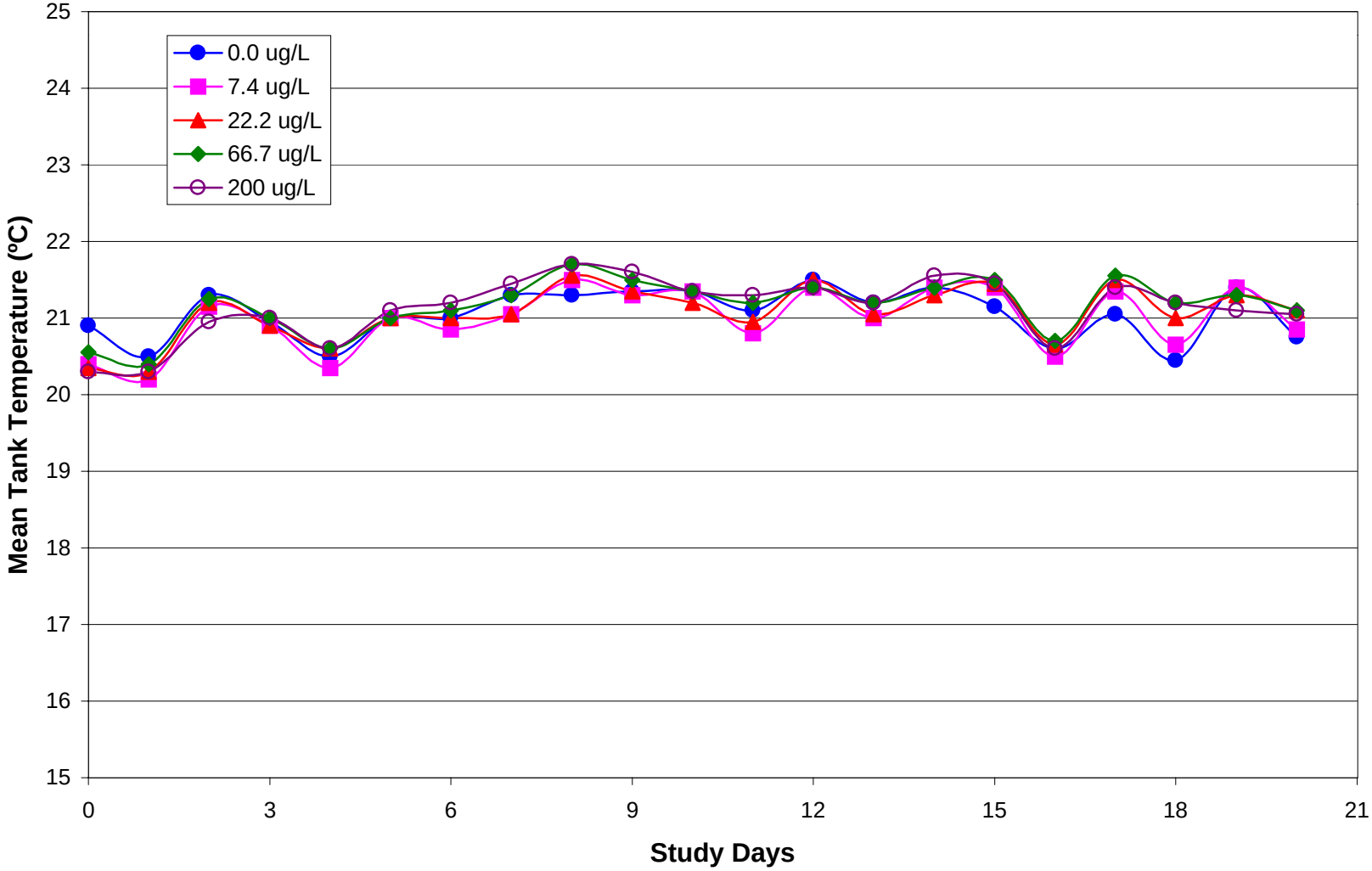
Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 200 ug/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc (ug/L)
		Initial No.= 20							**Initial No.= 20**												
0	10/14/04			100.0	20.3	7.4	8.6	1012			100.0	20.3	7.6	8.6	1053	100.0	20.3	7.5	8.6	1033	14.87
1	10/15/04		20	100.0	20.3	7.3	7.7	1095		20	100.0	20.3	7.3	7.7	1160	100.0	20.3	7.3	7.7	1128	
2	10/16/04		20	100.0	21.0	7.2	6.9	1006		20	100.0	20.9	7.2	6.9	1024	100.0	21.0	7.2	6.9	1015	
3	10/17/04		20	100.0	21.0	7.2	6.8	1037		20	100.0	21.0	7.2	6.9	1104	100.0	21.0	7.2	6.9	1071	
4	10/18/04		20	100.0	20.6	7.5	7.5	1049		20	100.0	20.6	7.5	7.6	1106	100.0	20.6	7.5	7.6	1078	
5	10/19/04		20	100.0	21.1	7.5	7.9	1046		20	100.0	21.1	7.5	8.0	1129	100.0	21.1	7.5	8.0	1088	
6	10/20/04		20	100.0	21.2	7.3	7.6	1040		20	100.0	21.2	7.4	7.5	1134	100.0	21.2	7.4	7.6	1087	13.05
7	10/21/04		20	100.0	21.4	7.2	7.1	1014		20	100.0	21.5	7.3	7.4	1124	100.0	21.5	7.3	7.3	1069	
8	10/22/04		20	100.0	21.7	7.4	7.7	1050		20	100.0	21.7	7.4	7.7	1130	100.0	21.7	7.4	7.7	1090	
9	10/23/04		20	100.0	21.6	7.2	7.4	1038		20	100.0	21.6	7.1	7.4	1062	100.0	21.6	7.2	7.4	1050	
10	10/24/04		20	100.0	21.4	7.3	7.5	1033		20	100.0	21.3	7.2	7.5	1044	100.0	21.4	7.3	7.5	1039	
11	10/25/04		20	100.0	21.3	7.3	7.7	1040		20	100.0	21.3	7.4	7.8	1080	100.0	21.3	7.4	7.8	1060	
12	10/26/04		20	100.0	21.4	7.3	7.9	1012		20	100.0	21.4	7.4	7.8	1147	100.0	21.4	7.4	7.9	1080	
13	10/27/04		20	100.0	21.2	7.4	7.8	1020		20	100.0	21.2	7.5	7.8	1130	100.0	21.2	7.5	7.8	1075	
14	10/28/04		20	100.0	21.5	7.5	7.9	1050		20	100.0	21.6	7.5	7.6	1131	100.0	21.6	7.5	7.8	1091	15.25
15	10/29/04		20	100.0	21.5	7.5	7.8	928		20	100.0	21.4	7.4	7.7	1099	100.0	21.5	7.5	7.8	1014	
16	10/30/04		20	100.0	20.6	7.5	8.0	1027		20	100.0	20.6	7.5	7.9	1127	100.0	20.6	7.5	8.0	1077	
17	10/31/04		20	100.0	21.4	7.5	8.0	1061		20	100.0	21.4	7.5	8.0	1168	100.0	21.4	7.5	8.0	1115	
18	11/01/04		20	100.0	21.2	7.4	7.7	1000		20	100.0	21.2	7.5	7.6	1130	100.0	21.2	7.5	7.7	1065	
19	11/02/04		20	100.0	21.1	7.3	7.6	1056		20	100.0	21.1	7.3	7.6	1144	100.0	21.1	7.3	7.6	1100	
20	11/03/04		20	100.0	21.0	7.3	7.7	1040		20	100.0	21.1	7.4	7.7	1150	100.0	21.1	7.4	7.7	1095	
21	11/04/04		20	100.0						20	100.0					100.0					
	Median:				**21.2**	**7.3**	**7.7**	**1038**				**21.2**	**7.4**	**7.7**	**1129**		**21.2**	**7.4**	**7.7**	**1077**	
	Mode:				**21.4**	**7.3**	**7.7**	**1040**				**21.1**	**7.5**	**7.7**	**1130**		**21.2**	**7.5**	**7.7**	**#N/A**	
	Mean:			**100.0**	**21.1**	**7.4**	**7.7**	**1031**			**100.0**	**21.1**	**7.4**	**7.7**	**1113**	**100.0**	**21.1**	**7.4**	**7.7**	**1072**	**14.390**
	SEM:			**0.00**	**0.09**	**0.02**	**0.09**	**7.00**			**0.00**	**0.09**	**0.03**	**0.08**	**8.62**	**0.00**	**0.09**	**0.03**	**0.08**	**6.45**	**0.68**
	% CV:			**0.00**	**8.63**	**4.13**	**14.21**	**99.86**			**0.00**	**8.71**	**4.84**	**13.01**	**118.43**	**0.00**	**8.65**	**4.26**	**13.46**	**90.32**	**31.00**

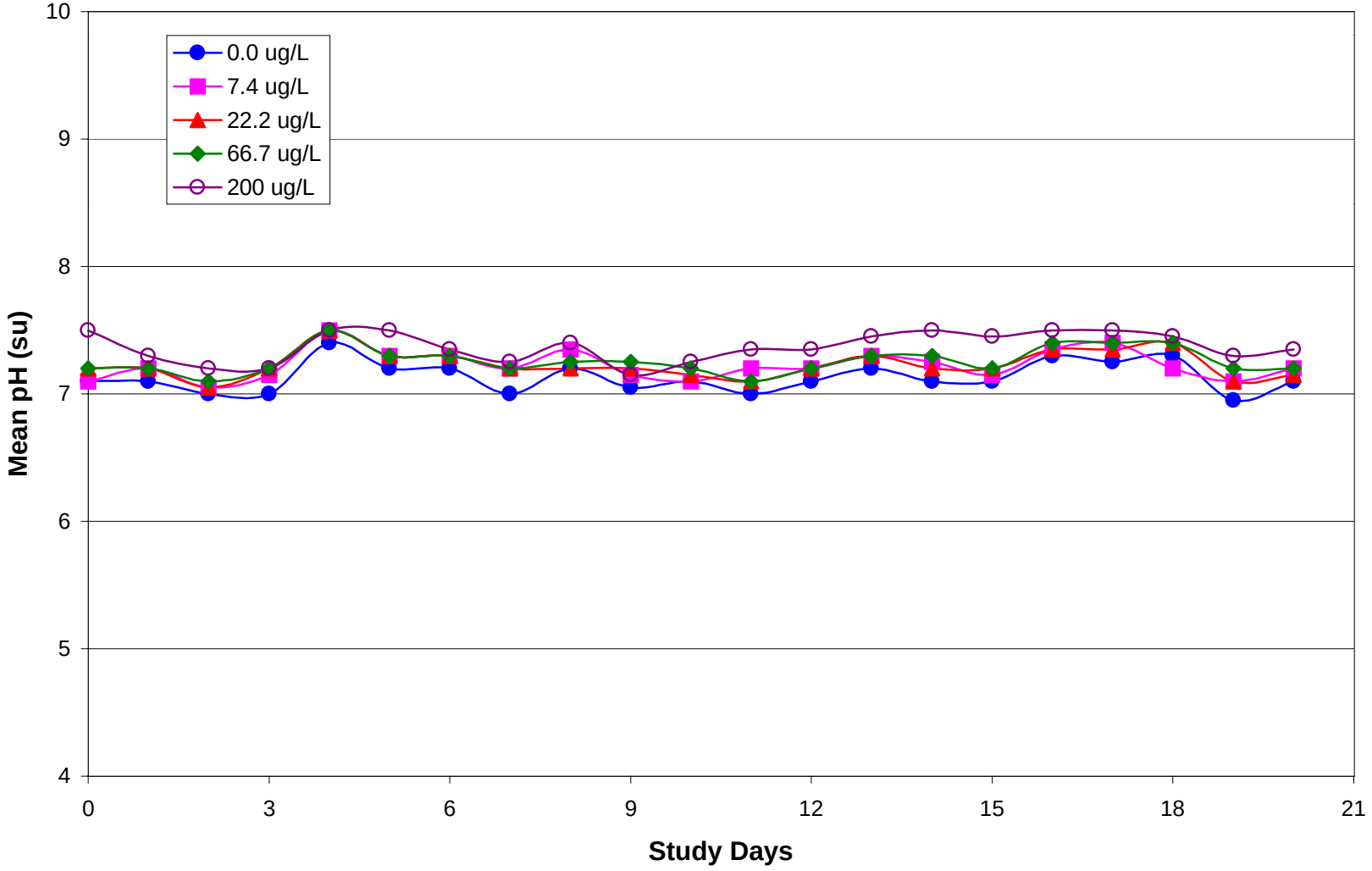
BATT02-00055
PCN - Renewal
Daily Average of Larval Survival for Initial Stage 51 Test



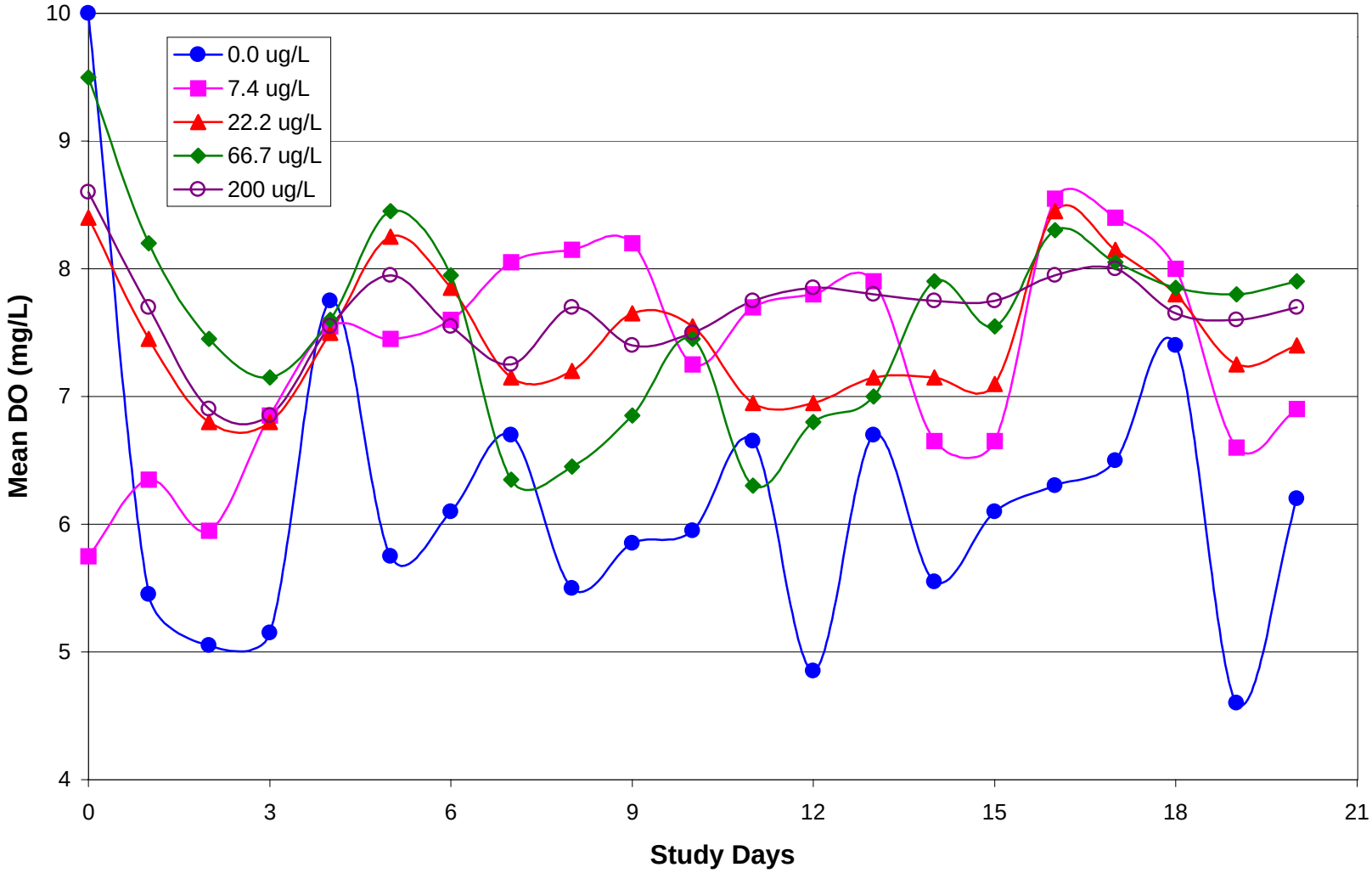
BATT02-00055
PCN - Renewal
Daily Average Tank Temperatures for Initial Stage 51 Test



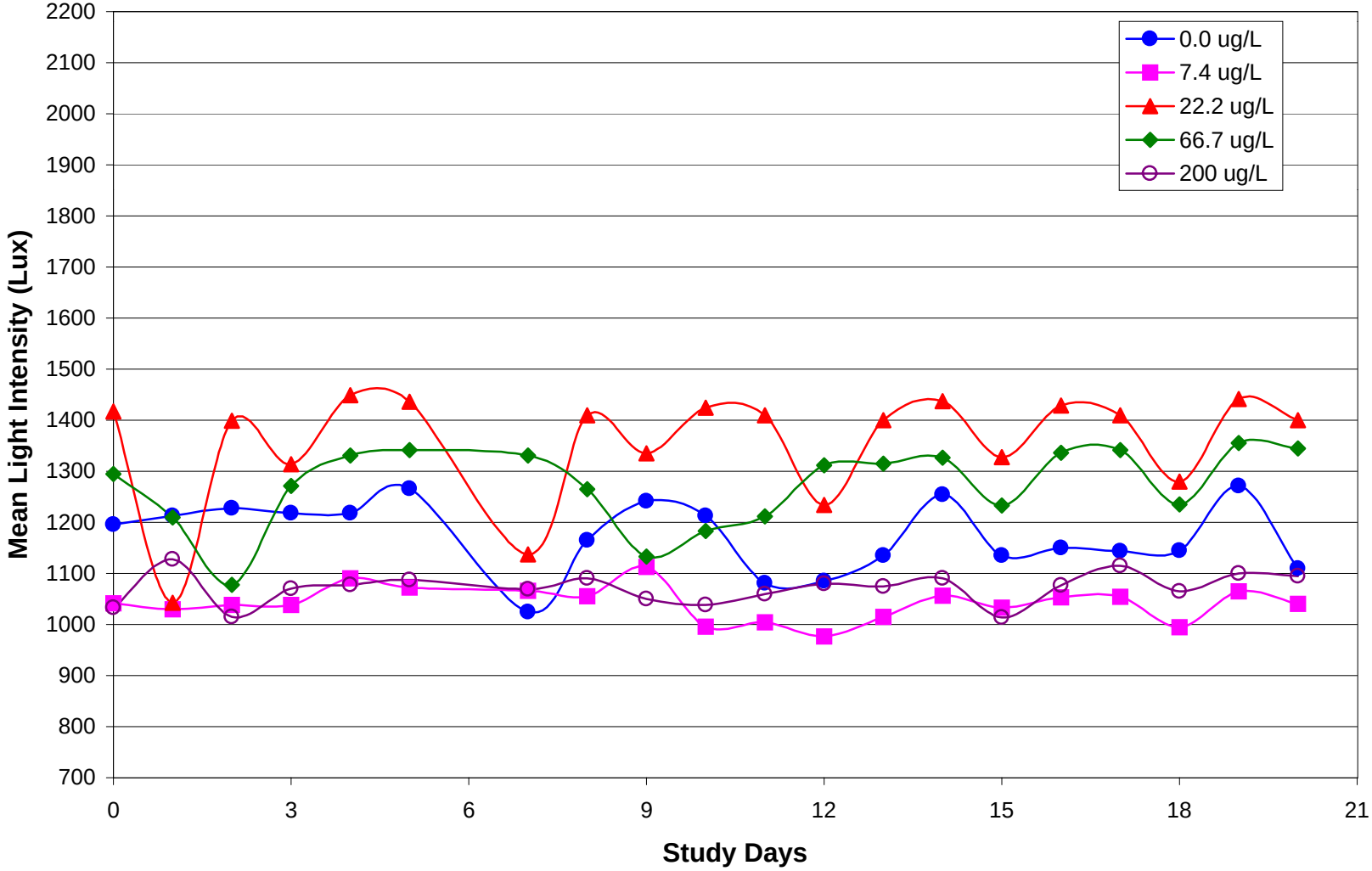
BATT02-00055
PCN - Renewal
Daily Average pH for Initial Stage 51 Test



BATT02-00055
PCN - Renewal
Daily Average Dissolved Oxygen (DO) for Initial Stage 51 Test



BATT02-00055
PCN - Renewal
Daily Average Light Intensity for Initial Stage 51 Test



WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA SPENT

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 0.0 mg/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	10/15/04		20	100.0	19.4	7.3	8.3	1175		20	100.0	19.4	7.3	8.4	1250	100.0	19.4	7.3	8.4	1213	
2	10/16/04		20	100.0	21.2	7.0	4.6	1204		20	100.0	21.2	7.0	4.5	1251	100.0	21.2	7.0	4.6	1228	
3	10/17/04		20	100.0	20.6	7.3	4.2	1183		20	100.0	20.6	7.1	4.1	1254	100.0	20.6	7.2	4.2	1219	
4	10/18/04		20	100.0	20.4	7.5	7.7	1236		20	100.0	20.4	7.6	8.2	1200	100.0	20.4	7.6	8.0	1218	
5	10/19/04		20	100.0	20.8	7.6	8.1	1273		20	100.0	20.8	7.5	7.8	1259	100.0	20.8	7.6	8.0	1266	
6	10/20/04		20	100.0	21.0	7.6	7.7	1211		20	100.0	21.0	7.5	7.4	1140	100.0	21.0	7.6	7.6	1176	
7	10/21/04		20	100.0	21.0	7.4	7.5	1040		20	100.0	21.0	7.4	7.2	1010	100.0	21.0	7.4	7.4	1025	
8	10/22/04		20	100.0	21.1	7.4	7.0	1180		20	100.0	20.9	7.5	7.9	1150	100.0	21.0	7.5	7.5	1165	
9	10/23/04		20	100.0	21.0	7.4	7.4	1248		20	100.0	21.1	7.5	7.7	1234	100.0	21.1	7.5	7.6	1241	
10	10/24/04		20	100.0	20.8	7.3	7.9	1172		20	100.0	20.8	7.3	7.6	1253	100.0	20.8	7.3	7.8	1213	
11	10/25/04		20	100.0	21.5	7.4	7.1	1088		20	100.0	21.1	7.6	7.9	1074	100.0	21.3	7.5	7.5	1081	
12	10/26/04		20	100.0	21.6	7.4	7.0	1099		20	100.0	21.4	7.5	7.7	1071	100.0	21.5	7.5	7.4	1085	
13	10/27/04		20	100.0	21.2	7.3	6.8	1080		20	100.0	21.2	7.2	4.8	1190	100.0	21.2	7.3	5.8	1135	
14	10/28/04		20	100.0	21.2	7.4	7.1	1260		20	100.0	21.2	7.4	7.7	1249	100.0	21.2	7.4	7.4	1255	
15	10/29/04		20	100.0	21.2	7.4	7.2	1119		20	100.0	21.2	7.4	7.3	1152	100.0	21.2	7.4	7.3	1136	
16	10/30/04		20	100.0	20.6	7.4	7.4	1120		20	100.0	20.6	7.5	7.4	1179	100.0	20.6	7.5	7.4	1150	
17	10/31/04		20	100.0	21.1	7.5	7.2	1123		20	100.0	21.1	7.4	7.7	1164	100.0	21.1	7.5	7.5	1144	
18	11/01/04		20	100.0	21.1	7.4	6.6	1130		20	100.0	21.0	7.6	7.7	1160	100.0	21.1	7.5	7.2	1145	
19	11/02/04		20	100.0	21.0	7.4	6.9	1284		20	100.0	21.0	7.5	7.6	1258	100.0	21.0	7.5	7.3	1271	
20	11/03/04		20	100.0	20.6	7.5	7.6	1100		20	100.0	20.4	7.6	7.9	1120	100.0	20.5	7.6	7.8	1110	
21	11/04/04		20	100.0	21.0	7.6	8.0	1058		20	100.0	21.6	7.7	8.2	1112	100.0	21.3	7.7	8.1	1085	
	Median:				**21.0**	**7.4**	**7.2**	**1172**				**21.0**	**7.5**	**7.7**	**1179**		**21.0**	**7.5**	**7.5**	**1165**	
	Mode:				**21.0**	**7.4**	**7.7**	**#N/A**				**21.2**	**7.5**	**7.7**	**#N/A**		**21.2**	**7.5**	**8.0**	**1213**	
	Mean:			**100.0**	**20.9**	**7.4**	**7.1**	**1161**			**100.0**	**20.9**	**7.4**	**7.3**	**1178**	**100.0**	**20.9**	**7.4**	**7.2**	**1169**	
	SEM:			**0.00**	**0.10**	**0.03**	**0.22**	**15.94**			**0.00**	**0.10**	**0.04**	**0.26**	**15.83**	**0.00**	**0.10**	**0.03**	**0.23**	**14.99**	
	% CV:			**0.00**	**9.97**	**4.86**	**37.76**	**214.39**			**0.00**	**10.03**	**6.39**	**45.03**	**211.46**	**0.00**	**9.80**	**5.34**	**39.76**	**200.89**	

DAILY DATA SPENT

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 7.4 ug/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	10/15/04		20	100.0	19.3	7.3	6.4	1185		20	100.0	19.3	7.3	6.5	875	100.0	19.3	7.3	6.5	1030	
2	10/16/04		20	100.0	21.0	7.1	4.3	1192		20	100.0	21.0	7.1	4.4	884	100.0	21.0	7.1	4.4	1038	
3	10/17/04		20	100.0	20.6	7.0	4.1	1187		20	100.0	20.7	7.0	4.3	890	100.0	20.7	7.0	4.2	1039	
4	10/18/04		20	100.0	20.2	7.3	6.5	1240		20	100.0	20.2	7.2	4.6	941	100.0	20.2	7.3	5.6	1091	
5	10/19/04		20	100.0	20.8	7.5	7.6	1198		20	100.0	20.8	7.5	7.6	947	100.0	20.8	7.5	7.6	1073	
6	10/20/04		20	100.0	20.9	7.5	7.3	1181		20	100.0	21.0	7.4	6.7	1201	100.0	21.0	7.5	7.0	1191	
7	10/21/04		20	100.0	20.8	7.3	7.1	1212		20	100.0	20.8	7.4	7.7	920	100.0	20.8	7.4	7.4	1066	
8	10/22/04		20	100.0	21.2	7.0	6.5	910		20	100.0	21.1	7.2	7.2	1200	100.0	21.2	7.1	6.9	1055	
9	10/23/04		20	100.0	21.1	7.3	7.1	1197		20	100.0	20.9	7.2	7.5	1029	100.0	21.0	7.3	7.3	1113	
10	10/24/04		20	100.0	20.8	7.4	8.1	1089		20	100.0	20.9	7.3	7.8	902	100.0	20.9	7.4	8.0	996	
11	10/25/04		20	100.0	21.0	7.6	7.9	1123		20	100.0	21.0	7.6	7.9	886	100.0	21.0	7.6	7.9	1005	
12	10/26/04		20	100.0	21.1	7.6	7.8	1022		20	100.0	21.1	7.6	7.8	932	100.0	21.1	7.6	7.8	977	
13	10/27/04		20	100.0	20.8	7.2	7.6	1150		20	100.0	20.8	7.3	7.6	880	100.0	20.8	7.3	7.6	1015	
14	10/28/04		20	100.0	21.0	7.5	7.7	1185		20	100.0	20.9	7.5	7.7	927	100.0	21.0	7.5	7.7	1056	
15	10/29/04		20	100.0	21.0	7.0	7.0	1160		20	100.0	21.2	7.2	7.9	905	100.0	21.1	7.1	7.5	1033	
16	10/30/04		20	100.0	20.5	7.5	7.9	1197		20	100.0	20.4	7.5	7.6	910	100.0	20.5	7.5	7.8	1054	
17	10/31/04		20	100.0	20.9	7.5	7.6	1193		20	100.0	20.9	7.5	7.3	916	100.0	20.9	7.5	7.5	1055	
18	11/01/04		20	100.0	20.9	7.5	7.4	1070		20	100.0	21.0	7.4	7.2	920	100.0	21.0	7.5	7.3	995	
19	11/02/04		20	100.0	20.9	7.4	7.2	1219		20	100.0	20.8	7.4	7.1	910	100.0	20.9	7.4	7.2	1065	
20	11/03/04		20	100.0	20.6	7.5	7.5	1180	1	19	95.0	20.6	7.5	7.5	900	97.5	20.6	7.5	7.5	1040	
21	11/04/04		20	100.0	20.8	7.8	8.1	1155		19	95.0	20.8	7.8	8.3	875	97.5	20.8	7.8	8.2	1015	
	Median:				**20.9**	**7.4**	**7.4**	**1185**				**20.9**	**7.4**	**7.5**	**910**		**20.9**	**7.4**	**7.5**	**1040**	
	Mode:				**20.8**	**7.5**	**7.6**	**1185**				**20.8**	**7.5**	**7.6**	**875**		**20.8**	**7.5**	**7.6**	**1015**	
	Mean:			**100.0**	**20.8**	**7.4**	**7.1**	**1155**			**99.5**	**20.8**	**7.4**	**7.1**	**940**	**99.8**	**20.8**	**7.4**	**7.1**	**1048**	
	SEM:			**0.00**	**0.09**	**0.05**	**0.24**	**16.71**			**0.33**	**0.09**	**0.04**	**0.26**	**20.23**	**0.16**	**0.09**	**0.04**	**0.24**	**10.16**	
	% CV:			**0.00**	**8.92**	**8.00**	**40.50**	**225.34**			**15.08**	**8.97**	**6.98**	**44.07**	**302.37**	**7.53**	**8.89**	**7.33**	**41.01**	**143.85**	

DAILY DATA SPENT

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 22.2 ug/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	10/15/04		20	100.0	19.4	7.3	7.3	975		20	100.0	19.4	7.3	7.0	1110	100.0	19.4	7.3	7.2	1043	
2	10/16/04		20	100.0	21.0	7.0	4.6	1410		20	100.0	21.0	7.0	4.4	1387	100.0	21.0	7.0	4.5	1399	
3	10/17/04		20	100.0	20.6	7.0	4.1	1221		20	100.0	20.6	7.0	4.1	1406	100.0	20.6	7.0	4.1	1314	
4	10/18/04		20	100.0	20.3	7.3	6.1	1456		20	100.0	20.3	7.2	4.7	1442	100.0	20.3	7.3	5.4	1449	
5	10/19/04		20	100.0	20.8	7.6	7.6	1436		20	100.0	20.8	7.5	7.3	1436	100.0	20.8	7.6	7.5	1436	
6	10/20/04		20	100.0	21.0	7.4	6.9	1450		20	100.0	21.0	7.4	7.0	1446	100.0	21.0	7.4	7.0	1448	
7	10/21/04		20	100.0	20.9	7.4	7.6	1158		20	100.0	20.9	7.4	7.0	1117	100.0	20.9	7.4	7.3	1138	
8	10/22/04		20	100.0	21.0	7.3	7.2	1390		20	100.0	20.7	7.4	6.4	1430	100.0	20.9	7.4	6.8	1410	
9	10/23/04		20	100.0	21.0	7.1	8.1	1441		20	100.0	21.1	7.3	7.7	1230	100.0	21.1	7.2	7.9	1336	
10	10/24/04		20	100.0	20.8	7.4	7.7	1437		20	100.0	21.1	7.5	7.8	1412	100.0	21.0	7.5	7.8	1425	
11	10/25/04		20	100.0	21.1	7.4	7.4	1416		20	100.0	21.1	7.4	7.3	1403	100.0	21.1	7.4	7.4	1410	
12	10/26/04		20	100.0	21.2	7.4	7.1	1220		20	100.0	21.2	7.4	6.9	1248	100.0	21.2	7.4	7.0	1234	
13	10/27/04		20	100.0	20.8	7.4	7.0	1430		20	100.0	20.9	7.4	6.7	1370	100.0	20.9	7.4	6.9	1400	
14	10/28/04		20	100.0	21.0	7.4	6.9	1455		20	100.0	21.1	7.4	6.8	1420	100.0	21.1	7.4	6.9	1438	
15	10/29/04		20	100.0	21.1	7.3	7.1	1305		20	100.0	21.1	7.3	7.2	1350	100.0	21.1	7.3	7.2	1328	
16	10/30/04		20	100.0	20.5	7.4	7.0	1430		20	100.0	20.5	7.4	6.7	1428	100.0	20.5	7.4	6.9	1429	
17	10/31/04		20	100.0	21.0	7.5	7.9	1422		20	100.0	21.1	7.4	7.1	1398	100.0	21.1	7.5	7.5	1410	
18	11/01/04		20	100.0	21.0	7.3	6.4	1240		20	100.0	21.0	7.3	6.4	1320	100.0	21.0	7.3	6.4	1280	
19	11/02/04		20	100.0	21.0	7.3	6.4	1445		20	100.0	21.0	7.3	6.7	1439	100.0	21.0	7.3	6.6	1442	
20	11/03/04		20	100.0	20.7	7.4	6.9	1410		20	100.0	20.7	7.4	6.9	1390	100.0	20.7	7.4	6.9	1400	
21	11/04/04		20	100.0	20.8	7.7	8.3	1329		20	100.0	20.8	7.7	7.9	1388	100.0	20.8	7.7	8.1	1359	
	Median:				**21.0**	**7.4**	**7.1**	**1416**				**21.0**	**7.4**	**6.9**	**1398**		**21.0**	**7.4**	**7.0**	**1400**	
	Mode:				**21.0**	**7.4**	**6.9**	**1410**				**21.1**	**7.4**	**7.0**	**#N/A**		**21.0**	**7.4**	**6.9**	**1410**	
	Mean:			**100.0**	**20.8**	**7.3**	**6.9**	**1356**			**100.0**	**20.8**	**7.4**	**6.7**	**1360**	**100.0**	**20.8**	**7.4**	**6.8**	**1358**	
	SEM:			**0.00**	**0.08**	**0.04**	**0.22**	**27.62**			**0.00**	**0.09**	**0.03**	**0.23**	**21.96**	**0.00**	**0.09**	**0.03**	**0.22**	**23.48**	
	% CV:			**0.00**	**8.52**	**6.13**	**38.79**	**343.78**			**0.00**	**8.80**	**5.67**	**40.01**	**272.86**	**0.00**	**8.58**	**5.77**	**38.69**	**291.97**	

DAILY DATA SPENT

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 66.7 ug/L**

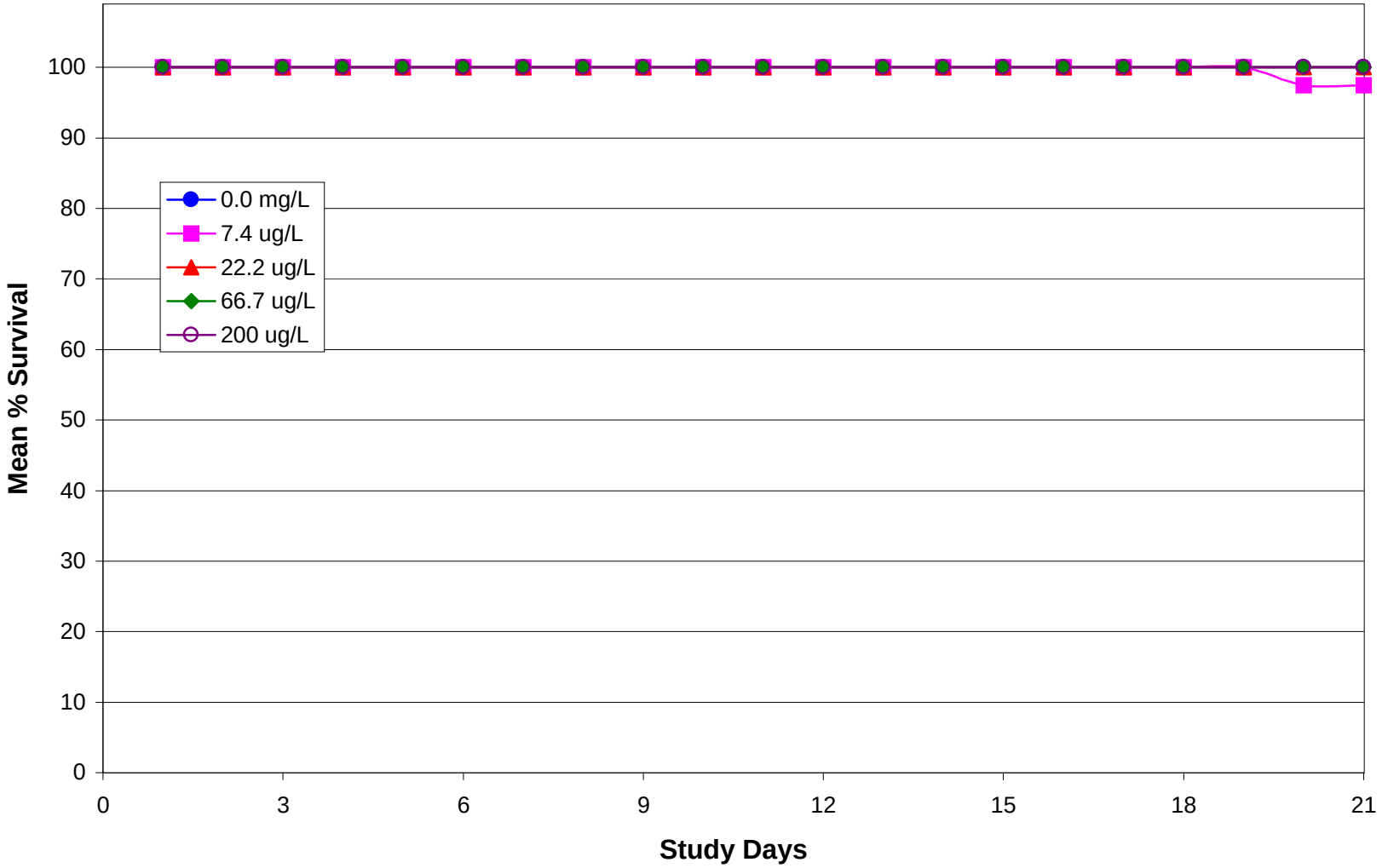
		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	10/15/04		20	100.0	19.4	7.3	7.7	1220		20	100.0	19.4	7.3	7.7	1200	100.0	19.4	7.3	7.7	1210	
2	10/16/04		20	100.0	20.9	7.1	4.9	1080		20	100.0	21.1	7.0	5.0	1075	100.0	21.0	7.1	5.0	1078	
3	10/17/04		20	100.0	20.6	7.0	4.3	1242		20	100.0	20.6	7.0	4.2	1301	100.0	20.6	7.0	4.3	1272	
4	10/18/04		20	100.0	20.3	7.1	4.7	1323		20	100.0	20.3	7.2	6.7	1339	100.0	20.3	7.2	5.7	1331	
5	10/19/04		20	100.0	20.9	7.5	7.6	1325		20	100.0	20.8	7.5	7.8	1358	100.0	20.9	7.5	7.7	1342	
6	10/20/04		20	100.0	21.0	7.5	7.6	1338		20	100.0	21.1	7.4	7.3	1361	100.0	21.1	7.5	7.5	1350	
7	10/21/04		20	100.0	20.9	7.3	7.3	1325		20	100.0	20.9	7.3	7.1	1336	100.0	20.9	7.3	7.2	1331	
8	10/22/04		20	100.0	20.9	7.4	7.3	1230		20	100.0	21.0	7.4	6.6	1300	100.0	21.0	7.4	7.0	1265	
9	10/23/04		20	100.0	20.8	7.4	6.9	1208		20	100.0	21.0	7.4	7.3	1058	100.0	20.9	7.4	7.1	1133	
10	10/24/04		20	100.0	21.0	7.3	7.9	1272		20	100.0	21.0	7.3	7.5	1093	100.0	21.0	7.3	7.7	1183	
11	10/25/04		20	100.0	21.1	7.5	7.9	1180		20	100.0	21.1	7.5	7.7	1244	100.0	21.1	7.5	7.8	1212	
12	10/26/04		20	100.0	21.1	7.6	7.8	1304		20	100.0	21.2	7.6	7.6	1320	100.0	21.2	7.6	7.7	1312	
13	10/27/04		20	100.0	20.9	7.4	7.6	1300		20	100.0	21.0	7.5	7.5	1330	100.0	21.0	7.5	7.6	1315	
14	10/28/04		20	100.0	21.1	7.4	7.5	1311		20	100.0	21.1	7.4	7.2	1342	100.0	21.1	7.4	7.4	1327	
15	10/29/04		20	100.0	21.1	7.4	7.5	1190		20	100.0	21.1	7.4	7.4	1277	100.0	21.1	7.4	7.5	1234	
16	10/30/04		20	100.0	20.5	7.4	7.8	1327		20	100.0	20.5	7.4	7.5	1345	100.0	20.5	7.4	7.7	1336	
17	10/31/04		20	100.0	21.1	7.4	7.8	1333		20	100.0	21.1	7.4	7.1	1350	100.0	21.1	7.4	7.5	1342	
18	11/01/04		20	100.0	21.0	7.4	7.2	1190		20	100.0	21.0	7.4	6.8	1280	100.0	21.0	7.4	7.0	1235	
19	11/02/04		20	100.0	21.0	7.3	7.2	1336		20	100.0	21.0	7.3	6.8	1375	100.0	21.0	7.3	7.0	1356	
20	11/03/04		20	100.0	20.6	7.4	7.4	1320		20	100.0	20.6	7.4	7.5	1370	100.0	20.6	7.4	7.5	1345	
21	11/04/04		20	100.0	20.7	7.6	7.9	1046		20	100.0	20.6	7.6	7.9	1146	100.0	20.7	7.6	7.9	1096	
	Median:				**20.9**	**7.4**	**7.5**	**1300**				**21.0**	**7.4**	**7.3**	**1320**		**21.0**	**7.4**	**7.5**	**1312**	
	Mode:				**20.9**	**7.4**	**7.6**	**1325**				**21.1**	**7.4**	**7.5**	**#N/A**		**21.0**	**7.4**	**7.7**	**1342**	
	Mean:			**100.0**	**20.8**	**7.4**	**7.1**	**1257**			**100.0**	**20.8**	**7.4**	**7.1**	**1276**	**100.0**	**20.8**	**7.4**	**7.1**	**1267**	
	SEM:			**0.00**	**0.09**	**0.03**	**0.24**	**18.50**			**0.00**	**0.09**	**0.03**	**0.20**	**22.14**	**0.00**	**0.09**	**0.03**	**0.21**	**18.98**	
	% CV:			**0.00**	**8.56**	**5.63**	**40.56**	**239.09**			**0.00**	**8.96**	**5.75**	**33.97**	**284.05**	**0.00**	**8.72**	**5.63**	**35.99**	**244.36**	

DAILY DATA SPENT

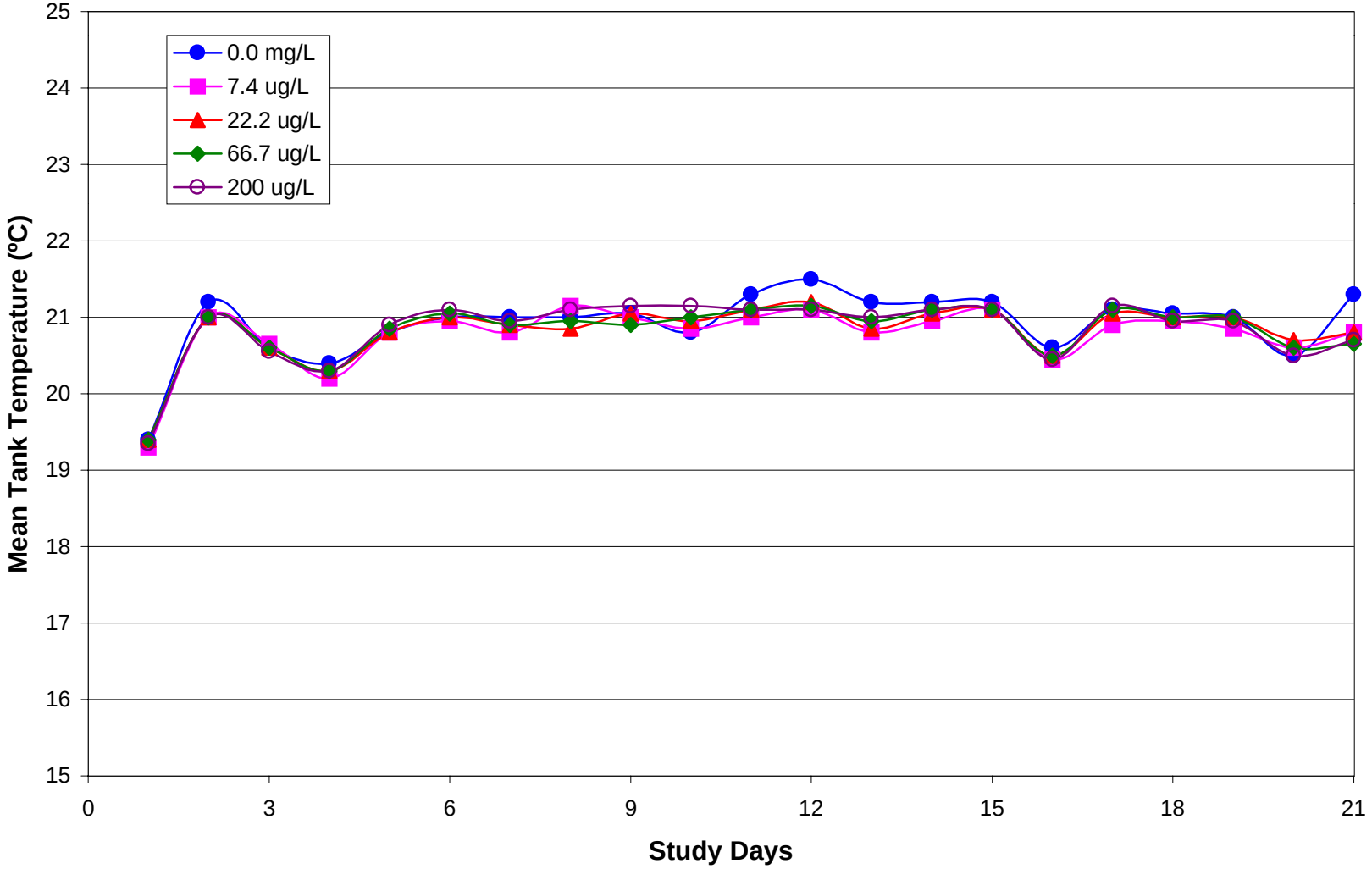
Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 200 ug/L**

		INITIAL STAGE 51 REPLICATE A							INITIAL STAGE 51 REPLICATE B							INITIAL STAGE 51 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	10/15/04		20	100.0	19.4	7.4	7.2	1095		20	100.0	19.3	7.4	7.2	1160	100.0	19.4	7.4	7.2	1128	
2	10/16/04		20	100.0	21.0	7.0	4.6	1006		20	100.0	21.0	7.0	4.5	1024	100.0	21.0	7.0	4.6	1015	
3	10/17/04		20	100.0	20.5	7.0	4.2	1037		20	100.0	20.6	7.0	4.4	1104	100.0	20.6	7.0	4.3	1071	
4	10/18/04		20	100.0	20.3	7.2	4.8	1049		20	100.0	20.3	7.2	6.2	1106	100.0	20.3	7.2	5.5	1078	
5	10/19/04		20	100.0	20.9	7.5	7.6	1046		20	100.0	20.9	7.5	7.7	1129	100.0	20.9	7.5	7.7	1088	
6	10/20/04		20	100.0	21.1	7.4	7.0	1040		20	100.0	21.1	7.3	7.0	1134	100.0	21.1	7.4	7.0	1087	
7	10/21/04		20	100.0	20.9	7.2	6.6	1014		20	100.0	21.0	7.2	6.8	1124	100.0	21.0	7.2	6.7	1069	
8	10/22/04		20	100.0	21.1	7.4	7.2	1050		20	100.0	21.1	7.4	7.2	1130	100.0	21.1	7.4	7.2	1090	
9	10/23/04		20	100.0	21.2	7.5	7.1	1038		20	100.0	21.1	7.4	7.0	1062	100.0	21.2	7.5	7.1	1050	
10	10/24/04		20	100.0	21.2	7.5	7.6	1033		20	100.0	21.1	7.6	7.8	1044	100.0	21.2	7.6	7.7	1039	
11	10/25/04		20	100.0	21.1	7.4	7.5	1040		20	100.0	21.1	7.4	7.5	1080	100.0	21.1	7.4	7.5	1060	
12	10/26/04		20	100.0	21.1	7.5	7.5	1012		20	100.0	21.1	7.5	7.5	1147	100.0	21.1	7.5	7.5	1080	
13	10/27/04		20	100.0	21.0	7.5	7.4	1020		20	100.0	21.0	7.5	7.3	1130	100.0	21.0	7.5	7.4	1075	
14	10/28/04		20	100.0	21.1	7.5	7.4	1050		20	100.0	21.1	7.5	7.4	1131	100.0	21.1	7.5	7.4	1091	
15	10/29/04		20	100.0	21.1	7.4	7.6	928		20	100.0	21.1	7.5	7.7	1099	100.0	21.1	7.5	7.7	1014	
16	10/30/04		20	100.0	20.5	7.5	7.9	1027		20	100.0	20.4	7.5	7.9	1127	100.0	20.5	7.5	7.9	1077	
17	10/31/04		20	100.0	21.2	7.6	6.5	1061		20	100.0	21.1	7.5	7.8	1168	100.0	21.2	7.6	7.2	1115	
18	11/01/04		20	100.0	20.9	7.4	7.4	1000		20	100.0	21.0	7.4	7.2	1130	100.0	21.0	7.4	7.3	1065	
19	11/02/04		20	100.0	21.0	7.3	7.4	1056		20	100.0	20.9	7.3	7.3	1144	100.0	21.0	7.3	7.4	1100	
20	11/03/04		20	100.0	20.5	7.4	7.6	1040		20	100.0	20.5	7.4	7.4	1150	100.0	20.5	7.4	7.5	1095	
21	11/04/04		20	100.0	20.7	7.7	8.0	935		20	100.0	20.7	7.7	8.0	1110	100.0	20.7	7.7	8.0	1023	
	Median:				**21.0**	**7.4**	**7.4**	**1038**				**21.0**	**7.4**	**7.3**	**1129**		**21.0**	**7.4**	**7.4**	**1077**	
	Mode:				**21.1**	**7.4**	**7.6**	**1040**				**21.1**	**7.5**	**7.2**	**1130**		**21.1**	**7.4**	**7.5**	**#N/A**	
	Mean:			**100.0**	**20.8**	**7.4**	**7.0**	**1027**			**100.0**	**20.8**	**7.4**	**7.1**	**1116**	**100.0**	**20.8**	**7.4**	**7.0**	**1072**	
	SEM:			**0.00**	**0.09**	**0.04**	**0.24**	**8.33**			**0.00**	**0.09**	**0.04**	**0.21**	**8.09**	**0.00**	**0.09**	**0.04**	**0.22**	**6.62**	
	% CV:			**0.00**	**9.31**	**6.42**	**40.91**	**119.12**			**0.00**	**9.51**	**6.47**	**36.35**	**110.93**	**0.00**	**9.38**	**6.38**	**37.85**	**92.61**	

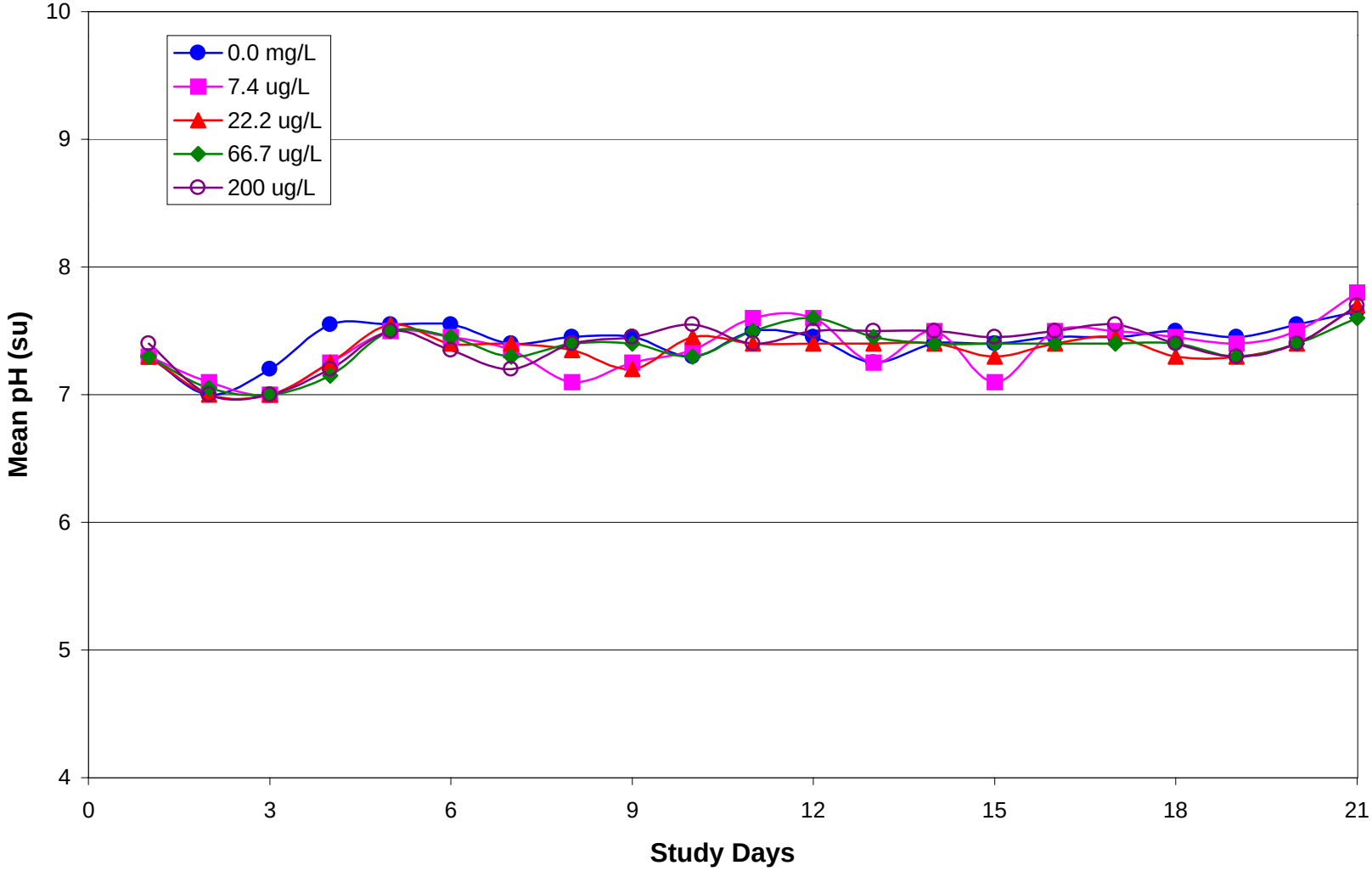
BATT02-00055
PCN - Spent
Daily Average of Larval Survival for Initial Stage 51 Test



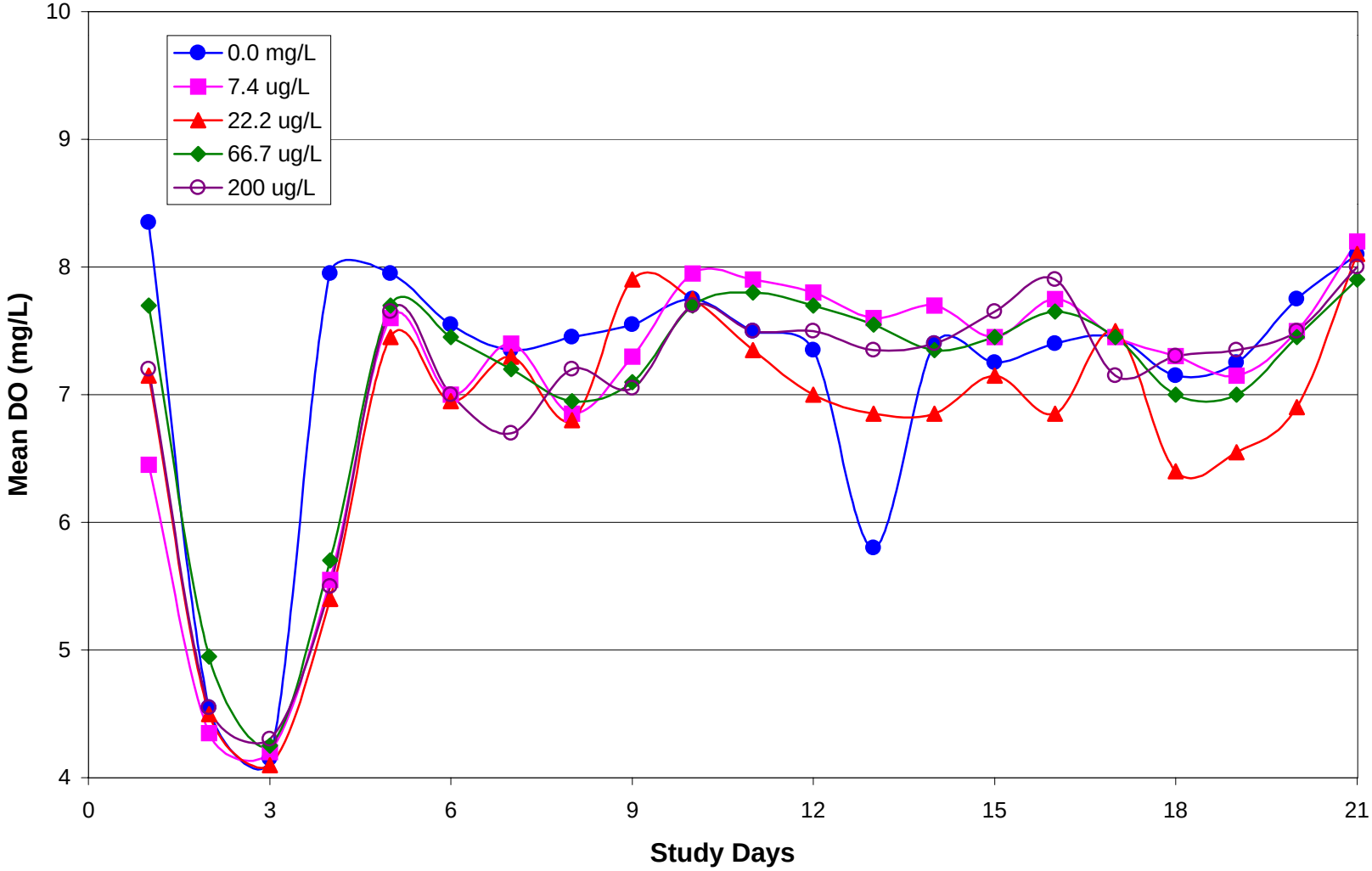
BATT02-00055
PCN - Spent
Daily Average Tank Temperatures for Initial Stage 51 Test



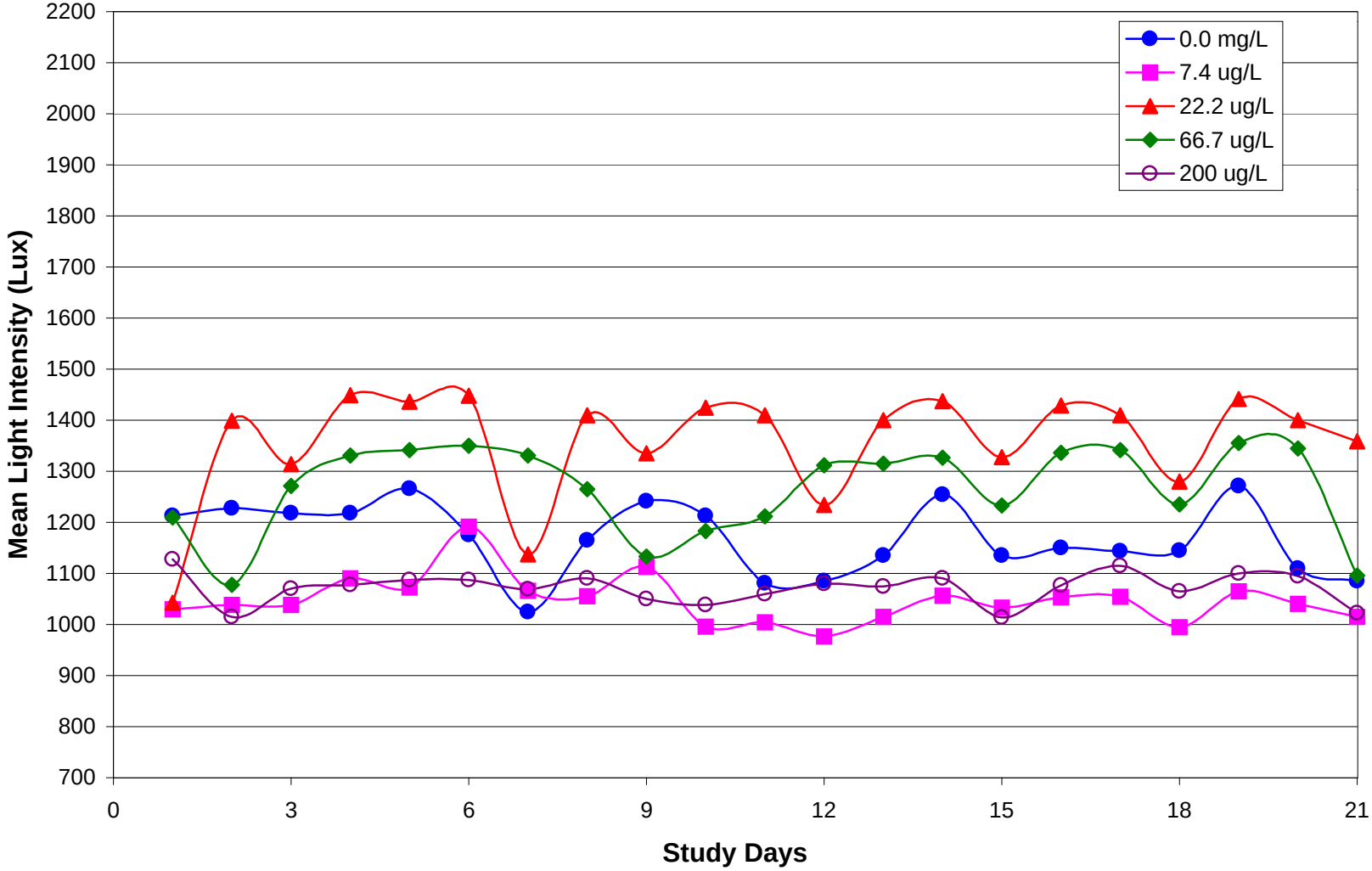
BATT02-00055
PCN - Spent
Daily Average pH for Initial Stage 51 Test



BATT02-00055
PCN - Spent
Daily Average Dissolved Oxygen (DO) for Initial Stage 51 Test



BATT02-00055
PCN - Spent
Daily Average Light Intensity for Initial Stage 51 Test



WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA RENEW

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 0.0 ug/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					Average Measured
		Daily	Cumul.		Tank			Light	Daily	Cumul.		Tank			Light		Tank			Light	Test
Study Day	Study Date	No. Dead	No. Alive	% Survival	Temp (°C)	pH (su)	DO (mg/L)	Intensity (Lux)	No. Dead	No. Alive	% Survival	Temp (°C)	pH (su)	DO (mg/L)	Intensity (Lux)	% Survival	Temp (°C)	pH (su)	DO (mg/L)	Intensity (Lux)	Conc[1] (ug/L)
		Initial No.= 20							**Initial No.= 20**												
0	10/20/04			100.0	21.0	7.7	8.3	1057			100.0	21.0	7.5	6.4	1229	100.0	21.0	7.6	7.4	1143	0.51
1	10/21/04		20	100.0	21.1	7.1	7.4	1144		20	100.0	21.1	7.2	7.6	1240	100.0	21.1	7.2	7.5	1192	
2	10/22/04		20	100.0	21.2	7.3	5.9	1210		20	100.0	21.2	7.4	6.2	1230	100.0	21.2	7.4	6.1	1220	
3	10/23/04		20	100.0	21.3	7.2	5.9	1181		20	100.0	21.3	7.1	6.3	1240	100.0	21.3	7.2	6.1	1211	
4	10/24/04		20	100.0	21.4	7.1	5.8	1179		20	100.0	21.4	7.0	5.9	1242	100.0	21.4	7.1	5.9	1211	
5	10/25/04		20	100.0	20.9	7.1	6.7	1120		20	100.0	20.9	7.1	6.9	900	100.0	20.9	7.1	6.8	1010	
6	10/26/04		20	100.0	21.3	7.2	5.4	954		20	100.0	21.3	7.2	5.8	1099	100.0	21.3	7.2	5.6	1027	
7	10/27/04		20	100.0	21.0	7.3	7.1	1300		20	100.0	21.0	7.4	7.6	1240	100.0	21.0	7.4	7.4	1270	
8	10/28/04		20	100.0	21.2	7.3	6.0	1272		20	100.0	21.2	7.3	6.2	1250	100.0	21.2	7.3	6.1	1261	0.51
9	10/29/04		20	100.0	21.0	7.1	6.4	1150		20	100.0	21.0	7.1	6.9	1230	100.0	21.0	7.1	6.7	1190	
10	10/30/04		20	100.0	20.6	7.3	6.8	1150		20	100.0	20.6	7.3	6.0	1133	100.0	20.6	7.3	6.4	1142	
11	10/31/04		20	100.0	21.0	7.4	6.7	1225		20	100.0	21.0	7.3	6.4	1241	100.0	21.0	7.4	6.6	1233	
12	11/01/04		20	100.0	20.6	7.3	5.3	1200		20	100.0	20.8	7.4	5.1	1190	100.0	20.7	7.4	5.2	1195	
13	11/02/04		20	100.0	21.3	7.1	5.8	1292		20	100.0	21.4	7.1	5.7	1330	100.0	21.4	7.1	5.8	1311	
14	11/03/04		20	100.0						20	100.0					100.0					
	Median:				**21.1**	**7.3**	**6.2**	**1180**				**21.1**	**7.3**	**6.3**	**1235**		**21.1**	**7.3**	**6.3**	**1203**	
	Mode:				**21.0**	**7.1**	**5.9**	**1150**				**21.0**	**7.1**	**6.4**	**1240**		**21.0**	**7.4**	**6.1**	**1211**	
	Mean:			**100.0**	**21.1**	**7.3**	**6.4**	**1174**			**100.0**	**21.1**	**7.2**	**6.4**	**1200**	**100.0**	**21.1**	**7.2**	**6.4**	**1187**	**0.510**
	SEM:			**0.00**	**0.07**	**0.04**	**0.22**	**24.71**			**0.00**	**0.06**	**0.04**	**0.19**	**27.24**	**0.00**	**0.06**	**0.04**	**0.19**	**22.63**	**0.00**
	% CV:			**0.00**	**5.38**	**6.14**	**32.96**	**269.83**			**0.00**	**5.04**	**5.59**	**27.82**	**294.31**	**0.00**	**5.18**	**5.62**	**27.70**	**245.85**	**0.00**

[1]All measured test concentrations reported by Battelle as less than limit of detection (LOD) [1.018 µg/L] or none detected (ND) are expressed as 0.51 µg/L (1/2 of LOD).

Results from samples collected on 11/03/04 are not reported pending further QA review.

DAILY DATA RENEW

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 7.4 ug/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					Average Measured
		Daily	Cumul.		Tank			Light	Daily	Cumul.		Tank			Light		Tank			Light	Test
Study Day	Study Date	No. Dead	No. Alive	% Survival	Temp (°C)	pH (su)	DO (mg/L)	Intensity (Lux)	No. Dead	No. Alive	% Survival	Temp (°C)	pH (su)	DO (mg/L)	Intensity (Lux)	% Survival	Temp (°C)	pH (su)	DO (mg/L)	Intensity (Lux)	Conc[1] (ug/L)
		Initial No.= 20							**Initial No.= 20**												
0	10/20/04			100.0	21.1	7.4	7.8	881			100.0	21.1	7.5	8.2	1003	100.0	21.1	7.5	8.0	942	0.51
1	10/21/04		20	100.0	21.0	7.2	8.2	822		20	100.0	21.0	7.2	8.2	1043	100.0	21.0	7.2	8.2	933	
2	10/22/04		20	100.0	21.6	7.3	8.2	840		20	100.0	21.6	7.2	8.2	990	100.0	21.6	7.3	8.2	915	
3	10/23/04		20	100.0	21.3	7.2	8.3	808		20	100.0	21.3	7.2	8.2	1088	100.0	21.3	7.2	8.3	948	
4	10/24/04		20	100.0	21.4	7.2	7.2	799		20	100.0	21.3	7.2	7.2	1079	100.0	21.4	7.2	7.2	939	
5	10/25/04		20	100.0	20.8	7.2	7.6	895		20	100.0	20.8	7.1	7.4	1081	100.0	20.8	7.2	7.5	988	
6	10/26/04		20	100.0	21.4	7.2	7.8	820		20	100.0	21.5	7.2	7.3	953	100.0	21.5	7.2	7.6	887	
7	10/27/04		20	100.0	21.1	7.3	7.7	870		20	100.0	21.1	7.3	6.9	1080	100.0	21.1	7.3	7.3	975	
8	10/28/04		20	100.0	21.4	7.2	6.7	881		20	100.0	21.4	7.3	5.7	1075	100.0	21.4	7.3	6.2	978	0.51
9	10/29/04		20	100.0	21.3	7.2	7.1	760		20	100.0	21.4	7.2	6.0	1068	100.0	21.4	7.2	6.6	914	
10	10/30/04		20	100.0	20.7	7.4	8.5	974		20	100.0	20.7	7.3	8.3	1119	100.0	20.7	7.4	8.4	1047	
11	10/31/04		20	100.0	21.3	7.4	8.4	967		20	100.0	21.4	7.4	8.2	1108	100.0	21.4	7.4	8.3	1038	
12	11/01/04		20	100.0	20.9	7.3	7.8	790		20	100.0	21.0	7.3	7.2	1020	100.0	21.0	7.3	7.5	905	
13	11/02/04		20	100.0	21.4	7.1	6.5	821		20	100.0	21.4	7.1	6.1	1105	100.0	21.4	7.1	6.3	963	
14	11/03/04		20	100.0						20	100.0					100.0					
	Median:				**21.3**	**7.2**	**7.8**	**831**				**21.3**	**7.2**	**7.4**	**1077**		**21.3**	**7.2**	**7.5**	**945**	
	Mode:				**21.4**	**7.2**	**7.8**	**881**				**21.4**	**7.2**	**8.2**	**#N/A**		**21.4**	**7.2**	**8.2**	**#N/A**	
	Mean:			**100.0**	**21.2**	**7.3**	**7.7**	**852**			**100.0**	**21.2**	**7.3**	**7.4**	**1058**	**100.0**	**21.2**	**7.3**	**7.5**	**955**	**0.510**
	SEM:			**0.00**	**0.07**	**0.03**	**0.17**	**16.91**			**0.00**	**0.07**	**0.03**	**0.24**	**13.16**	**0.00**	**0.07**	**0.03**	**0.20**	**12.56**	**0.00**
	% CV:			**0.00**	**5.74**	**3.48**	**22.53**	**216.77**			**0.00**	**5.83**	**4.06**	**33.70**	**151.37**	**0.00**	**5.75**	**3.60**	**27.48**	**152.08**	**0.00**

[1]All measured test concentrations reported by Battelle as less than limit of detection (LOD) [1.018 µg/L] or none detected (ND) are expressed as 0.51 µg/L (1/2 of LOD).

Results from samples collected on 11/03/04 are not reported pending further QA review.

DAILY DATA RENEW

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 22.2 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc[1] (ug/L)
		Initial No.= 20								**Initial No.= 20**												
0	10/20/04			100.0	21.1	7.8	8.4	1212				100.0	21.1	7.8	8.4	1170	100.0	21.1	7.8	8.4	1191	0.51
1	10/21/04		20	100.0	21.0	7.2	7.3	1173			20	100.0	21.1	7.2	7.2	1243	100.0	21.1	7.2	7.3	1208	
2	10/22/04		20	100.0	21.6	7.2	7.4	1160			20	100.0	21.6	7.2	7.2	1210	100.0	21.6	7.2	7.3	1185	
3	10/23/04		20	100.0	21.4	7.2	7.2	1220			20	100.0	21.4	7.3	7.3	1165	100.0	21.4	7.3	7.3	1193	
4	10/24/04		20	100.0	21.2	7.2	7.4	1205			20	100.0	21.3	7.2	7.6	1260	100.0	21.3	7.2	7.5	1233	
5	10/25/04		20	100.0	20.8	7.1	7.2	1286			20	100.0	21.0	7.1	6.9	1317	100.0	20.9	7.1	7.1	1302	
6	10/26/04		20	100.0	21.5	7.2	7.4	1081			20	100.0	21.5	7.2	7.2	1171	100.0	21.5	7.2	7.3	1126	
7	10/27/04		20	100.0	21.1	7.3	7.6	1140			20	100.0	21.1	7.3	7.4	1230	100.0	21.1	7.3	7.5	1185	
8	10/28/04		20	100.0	21.4	7.2	7.3	1236			20	100.0	21.3	7.2	7.5	1258	100.0	21.4	7.2	7.4	1247	0.51
9	10/29/04		20	100.0	21.5	7.3	7.5	1193			20	100.0	21.5	7.3	7.4	1221	100.0	21.5	7.3	7.5	1207	
10	10/30/04		20	100.0	20.7	7.4	8.5	1268			20	100.0	20.8	7.4	8.6	1234	100.0	20.8	7.4	8.6	1251	
11	10/31/04		20	100.0	21.4	7.4	8.3	1232			20	100.0	21.4	7.5	8.2	1260	100.0	21.4	7.5	8.3	1246	
12	11/01/04		20	100.0	21.1	7.3	7.9	1200			20	100.0	21.1	7.3	8.0	1200	100.0	21.1	7.3	8.0	1200	
13	11/02/04		20	100.0	21.4	7.1	7.5	1345			20	100.0	21.3	7.1	7.6	1264	100.0	21.4	7.1	7.6	1305	
14	11/03/04		20	100.0							20	100.0					100.0					
	Median:				**21.3**	**7.2**	**7.5**	**1209**					**21.3**	**7.3**	**7.5**	**1232**		**21.3**	**7.2**	**7.5**	**1208**	
	Mode:				**21.4**	**7.2**	**7.4**	**#N/A**					**21.1**	**7.2**	**7.2**	**1260**		**21.1**	**7.2**	**7.3**	**1185**	
	Mean:			**100.0**	**21.2**	**7.3**	**7.6**	**1211**				**100.0**	**21.3**	**7.3**	**7.6**	**1229**	**100.0**	**21.2**	**7.3**	**7.6**	**1220**	**0.510**
	SEM:			**0.00**	**0.07**	**0.05**	**0.12**	**17.30**				**0.00**	**0.06**	**0.05**	**0.13**	**11.52**	**0.00**	**0.07**	**0.05**	**0.13**	**12.82**	**0.00**
	% CV:			**0.00**	**5.92**	**6.53**	**16.35**	**185.98**				**0.00**	**4.87**	**6.73**	**18.29**	**122.98**	**0.00**	**5.35**	**6.60**	**17.09**	**137.32**	**0.00**

[1]All measured test concentrations reported by Battelle as less than limit of detection (LOD) [1.018 µg/L] or none detected (ND) are expressed as 0.51 µg/L (1/2 of LOD).

Results from samples collected on 11/03/04 are not reported pending further QA review.

DAILY DATA RENEW

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 66.7 ug/L**

		INITIAL STAGE 54 REPLICATE C								INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					Average Measured
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Flow Rate (mL/min)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Test Conc[1] (ug/L)
		Initial No.= 20								**Initial No.= 20**												
0	10/20/04			100.0	21.1	7.7	8.6	1127				100.0	21.1	7.7	8.5	1110	100.0	21.1	7.7	8.6	1119	2.20
1	10/21/04		20	100.0	21.3	7.2	6.9	1057			20	100.0	21.4	7.2	5.8	1093	100.0	21.4	7.2	6.4	1075	
2	10/22/04		20	100.0	21.6	7.3	6.3	1180			20	100.0	21.6	7.3	6.6	1150	100.0	21.6	7.3	6.5	1165	
3	10/23/04		20	100.0	21.6	7.2	6.8	1128			20	100.0	21.6	7.1	7.4	1150	100.0	21.6	7.2	7.1	1139	
4	10/24/04		20	100.0	21.4	7.2	7.4	1132			20	100.0	21.4	7.2	7.7	1140	100.0	21.4	7.2	7.6	1136	
5	10/25/04		20	100.0	21.3	7.1	6.0	1164			20	100.0	21.3	7.1	5.8	1161	100.0	21.3	7.1	5.9	1163	
6	10/26/04		20	100.0	21.4	7.2	6.6	992			20	100.0	21.4	7.2	6.4	996	100.0	21.4	7.2	6.5	994	
7	10/27/04		20	100.0	21.1	7.3	6.9	1090			20	100.0	21.4	7.3	6.5	1100	100.0	21.3	7.3	6.7	1095	
8	10/28/04		20	100.0	21.4	7.3	7.6	1265			20	100.0	21.4	7.3	7.8	1254	100.0	21.4	7.3	7.7	1260	4.74
9	10/29/04		20	100.0	21.5	7.3	7.6	1084			20	100.0	21.5	7.3	7.4	1120	100.0	21.5	7.3	7.5	1102	
10	10/30/04		20	100.0	20.8	7.4	8.5	1263			20	100.0	20.8	7.5	8.4	1240	100.0	20.8	7.5	8.5	1252	
11	10/31/04		20	100.0	21.4	7.4	8.1	1252			20	100.0	21.5	7.4	8.1	1259	100.0	21.5	7.4	8.1	1256	
12	11/01/04		20	100.0	21.1	7.4	7.9	1130			20	100.0	21.1	7.4	8.0	1050	100.0	21.1	7.4	8.0	1090	
13	11/02/04		20	100.0	21.2	7.2	8.1	1064			20	100.0	21.2	7.2	8.2	1109	100.0	21.2	7.2	8.2	1087	
14	11/03/04		20	100.0							20	100.0					100.0					
	Median:				**21.4**	**7.3**	**7.5**	**1129**					**21.4**	**7.3**	**7.6**	**1130**		**21.4**	**7.3**	**7.5**	**1127**	
	Mode:				**21.4**	**7.2**	**6.9**	**#N/A**					**21.4**	**7.2**	**5.8**	**1150**		**21.4**	**7.2**	**#N/A**	**#N/A**	
	Mean:			**100.0**	**21.3**	**7.3**	**7.4**	**1138**				**100.0**	**21.3**	**7.3**	**7.3**	**1138**	**100.0**	**21.3**	**7.3**	**7.4**	**1138**	**3.468**
	SEM:			**0.00**	**0.06**	**0.04**	**0.22**	**21.75**				**0.00**	**0.06**	**0.04**	**0.25**	**19.98**	**0.00**	**0.06**	**0.04**	**0.23**	**20.43**	**1.27**
	% CV:			**0.00**	**4.81**	**5.43**	**29.98**	**241.25**				**0.00**	**4.77**	**5.99**	**34.67**	**221.60**	**0.00**	**4.70**	**5.67**	**31.55**	**226.63**	**96.64**

Results from samples collected on 11/03/04 are not reported pending further QA review.

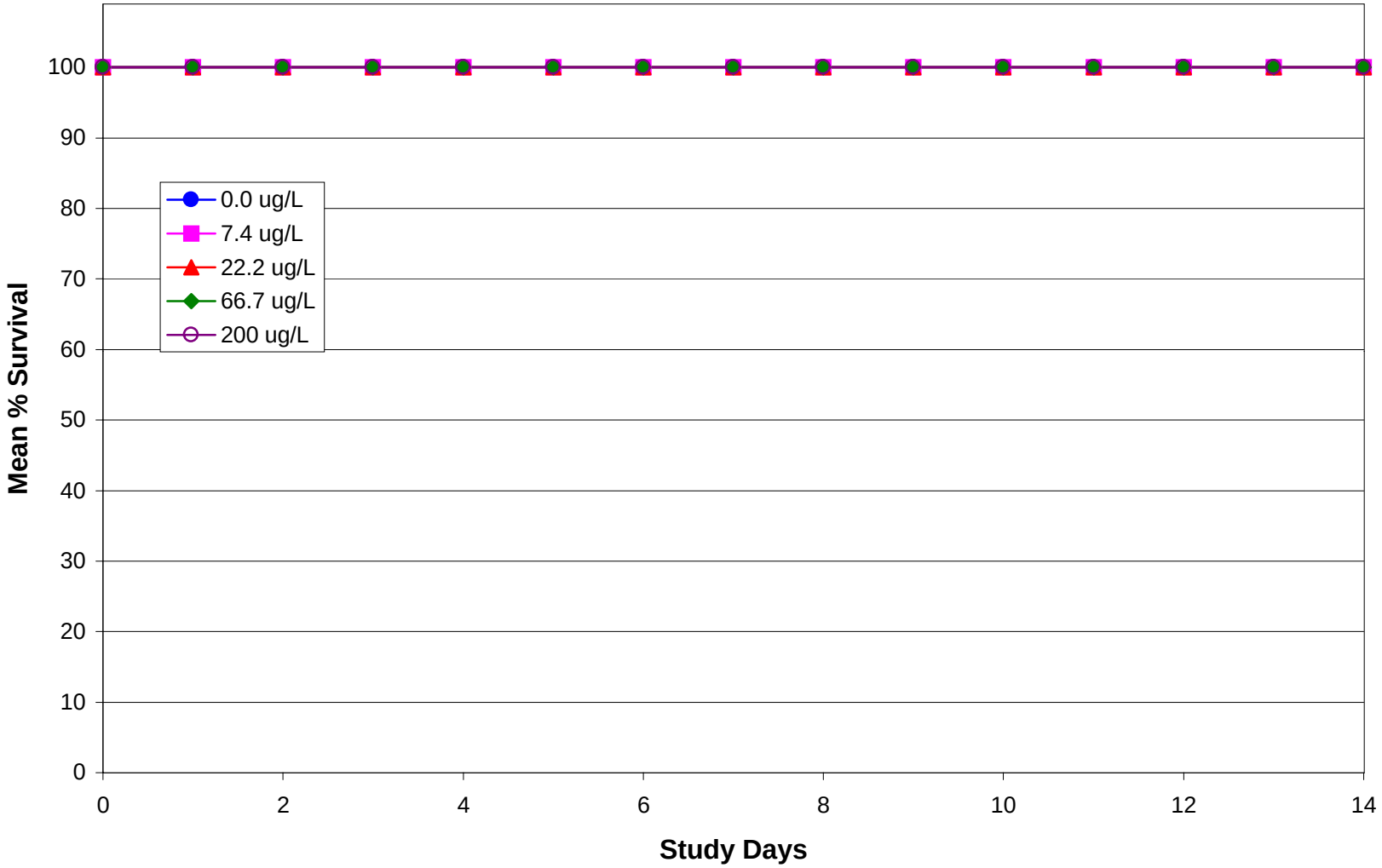
DAILY DATA RENEW

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 200 ug/L**

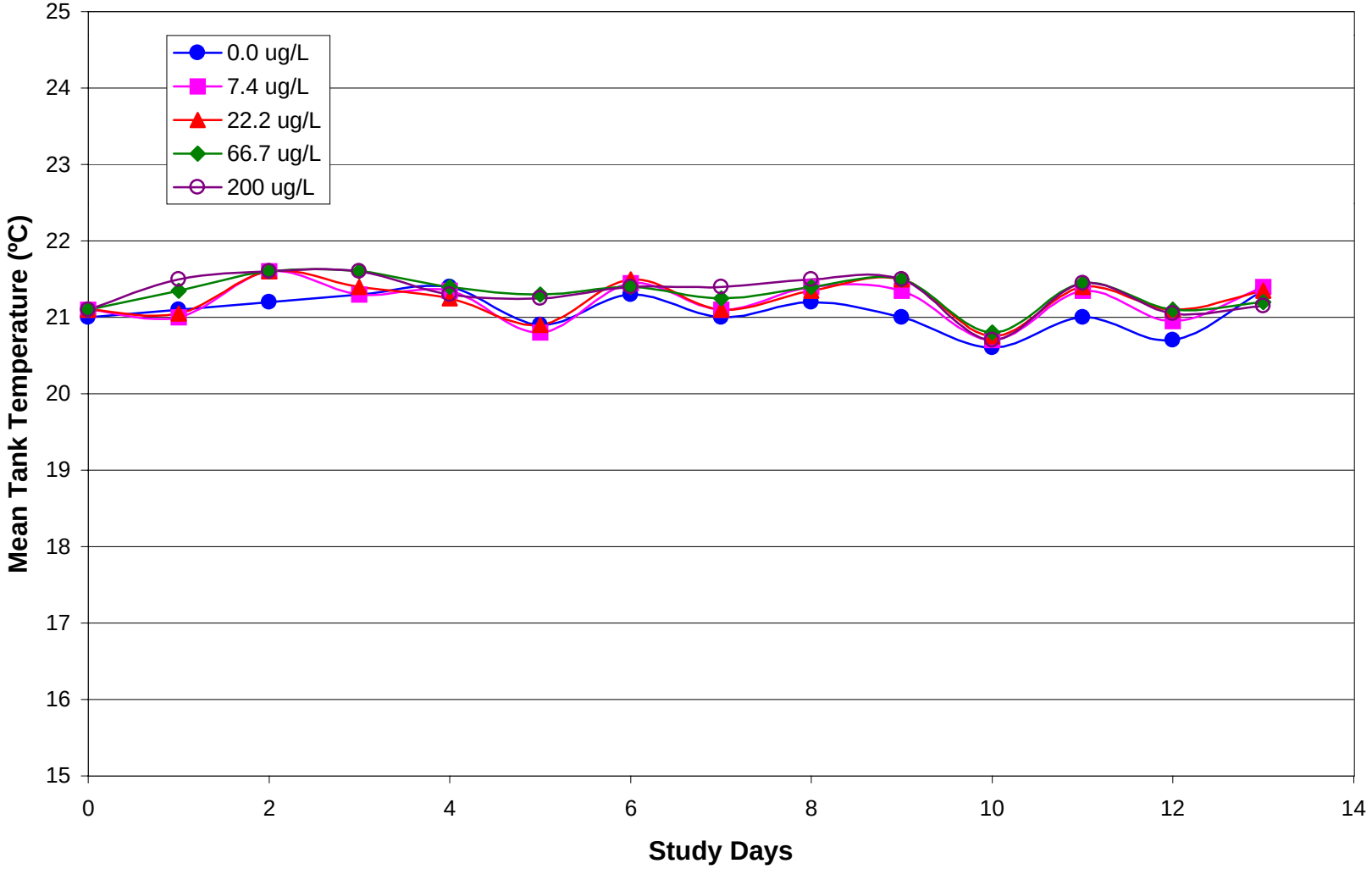
		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					Average Measured Test
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Conc[1] (ug/L)
		Initial No.= 20							**Initial No.= 20**												
0	10/20/04			100.0	21.1	7.7	8.4	971			100.0	21.1	7.7	8.2	918	100.0	21.1	7.7	8.3	945	13.05
1	10/21/04		20	100.0	21.5	7.2	7.5	927		20	100.0	21.5	7.3	7.3	857	100.0	21.5	7.3	7.4	892	
2	10/22/04		20	100.0	21.6	7.4	7.8	1150		20	100.0	21.6	7.4	7.8	1150	100.0	21.6	7.4	7.8	1150	
3	10/23/04		20	100.0	21.6	7.1	7.4	968		20	100.0	21.6	7.2	7.3	934	100.0	21.6	7.2	7.4	951	
4	10/24/04		20	100.0	21.3	7.3	7.4	983		20	100.0	21.3	7.2	7.5	960	100.0	21.3	7.3	7.5	972	
5	10/25/04		20	100.0	21.3	7.4	7.6	1026		20	100.0	21.2	7.5	7.9	990	100.0	21.3	7.5	7.8	1008	
6	10/26/04		20	100.0	21.4	7.5	7.8	946		20	100.0	21.4	7.5	8.0	859	100.0	21.4	7.5	7.9	903	
7	10/27/04		20	100.0	21.4	7.5	7.7	970		20	100.0	21.4	7.6	8.0	970	100.0	21.4	7.6	7.9	970	
8	10/28/04		20	100.0	21.5	7.4	8.0	992		20	100.0	21.5	7.4	7.9	896	100.0	21.5	7.4	8.0	944	15.25
9	10/29/04		20	100.0	21.5	7.3	7.6	974		20	100.0	21.5	7.4	7.8	982	100.0	21.5	7.4	7.7	978	
10	10/30/04		20	100.0	20.7	7.5	8.2	1006		20	100.0	20.7	7.5	8.0	932	100.0	20.7	7.5	8.1	969	
11	10/31/04		20	100.0	21.5	7.5	8.0	993		20	100.0	21.4	7.5	7.9	954	100.0	21.5	7.5	8.0	974	
12	11/01/04		20	100.0	21.0	7.5	8.0	930		20	100.0	21.1	7.5	7.6	930	100.0	21.1	7.5	7.8	930	
13	11/02/04		20	100.0	21.1	7.4	7.9	959		20	100.0	21.2	7.4	7.4	884	100.0	21.2	7.4	7.7	922	
14	11/03/04		20	100.0						20	100.0					100.0					
	Median:				**21.4**	**7.4**	**7.8**	**973**				**21.4**	**7.5**	**7.9**	**933**		**21.4**	**7.4**	**7.8**	**960**	
	Mode:				**21.5**	**7.5**	**8.0**	**#N/A**				**21.5**	**7.5**	**7.9**	**#N/A**		**21.5**	**7.5**	**7.8**	**#N/A**	
	Mean:			**100.0**	**21.3**	**7.4**	**7.8**	**985**			**100.0**	**21.3**	**7.4**	**7.8**	**944**	**100.0**	**21.3**	**7.4**	**7.8**	**965**	**14.150**
	SEM:			**0.00**	**0.07**	**0.04**	**0.08**	**14.62**			**0.00**	**0.07**	**0.04**	**0.08**	**19.45**	**0.00**	**0.07**	**0.04**	**0.07**	**16.54**	**1.10**
	% CV:			**0.00**	**5.65**	**5.48**	**10.64**	**174.22**			**0.00**	**5.32**	**5.11**	**10.32**	**236.85**	**0.00**	**5.45**	**5.18**	**9.50**	**199.29**	**41.36**

Results from samples collected on 11/03/04 are not reported pending further QA review.

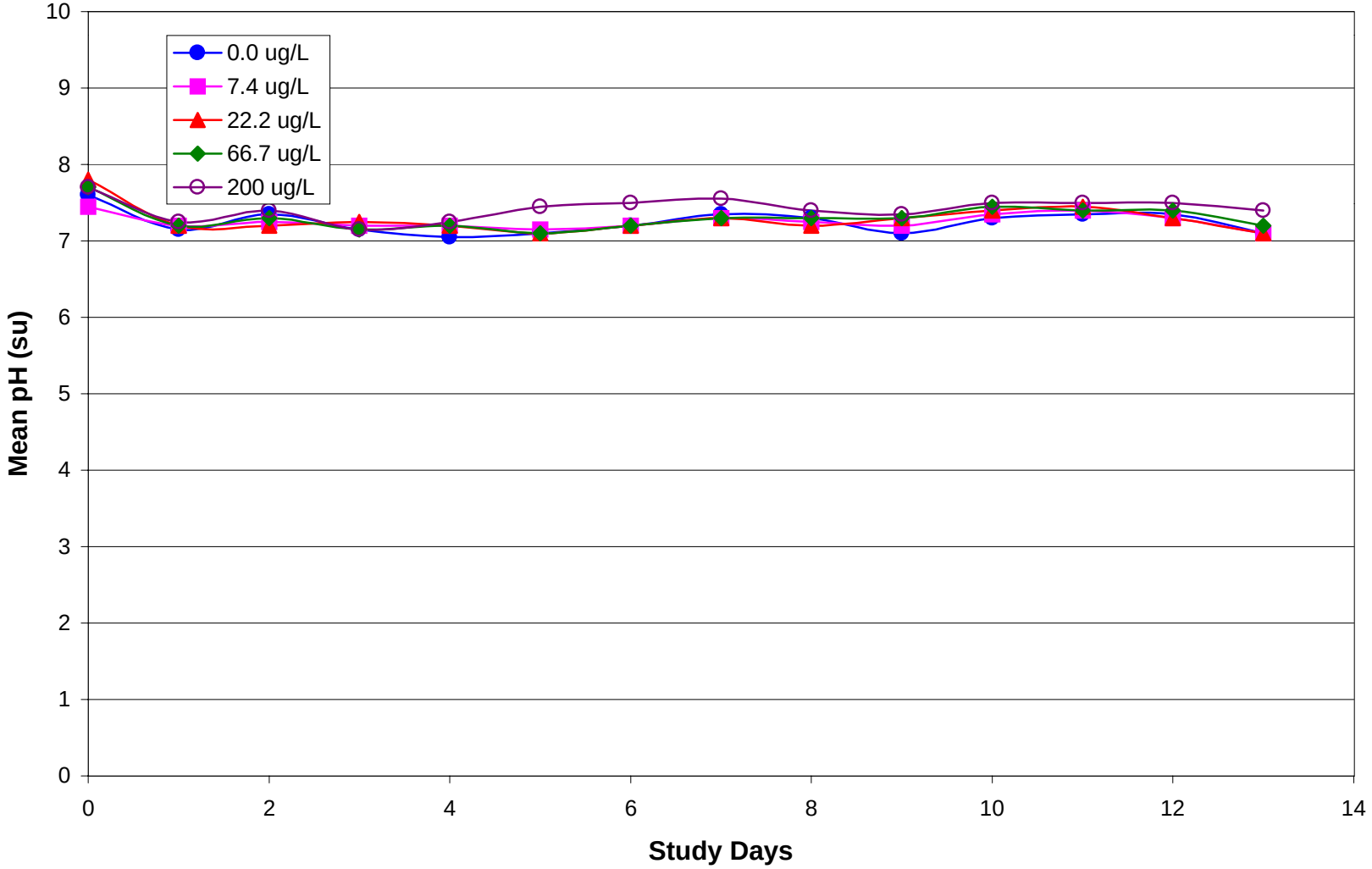
BATT02-00055
PCN - Renewal
Daily Average of Larval Survival for Initial Stage 54 Test



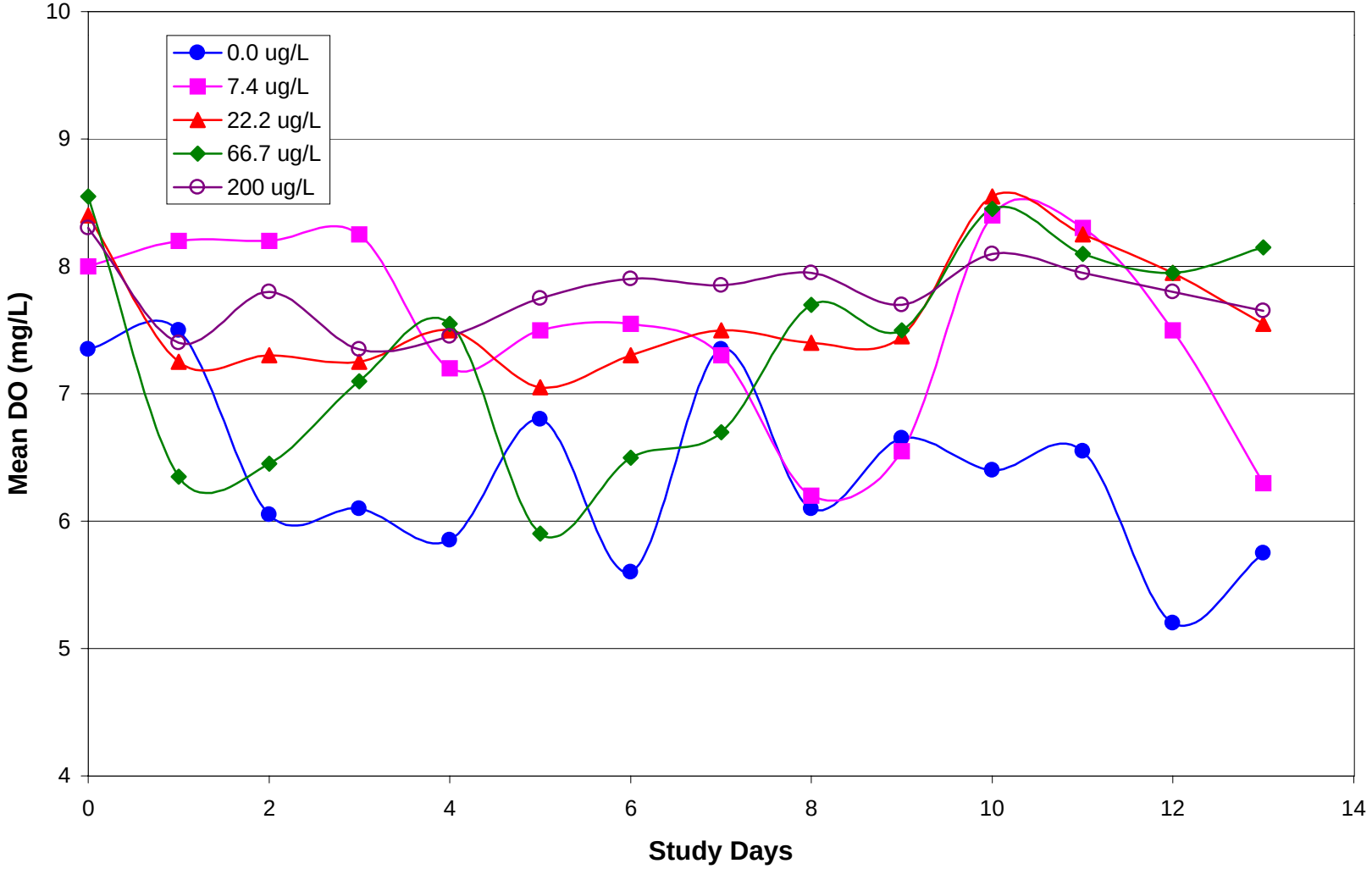
BATT02-00055
PCN - Renewal
Daily Average Tank Temperatures for Initial Stage 54 Test



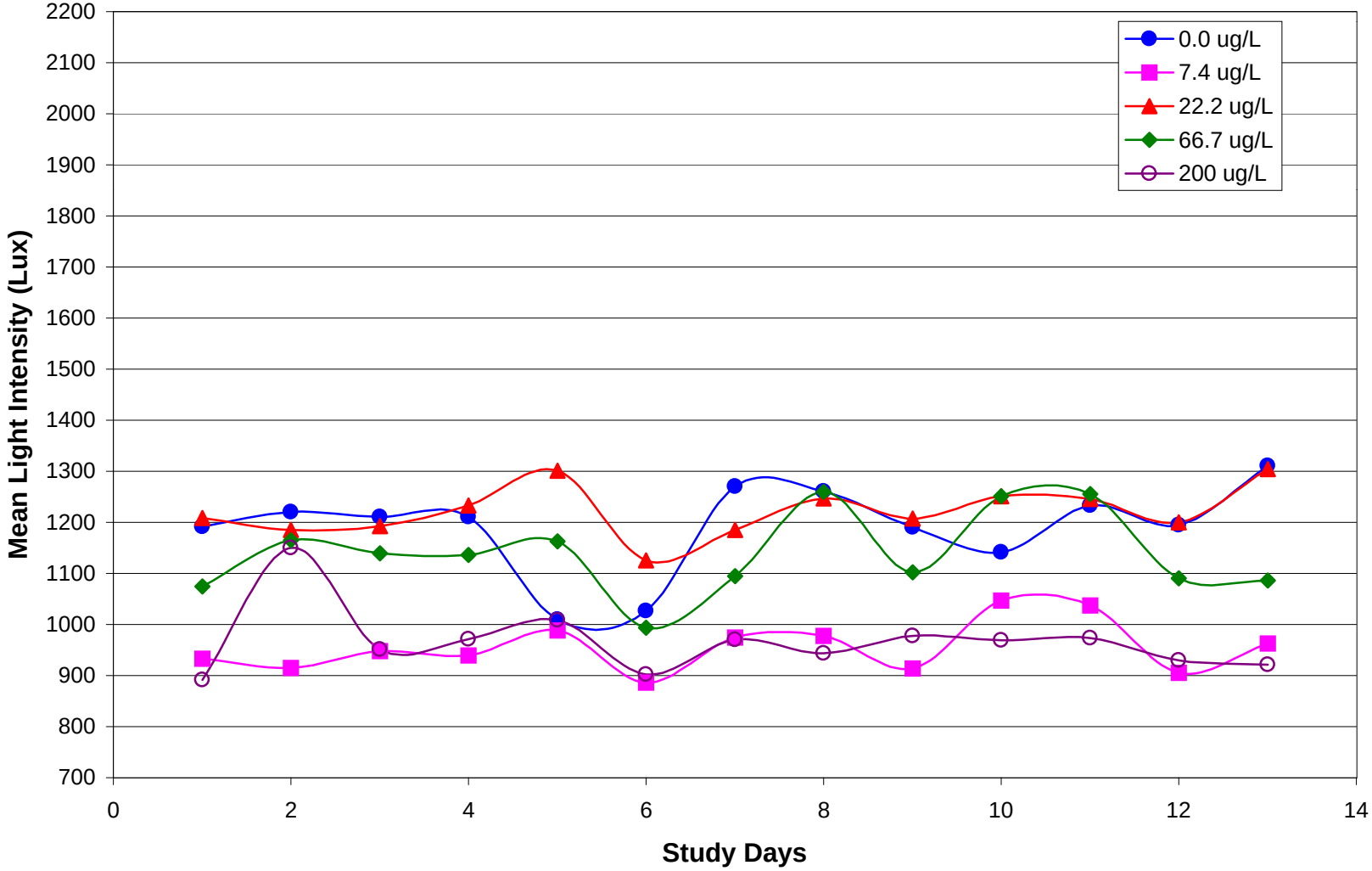
BATT02-00055
PCN - Renewal
Daily Average pH for Initial Stage 54 Test



BATT02-00055
PCN - Renewal
Daily Average Dissolved Oxygen (DO) for Initial Stage 54 Test



BATT02-00055
PCN - Renewal
Daily Average Light Intensity for Initial Stage 54 Test



WA 3-11 Amphibian Metamorphosis
Assay Demonstration Study

FEL

DAILY DATA SPENT

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 0.0 ug/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	10/21/04		20	100.0	20.9	7.5	8.0	1144		20	100.0	20.9	7.5	8.0	1240	100.0	20.9	7.5	8.0	1192	
2	10/22/04		20	100.0	20.8	7.5	7.9	1210		20	100.0	20.8	7.6	8.1	1230	100.0	20.8	7.6	8.0	1220	
3	10/23/04		20	100.0	21.1	7.4	7.8	1181		20	100.0	21.1	7.4	7.5	1240	100.0	21.1	7.4	7.7	1211	
4	10/24/04		20	100.0	21.1	7.4	7.6	1179		20	100.0	20.8	7.3	7.4	1242	100.0	21.0	7.4	7.5	1211	
5	10/25/04		20	100.0	21.0	7.6	8.0	1120		20	100.0	21.1	7.6	8.0	900	100.0	21.1	7.6	8.0	1010	
6	10/26/04		20	100.0	21.0	7.6	8.0	954		20	100.0	21.1	7.6	8.1	1099	100.0	21.1	7.6	8.1	1027	
7	10/27/04		20	100.0	20.9	7.5	8.0	1300		20	100.0	20.8	7.6	8.0	1240	100.0	20.9	7.6	8.0	1270	
8	10/28/04		20	100.0	21.0	7.5	7.7	1272		20	100.0	20.9	7.6	7.8	1250	100.0	21.0	7.6	7.8	1261	
9	10/29/04		20	100.0	20.9	7.6	8.0	1150		20	100.0	21.0	7.5	8.0	1230	100.0	21.0	7.6	8.0	1190	
10	10/30/04		20	100.0	20.5	7.5	7.5	1150		20	100.0	20.5	7.5	7.3	1133	100.0	20.5	7.5	7.4	1142	
11	10/31/04		20	100.0	21.1	7.4	7.7	1225		20	100.0	21.1	7.4	7.8	1241	100.0	21.1	7.4	7.8	1233	
12	11/01/04		20	100.0	20.9	7.7	7.8	1200		20	100.0	20.9	7.6	7.4	1190	100.0	20.9	7.7	7.6	1195	
13	11/02/04		20	100.0	20.9	7.6	7.6	1292		20	100.0	20.9	7.6	7.7	1330	100.0	20.9	7.6	7.7	1311	
14	11/03/04		20	100.0	20.1	7.8	8.4	1200		20	100.0	20.2	7.8	8.4	1268	100.0	20.2	7.8	8.4	1234	
	Median:				**20.9**	**7.5**	**7.9**	**1191**				**20.9**	**7.6**	**7.9**	**1240**		**20.9**	**7.6**	**7.9**	**1211**	
	Mode:				**20.9**	**7.5**	**8.0**	**1150**				**20.9**	**7.6**	**8.0**	**1240**		**20.9**	**7.6**	**8.0**	**1211**	
	Mean:			**100.0**	**20.9**	**7.5**	**7.9**	**1184**			**100.0**	**20.9**	**7.5**	**7.8**	**1202**	**100.0**	**20.9**	**7.5**	**7.8**	**1193**	
	SEM:			**0.00**	**0.07**	**0.03**	**0.06**	**23.05**			**0.00**	**0.07**	**0.03**	**0.09**	**27.61**	**0.00**	**0.07**	**0.03**	**0.07**	**22.60**	
	% CV:			**0.00**	**5.91**	**4.22**	**8.36**	**250.61**			**0.00**	**5.54**	**4.45**	**11.58**	**297.97**	**0.00**	**5.61**	**4.15**	**9.61**	**244.82**	

DAILY DATA SPENT

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 7.4 ug/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	10/21/04		20	100.0	20.9	7.5	7.6	822		20	100.0	20.9	7.4	7.3	1043	100.0	20.9	7.5	7.5	933	
2	10/22/04		20	100.0	20.9	7.4	7.2	840		20	100.0	21.1	7.3	7.2	990	100.0	21.0	7.4	7.2	915	
3	10/23/04		20	100.0	21.0	7.2	7.7	808		20	100.0	21.0	7.2	7.6	1088	100.0	21.0	7.2	7.7	948	
4	10/24/04		20	100.0	20.9	7.3	8.3	799		20	100.0	21.0	7.3	7.8	1079	100.0	21.0	7.3	8.1	939	
5	10/25/04		20	100.0	21.1	7.5	7.4	895		20	100.0	21.1	7.4	7.1	1081	100.0	21.1	7.5	7.3	988	
6	10/26/04		20	100.0	21.1	7.5	7.8	820		20	100.0	21.2	7.4	6.5	953	100.0	21.2	7.5	7.2	887	
7	10/27/04		20	100.0	20.9	7.5	7.3	870		20	100.0	21.0	7.3	5.8	1080	100.0	21.0	7.4	6.6	975	
8	10/28/04		20	100.0	21.0	7.5	7.4	881		20	100.0	21.1	7.3	6.1	1075	100.0	21.1	7.4	6.8	978	
9	10/29/04		20	100.0	20.8	7.6	8.0	760		20	100.0	20.9	7.4	6.4	1068	100.0	20.9	7.5	7.2	914	
10	10/30/04		20	100.0	20.5	7.5	7.8	974		20	100.0	20.5	7.3	5.9	1119	100.0	20.5	7.4	6.9	1047	
11	10/31/04		20	100.0	21.0	7.5	6.8	967		20	100.0	20.8	7.4	7.0	1108	100.0	20.9	7.5	6.9	1038	
12	11/01/04		20	100.0	20.9	7.5	7.3	790		20	100.0	20.9	7.4	5.4	1020	100.0	20.9	7.5	6.4	905	
13	11/02/04		20	100.0	20.9	7.4	7.2	821		20	100.0	20.9	7.2	5.1	1105	100.0	20.9	7.3	6.2	963	
14	11/03/04		20	100.0	20.3	7.6	7.9	810		20	100.0	20.5	7.5	7.4	1040	100.0	20.4	7.6	7.7	925	
	Median:				**20.9**	**7.5**	**7.5**	**822**				**21.0**	**7.4**	**6.8**	**1077**		**20.9**	**7.4**	**7.2**	**944**	
	Mode:				**20.9**	**7.5**	**7.2**	**#N/A**				**20.9**	**7.4**	**#N/A**	**#N/A**		**20.9**	**7.5**	**7.2**	**#N/A**	
	Mean:			**100.0**	**20.9**	**7.5**	**7.6**	**847**			**100.0**	**20.9**	**7.3**	**6.6**	**1061**	**100.0**	**20.9**	**7.4**	**7.1**	**954**	
	SEM:			**0.00**	**0.06**	**0.03**	**0.11**	**17.00**			**0.00**	**0.06**	**0.02**	**0.23**	**12.56**	**0.00**	**0.06**	**0.02**	**0.14**	**12.72**	
	% CV:			**0.00**	**4.81**	**3.96**	**14.33**	**218.59**			**0.00**	**4.55**	**3.14**	**33.16**	**144.32**	**0.00**	**4.55**	**3.34**	**19.95**	**154.05**	

DAILY DATA SPENT

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 22.2 ug/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	10/21/04		20	100.0	20.8	7.4	7.6	1173		20	100.0	20.9	7.3	6.8	1243	100.0	20.9	7.4	7.2	1208	
2	10/22/04		20	100.0	21.1	7.4	7.3	1160		20	100.0	21.1	7.4	6.8	1210	100.0	21.1	7.4	7.1	1185	
3	10/23/04		20	100.0	20.9	7.2	7.3	1220		20	100.0	20.8	7.3	7.2	1165	100.0	20.9	7.3	7.3	1193	
4	10/24/04		20	100.0	21.1	7.4	7.6	1205		20	100.0	21.0	7.3	7.9	1260	100.0	21.1	7.4	7.8	1233	
5	10/25/04		20	100.0	21.0	7.4	7.8	1286		20	100.0	21.1	7.4	7.6	1317	100.0	21.1	7.4	7.7	1302	
6	10/26/04		20	100.0	21.2	7.5	7.8	1081		20	100.0	21.2	7.5	7.5	1171	100.0	21.2	7.5	7.7	1126	
7	10/27/04		20	100.0	21.0	7.4	7.5	1140		20	100.0	21.0	7.4	7.1	1230	100.0	21.0	7.4	7.3	1185	
8	10/28/04		20	100.0	21.1	7.4	7.3	1236		20	100.0	21.1	7.4	7.2	1258	100.0	21.1	7.4	7.3	1247	
9	10/29/04		20	100.0	21.0	7.4	7.7	1193		20	100.0	21.1	7.4	7.5	1221	100.0	21.1	7.4	7.6	1207	
10	10/30/04		20	100.0	20.6	7.4	7.8	1268		20	100.0	20.6	7.4	7.5	1234	100.0	20.6	7.4	7.7	1251	
11	10/31/04		20	100.0	21.1	7.5	7.3	1232		20	100.0	21.1	7.5	7.4	1260	100.0	21.1	7.5	7.4	1246	
12	11/01/04		20	100.0	21.0	7.4	7.2	1200		20	100.0	20.9	7.5	7.5	1200	100.0	21.0	7.5	7.4	1200	
13	11/02/04		20	100.0	20.9	7.3	7.1	1345		20	100.0	20.9	7.3	7.2	1264	100.0	20.9	7.3	7.2	1305	
14	11/03/04		20	100.0	20.5	7.5	7.7	1190		20	100.0	20.6	7.5	7.7	1200	100.0	20.6	7.5	7.7	1195	
	Median:				**21.0**	**7.4**	**7.6**	**1203**				**21.0**	**7.4**	**7.5**	**1232**		**21.0**	**7.4**	**7.4**	**1208**	
	Mode:				**21.1**	**7.4**	**7.3**	**#N/A**				**21.1**	**7.4**	**7.5**	**1260**		**21.1**	**7.4**	**7.3**	**1185**	
	Mean:			**100.0**	**21.0**	**7.4**	**7.5**	**1209**			**100.0**	**21.0**	**7.4**	**7.4**	**1231**	**100.0**	**21.0**	**7.4**	**7.4**	**1220**	
	SEM:			**0.00**	**0.05**	**0.02**	**0.07**	**17.36**			**0.00**	**0.05**	**0.02**	**0.09**	**10.86**	**0.00**	**0.05**	**0.02**	**0.06**	**12.77**	
	% CV:			**0.00**	**4.35**	**2.88**	**8.94**	**186.78**			**0.00**	**4.08**	**2.88**	**11.73**	**115.83**	**0.00**	**4.14**	**2.70**	**8.78**	**136.81**	

DAILY DATA SPENT

Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 66.7 ug/L**

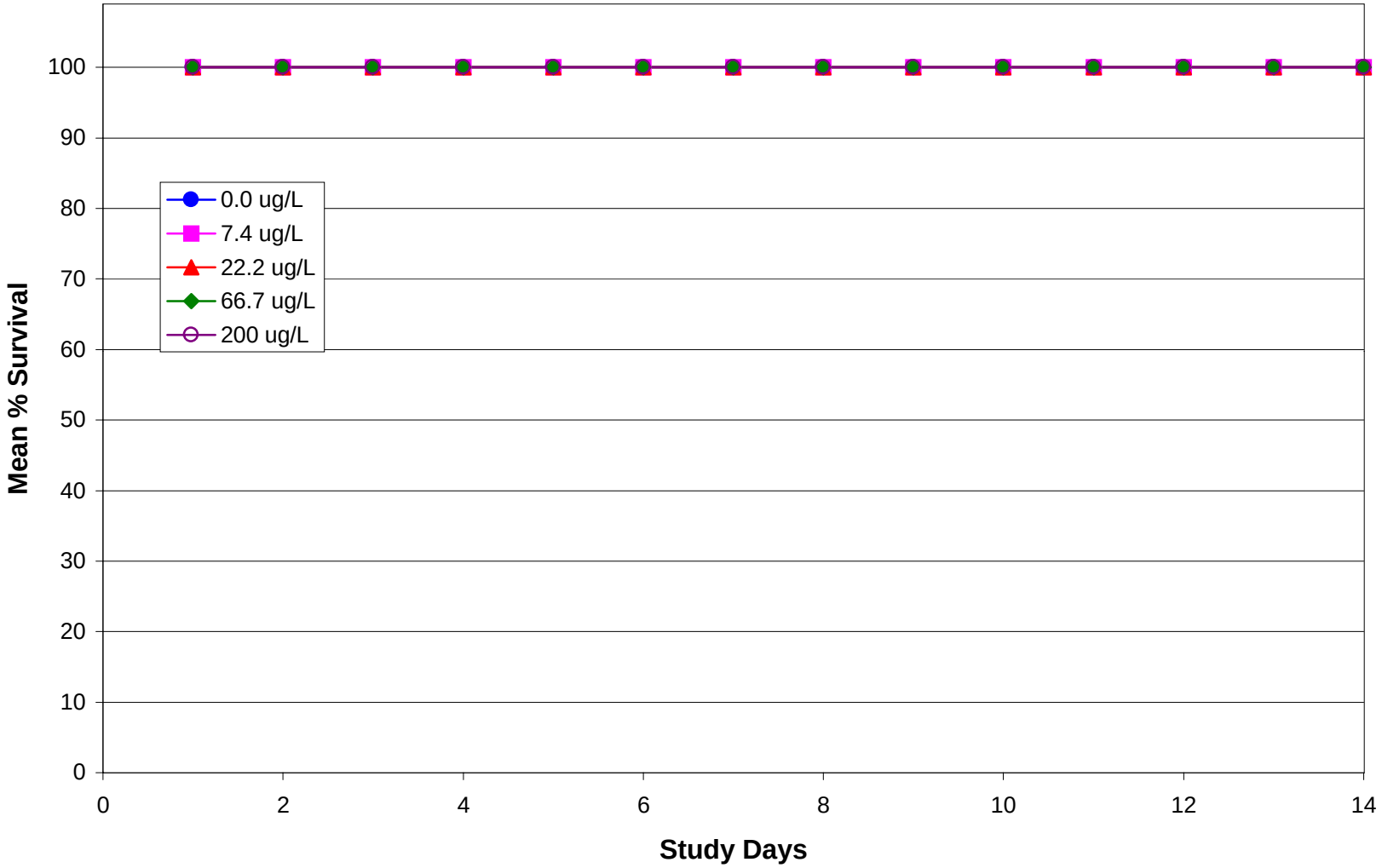
		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	10/21/04		20	100.0	21.0	7.3	7.7	1057		20	100.0	21.0	7.3	6.8	1093	100.0	21.0	7.3	7.3	1075	
2	10/22/04		20	100.0	21.1	7.4	7.5	1180		20	100.0	21.0	7.4	7.4	1150	100.0	21.1	7.4	7.5	1165	
3	10/23/04		20	100.0	20.9	7.4	7.4	1128		20	100.0	20.9	7.4	7.8	1150	100.0	20.9	7.4	7.6	1139	
4	10/24/04		20	100.0	21.2	7.4	7.5	1132		20	100.0	21.1	7.3	8.0	1140	100.0	21.2	7.4	7.8	1136	
5	10/25/04		20	100.0	21.1	7.4	7.6	1164		20	100.0	21.1	7.4	7.4	1161	100.0	21.1	7.4	7.5	1163	
6	10/26/04		20	100.0	21.2	7.5	7.6	992		20	100.0	21.2	7.5	7.4	996	100.0	21.2	7.5	7.5	994	
7	10/27/04		20	100.0	21.1	7.4	7.2	1090		20	100.0	21.1	7.4	6.3	1100	100.0	21.1	7.4	6.8	1095	
8	10/28/04		20	100.0	21.2	7.4	7.1	1265		20	100.0	21.2	7.4	6.7	1254	100.0	21.2	7.4	6.9	1260	
9	10/29/04		20	100.0	21.1	7.5	7.4	1084		20	100.0	21.2	7.4	7.0	1120	100.0	21.2	7.5	7.2	1102	
10	10/30/04		20	100.0	20.6	7.4	8.1	1263		20	100.0	20.5	7.5	8.2	1240	100.0	20.6	7.5	8.2	1252	
11	10/31/04		20	100.0	21.2	7.4	6.1	1252		20	100.0	21.1	7.5	7.8	1259	100.0	21.2	7.5	7.0	1256	
12	11/01/04		20	100.0	21.0	7.5	7.6	1130		20	100.0	20.9	7.6	7.9	1050	100.0	21.0	7.6	7.8	1090	
13	11/02/04		20	100.0	20.9	7.4	7.4	1064		20	100.0	20.8	7.5	7.7	1109	100.0	20.9	7.5	7.6	1087	
14	11/03/04		20	100.0	20.5	7.5	7.9	1170		20	100.0	20.4	7.6	8.0	1110	100.0	20.5	7.6	8.0	1140	
	Median:				**21.1**	**7.4**	**7.5**	**1131**				**21.1**	**7.4**	**7.6**	**1130**		**21.1**	**7.4**	**7.5**	**1138**	
	Mode:				**21.1**	**7.4**	**7.4**	**#N/A**				**21.1**	**7.4**	**7.4**	**1150**		**21.2**	**7.4**	**7.8**	**#N/A**	
	Mean:			**100.0**	**21.0**	**7.4**	**7.4**	**1141**			**100.0**	**21.0**	**7.4**	**7.5**	**1138**	**100.0**	**21.0**	**7.4**	**7.4**	**1139**	
	SEM:			**0.00**	**0.06**	**0.02**	**0.12**	**21.85**			**0.00**	**0.07**	**0.03**	**0.15**	**19.98**	**0.00**	**0.06**	**0.02**	**0.11**	**20.38**	
	% CV:			**0.00**	**4.80**	**2.13**	**16.93**	**242.04**			**0.00**	**5.46**	**3.44**	**20.80**	**221.60**	**0.00**	**5.09**	**2.55**	**14.74**	**225.87**	

DAILY DATA SPENT

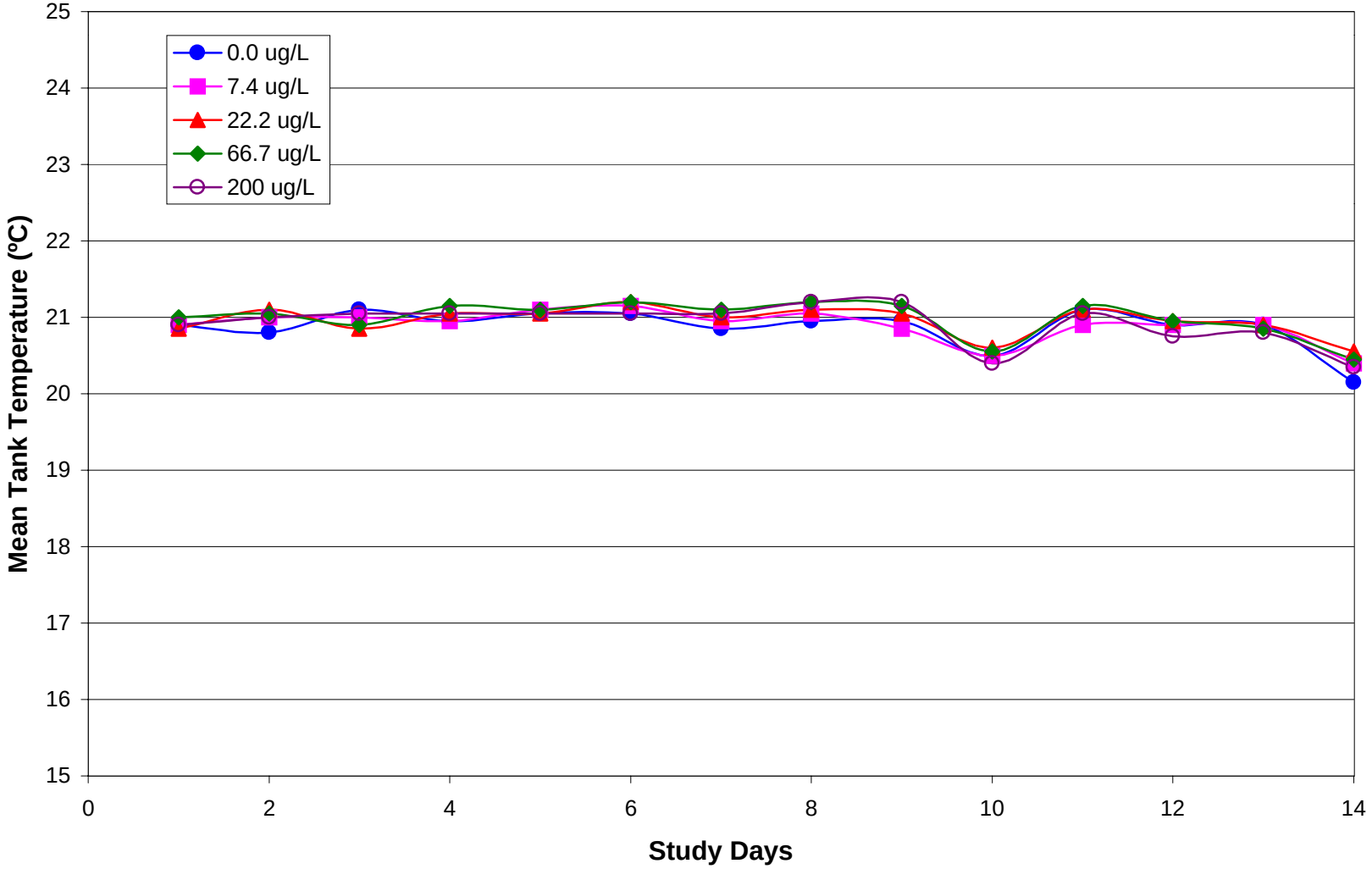
Workorder No: 00055 **Test No: 1**
Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 200 ug/L**

		INITIAL STAGE 54 REPLICATE C							INITIAL STAGE 54 REPLICATE D							INITIAL STAGE 54 DAILY MEANS OF REPLICATES					
Study Day	Study Date	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	Daily No. Dead	Cumul. No. Alive	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	% Survival	Tank Temp (°C)	pH (su)	DO (mg/L)	Light Intensity (Lux)	
		Initial No.= 20							**Initial No.= 20**												
1	10/21/04		20	100.0	20.9	7.3	7.6	927		20	100.0	20.9	7.3	6.7	857	100.0	20.9	7.3	7.2	892	
2	10/22/04		20	100.0	21.0	7.5	7.7	1150		20	100.0	21.0	7.5	7.5	1150	100.0	21.0	7.5	7.6	1150	
3	10/23/04		20	100.0	21.0	7.5	7.9	968		20	100.0	21.1	7.6	7.6	934	100.0	21.1	7.6	7.8	951	
4	10/24/04		20	100.0	21.0	7.5	7.6	983		20	100.0	21.1	7.6	7.7	960	100.0	21.1	7.6	7.7	972	
5	10/25/04		20	100.0	21.1	7.4	7.4	1026		20	100.0	21.0	7.5	7.9	990	100.0	21.1	7.5	7.7	1008	
6	10/26/04		20	100.0	21.1	7.5	7.4	946		20	100.0	21.0	7.6	8.0	859	100.0	21.1	7.6	7.7	903	
7	10/27/04		20	100.0	21.1	7.4	7.0	970		20	100.0	21.0	7.5	7.8	970	100.0	21.1	7.5	7.4	970	
8	10/28/04		20	100.0	21.2	7.4	7.3	992		20	100.0	21.2	7.4	7.6	896	100.0	21.2	7.4	7.5	944	
9	10/29/04		20	100.0	21.2	7.4	7.7	974		20	100.0	21.2	7.5	7.8	982	100.0	21.2	7.5	7.8	978	
10	10/30/04		20	100.0	20.4	7.6	8.4	1006		20	100.0	20.4	7.5	7.7	932	100.0	20.4	7.6	8.1	969	
11	10/31/04		20	100.0	21.0	7.5	8.2	993		20	100.0	21.1	7.6	8.0	954	100.0	21.1	7.6	8.1	974	
12	11/01/04		20	100.0	20.7	7.6	7.8	930		20	100.0	20.8	7.5	7.3	930	100.0	20.8	7.6	7.6	930	
13	11/02/04		20	100.0	20.8	7.4	7.8	959		20	100.0	20.8	7.4	7.1	884	100.0	20.8	7.4	7.5	922	
14	11/03/04		20	100.0	20.3	7.6	8.1	960		20	100.0	20.4	7.5	7.6	900	100.0	20.4	7.6	7.9	930	
	Median:				**21.0**	**7.5**	**7.7**	**972**				**21.0**	**7.5**	**7.7**	**933**		**21.1**	**7.5**	**7.7**	**960**	
	Mode:				**21.0**	**7.5**	**7.6**	**#N/A**				**21.0**	**7.5**	**7.6**	**#N/A**		**21.1**	**7.6**	**7.8**	**930**	
	Mean:			**100.0**	**20.9**	**7.5**	**7.7**	**985**			**100.0**	**20.9**	**7.5**	**7.6**	**943**	**100.0**	**20.9**	**7.5**	**7.7**	**964**	
	SEM:			**0.00**	**0.07**	**0.02**	**0.10**	**14.70**			**0.00**	**0.07**	**0.02**	**0.10**	**19.62**	**0.00**	**0.07**	**0.02**	**0.07**	**16.67**	
	% CV:			**0.00**	**6.06**	**3.34**	**13.44**	**175.24**			**0.00**	**5.58**	**3.20**	**13.00**	**239.13**	**0.00**	**5.76**	**2.91**	**9.14**	**200.95**	

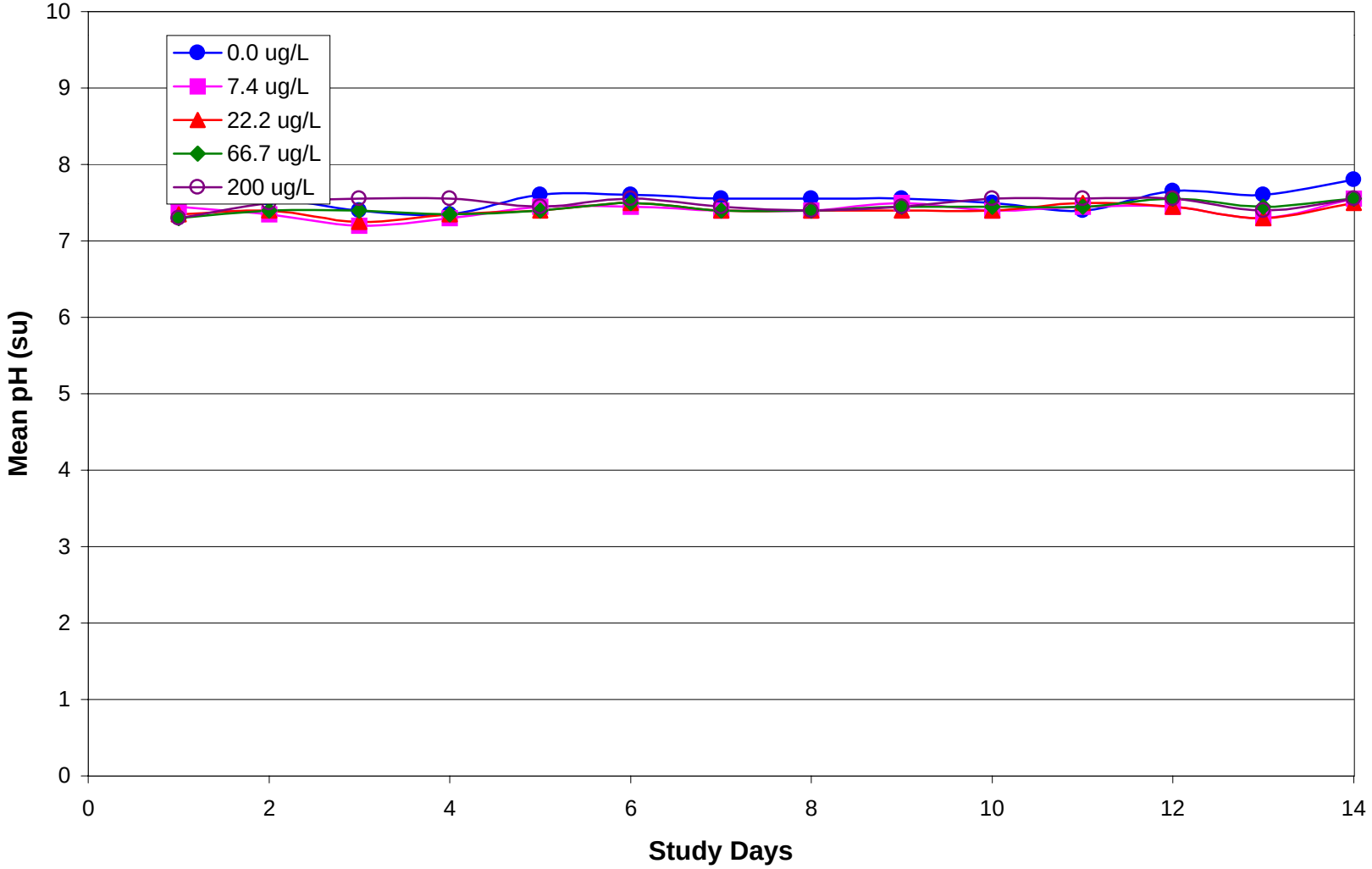
BATT02-00055
PCN - Spent
Daily Average of Larval Survival for Initial Stage 54 Test



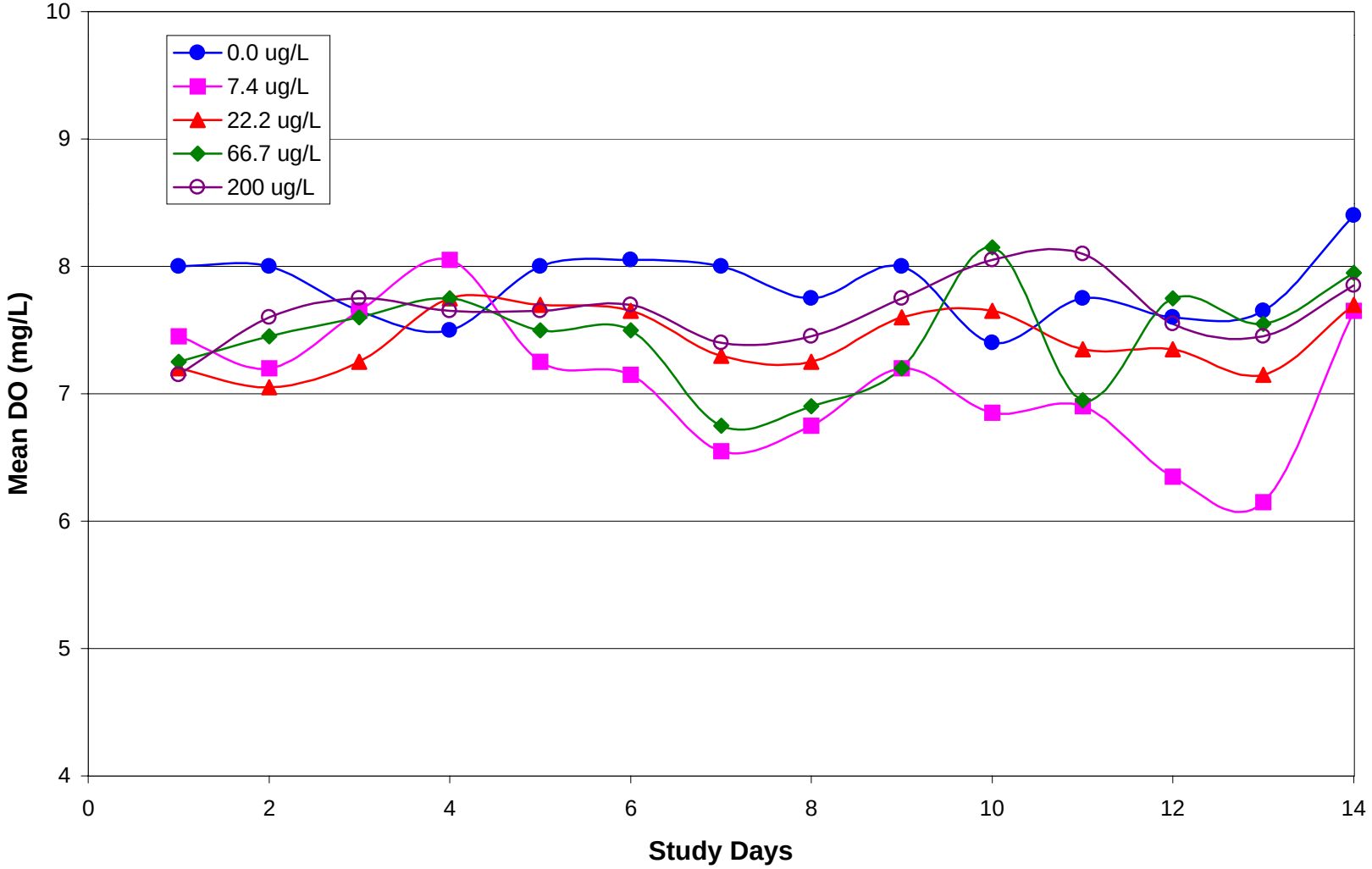
BATT02-00055
PCN - Spent
Daily Average Tank Temperatures for Initial Stage 54 Test



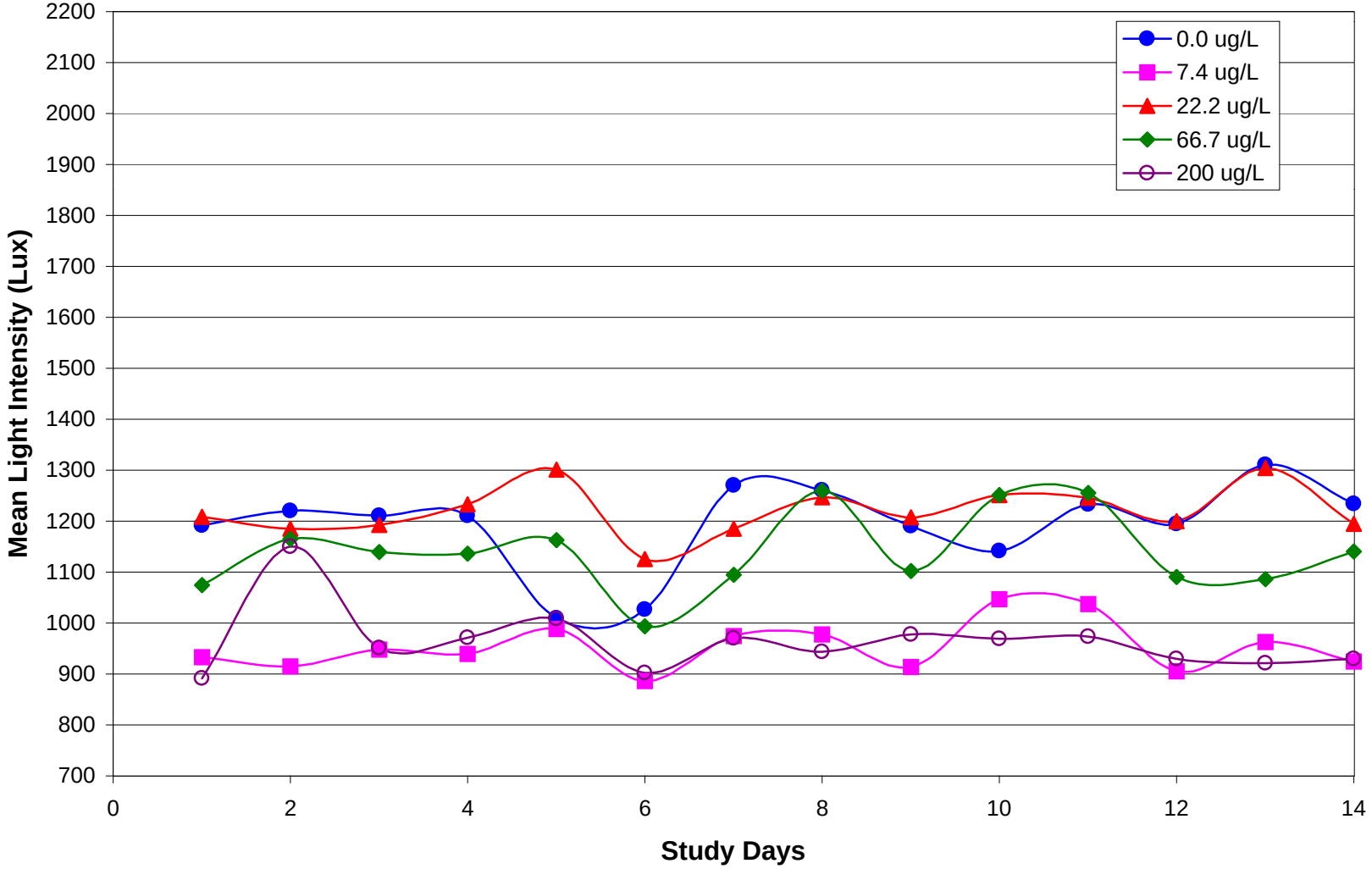
BATT02-00055
PCN - Spent
Daily Average pH for Initial Stage 54 Test



BATT02-00055
PCN - Spent
Daily Average Dissolved Oxygen (DO) for Initial Stage 54 Test



BATT02-00055
PCN - Spent
Daily Average Light Intensity for Initial Stage 54 Test



Pregnenolone-16-α-Carbonitrile (PCN) Metamorphosis Assay: Test Termination Data – Stage and Growth

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00055 **Test No: 1**

Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 0.0 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)
A	201	0.4268	56
	202	0.5634	56
	203	0.4822	56
	204	0.4765	56
	205	0.4576	56
	206	0.5966	56
	207	0.4571	56
	208	0.8576	59
	209	0.4948	57
	210	0.6147	57
	211	0.6709	59
	212	0.7793	59
	213	0.7266	57
	214	0.6407	57
	215	0.5798	57
	216	0.6594	57
	217	0.5920	57
	218	0.5240	57
	219	0.5720	57
	220	0.6821	57
	Median:	**0.586**	**57**
	Mode:		**57**
	Mean:	**0.593**	**56.950**
	SEM:	**0.026**	**0.223**
	%CV:	**14.897**	**13.234**
B	221	0.4203	57
	222	0.3471	55
	223	0.3153	55
	224	0.3034	55
	225	0.2625	55
	226	0.2632	55
	227	0.3583	55
	228	0.3752	55
	229	0.4103	56
	230	0.4082	56
	231	0.4574	56
	232	0.3649	56
	233	0.3936	56
	234	0.4085	56
	235	0.3596	56
	236	0.4027	56
	237	0.3929	56
	238	0.4204	56
	239	0.3633	56
	240	0.3639	56
	Median:	**0.370**	**56**
	Mode:		**56**
	Rep Mean:	**0.370**	**55.700**
	SEM:	**0.012**	**0.128**
	%CV:	**8.499**	**7.654**
	Median:	**0.442**	**56**
	Mode:		**56**
	Total Mean:	**0.481**	**56.325**
	SEM:	**0.023**	**0.162**
	%CV:	**20.630**	**13.624**

Gross Morphology

Comments:

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00055 **Test No: 1**

Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 7.4 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)
A	241	0.3654	56
	242	0.8080	59
	243	0.7665	57
	244	0.6911	57
	245	0.8600	57
	246	0.8743	59
	247	0.5683	57
	248	0.7967	57
	249	0.5804	59
	250	0.5504	57
	251	0.7919	57
	252	0.6618	57
	253	0.7120	59
	254	0.8725	59
	255	1.0252	59
	256	0.9172	59
	257	0.7871	57
	258	0.6725	57
	259	0.6527	57
	260	0.7733	57
	Median:	**0.770**	**57**
	Mode:		**57**
	Mean:	**0.736**	**57.650**
	SEM:	**0.034**	**0.233**
	%CV:	**17.526**	**13.697**
B	261	0.8858	59
	262	0.9718	59
	263	0.7878	59
	264	0.7347	57
	265	0.6939	57
	266	0.9361	59
	267	0.7724	58
	268	1.0318	57
	269	0.6878	59
	270	0.4308	56
	271	0.7853	57
	272	0.5724	57
	273	0.7407	57
	274	0.9003	59
	275	1.1019	58
	276	0.8193	59
	277	0.7721	57
	278	0.5723	56
	279	0.6338	57
	Median:	**0.772**	**57**
	Mode:		**57**
	Rep Mean:	**0.781**	**57.737**
	SEM:	**0.038**	**0.252**
	%CV:	**18.952**	**14.445**
	Median:	**0.772**	**57**
	Mode:		**57**
	Total Mean:	**0.758**	**57.692**
	SEM:	**0.025**	**0.169**
	%CV:	**18.195**	**13.892**

Gross Morphology

Comments:

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00055 **Test No: 1**

Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 22.2 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	280	0.3650	55	
	281	0.4431	55	
	282	0.5348	56	
	283	0.8532	57	
	284	0.6103	56	
	285	0.4883	56	
	286	0.5685	57	
	287	0.6412	57	
	288	0.5836	57	
	289	0.6666	57	
	290	0.7487	57	
	291	0.5959	57	
	292	0.6560	57	
	293	0.5524	56	
	294	0.7037	57	
	295	0.7175	57	
	296	0.5815	57	
	297	0.6665	57	
	298	0.7463	57	
	299	0.6915	57	
	Median:	**0.626**	**57**	
	Mode:		**57**	
	Mean:	**0.621**	**56.600**	
	SEM:	**0.025**	**0.152**	
	%CV:	**14.449**	**9.046**	
B	300	0.6483	57	
	301	0.5596	57	
	302	0.6193	59	
	303	0.8051	59	
	304	1.0216	57	
	305	0.6488	59	
	306	0.7552	57	
	307	0.7587	58	
	308	0.7592	59	
	309	0.7413	57	
	310	0.6700	59	
	311	0.6601	57	
	312	0.8849	59	
	313	0.5820	57	
	314	0.7992	57	
	315	0.8971	59	
	316	0.6989	59	
	317	0.8417	58	
	318	0.8254	59	
	319	0.6109	59	
	Median:	**0.748**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.739**	**58.100**	
	SEM:	**0.027**	**0.216**	
	%CV:	**13.857**	**12.698**	
	Median:	**0.667**	**57**	
	Mode:		**57**	
	Total Mean:	**0.680**	**57.350**	
	SEM:	**0.021**	**0.177**	
	%CV:	**15.736**	**14.816**	

TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00055 **Test No: 1**

Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 66.7ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)
A	320	0.7302	57
	321	0.5718	57
	322	0.6918	58
	323	0.8365	57
	324	0.7308	59
	325	0.8707	57
	326	0.7500	57
	327	0.7428	57
	328	0.9186	59
	329	0.6293	59
	330	0.7236	59
	331	0.8266	57
	332	0.6525	57
	333	0.5867	57
	334	0.7005	57
	335	0.5811	57
	336	0.6147	57
	337	0.5940	57
	338	0.6910	57
	339	0.8218	57
	Median:	**0.712**	**57**
	Mode:		**57**
	Mean:	**0.713**	**57.450**
	SEM:	**0.023**	**0.185**
	%CV:	**12.117**	**10.892**
B	340	0.8320	59
	341	0.8161	57
	342	0.9216	57
	343	0.6155	57
	344	0.8276	57
	345	0.6360	57
	346	0.8050	58
	347	0.7670	57
	348	0.6383	57
	349	0.8533	57
	350	0.6954	57
	351	0.8540	57
	352	0.7402	57
	353	0.6809	57
	354	0.7719	57
	355	0.6924	57
	356	0.7885	57
	357	0.6967	57
	358	0.6233	57
	359	0.7318	57
	Median:	**0.754**	**57**
	Mode:		**57**
	Rep Mean:	**0.749**	**57.150**
	SEM:	**0.020**	**0.109**
	%CV:	**10.170**	**6.473**
	Median:	**0.731**	**57**
	Mode:		**57**
	Total Mean:	**0.731**	**57.300**
	SEM:	**0.015**	**0.109**
	%CV:	**11.224**	**9.074**

Gross Morphology

Comments:

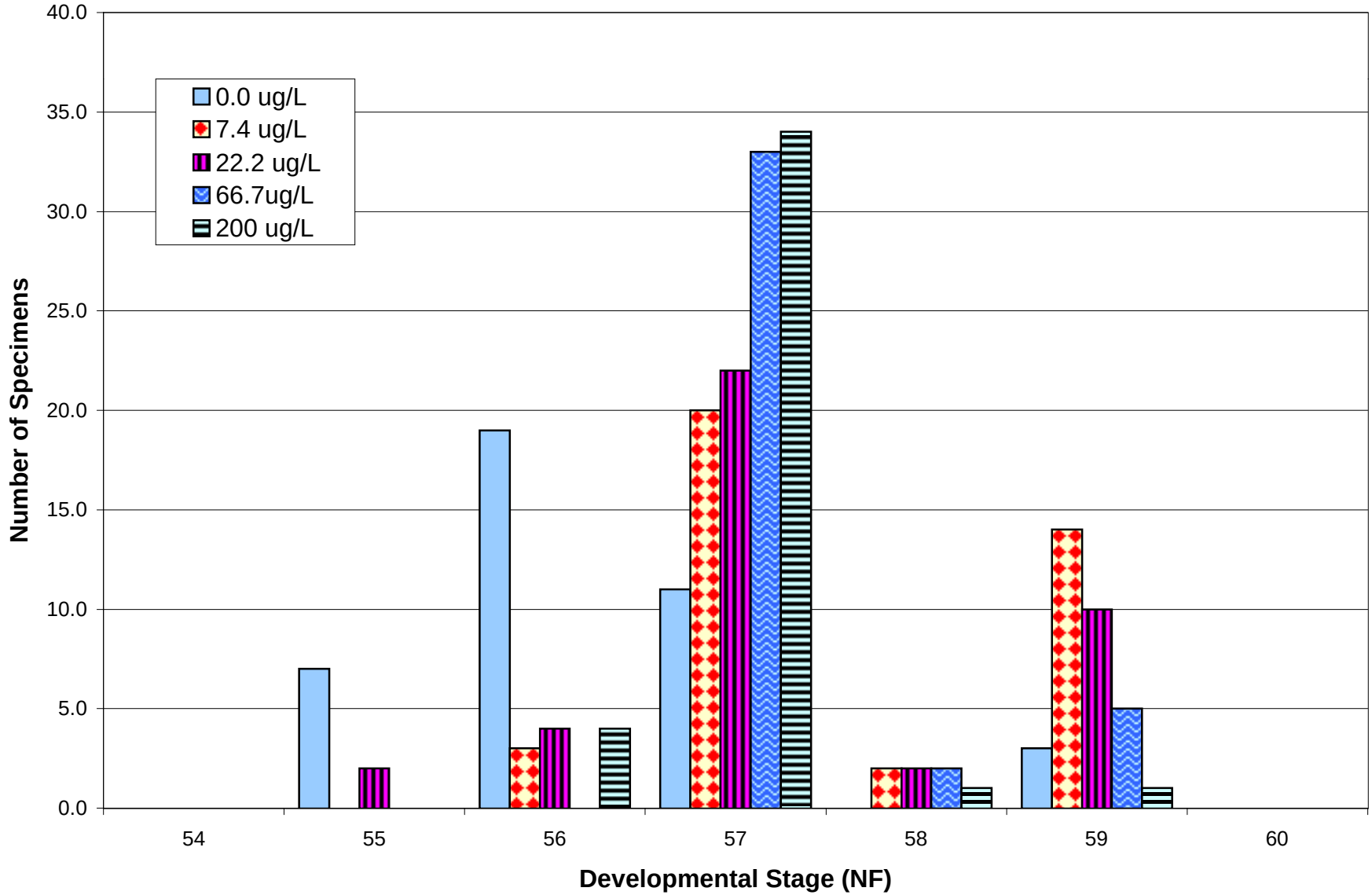
TEST TERMINATION DATA (Initial Stage 51)

Workorder No: 00055 **Test No: 1**

Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 200 ug/L**

Stage 51 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
A	360	0.5986	57	
	361	0.8748	57	
	362	0.7052	57	
	363	0.7367	57	
	364	0.7150	57	
	365	0.7211	57	
	366	0.6490	57	
	367	0.7589	57	
	368	0.7708	57	
	369	0.7260	57	
	370	0.7544	58	
	371	0.6917	57	
	372	0.6511	56	
	373	0.6498	57	
	374	0.6405	57	
	375	0.6379	57	
	376	0.7522	57	
	377	0.8341	57	
	378	0.7182	57	
	379	0.6858	57	
	Median:	**0.717**	**57**	
	Mode:		**57**	
	Mean:	**0.714**	**57.000**	
	SEM:	**0.015**	**0.073**	
	%CV:	**8.035**	**4.297**	
B	380	0.4639	56	
	381	0.7236	57	
	382	0.5814	57	
	383	0.6213	57	
	384	0.6188	57	
	385	0.5633	57	
	386	0.6058	57	
	387	0.6806	57	
	388	0.5488	57	
	389	0.4666	56	
	390	0.4402	56	
	391	0.6413	57	
	392	0.6308	59	
	393	0.5478	57	
	394	0.4671	57	
	395	0.5634	57	
	396	0.6589	57	
	397	0.7239	57	
	398	0.7088	57	
	399	0.4719	57	
	Median:	**0.594**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.586**	**56.950**	
	SEM:	**0.020**	**0.135**	
	%CV:	**11.853**	**8.014**	
	Median:	**0.650**	**57**	
	Mode:		**57**	
	Total Mean:	**0.650**	**56.975**	
	SEM:	**0.016**	**0.076**	
	%CV:	**12.652**	**6.355**	

BATT02-00055
Pregnenolone-16-a-Carbonitrile (PCN)
Stage Distribution for Initial Stage 51 Test



TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00055 **Test No: 1**

Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 0.0 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)
C	001	0.8200	59
	002	0.7575	59
	003	1.2389	59
	004	1.0141	57
	005	0.8378	59
	006	0.8978	59
	007	0.9622	58
	008	0.7525	57
	009	0.9282	57
	010	0.8016	57
	011	1.0707	59
	012	0.8370	59
	013	0.6236	57
	014	0.8052	57
	015	0.8165	57
	016	0.7300	57
	017	0.8189	59
	018	0.6822	57
	019	0.8071	59
	020	0.8336	59
	Median:	**0.819**	**59**
	Mode:		**59**
	Mean:	**0.852**	**58.050**
	SEM:	**0.031**	**0.223**
	%CV:	**15.109**	**13.108**
D	021	0.8977	59
	022	0.9382	59
	023	0.9577	59
	024	0.8563	59
	025	0.9868	59
	026	0.8421	57
	027	1.0150	59
	028	0.6389	57
	029	0.5739	57
	030	0.5872	57
	031	0.5491	57
	032	0.7358	57
	033	0.7064	57
	034	0.7914	59
	035	1.1709	59
	036	0.8466	59
	037	0.9513	57
	038	0.8113	57
	039	0.6270	57
	040	0.5582	57
	Median:	**0.827**	**57**
	Mode:		**57**
	Rep Mean:	**0.802**	**57.900**
	SEM:	**0.039**	**0.228**
	%CV:	**19.658**	**13.416**
	Median:	**0.819**	**58**
	Mode:		**57**
	Total Mean:	**0.827**	**57.975**
	SEM:	**0.025**	**0.158**
	%CV:	**17.459**	**13.129**

Gross Morphology

Comments:

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00055 **Test No: 1**

Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 7.4 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	041	1.1302	59	
	042	1.0715	59	
	043	0.7794	59	
	044	0.7404	59	
	045	0.9966	59	
	046	0.7314	57	
	047	0.6221	57	
	048	0.7388	57	
	049	0.8935	59	
	050	0.6447	57	
	051	0.6953	59	
	052	0.7900	57	
	053	0.5535	57	
	054	0.8805	58	
	055	0.9603	58	
	056	0.7883	59	
	057	1.0398	59	
	058	0.8458	57	
	059	0.8887	59	
	060	0.7730	59	
	Median:	**0.789**	**59**	
	Mode:		**59**	
	Mean:	**0.828**	**58.200**	
	SEM:	**0.035**	**0.213**	
	%CV:	**17.000**	**12.472**	
D	061	0.8561	59	
	062	0.8537	59	
	063	0.6163	59	
	064	0.9069	59	
	065	0.8151	59	
	066	1.0095	59	Kinked Tail
	067	0.8271	59	
	068	0.5858	57	
	069	0.5836	57	
	070	0.5341	57	
	071	0.8027	57	
	072	0.8294	59	
	073	0.7650	58	
	074	0.7080	57	
	075	0.6435	57	
	076	0.5752	56	
	077	0.8215	57	
	078	0.7014	57	
	079	0.6814	57	
	080	0.6508	57	
	Median:	**0.737**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.738**	**57.800**	
	SEM:	**0.029**	**0.236**	
	%CV:	**14.998**	**13.894**	
	Median:	**0.784**	**58**	
	Mode:		**59**	
	Total Mean:	**0.783**	**58.000**	
	SEM:	**0.023**	**0.160**	
	%CV:	**16.691**	**13.298**	

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00055 **Test No: 1**

Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 22.2 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)
C	081	0.9563	59
	082	0.9299	59
	083	0.9885	59
	084	0.8485	59
	085	0.7713	59
	086	1.0553	59
	087	0.8553	59
	088	0.7642	59
	089	0.8507	58
	090	0.7182	57
	091	0.7963	59
	092	0.6982	59
	093	0.9835	57
	094	0.7160	57
	095	0.7986	57
	096	0.5564	57
	097	0.9651	59
	098	0.9645	59
	099	0.7472	59
	100	0.9412	59
	Median:	**0.850**	**59**
	Mode:		**59**
	Mean:	**0.845**	**58.450**
	SEM:	**0.028**	**0.198**
	%CV:	**13.788**	**11.602**
D	101	0.9376	57
	102	0.9476	59
	103	0.6507	59
	104	0.8121	59
	105	0.8955	59
	106	0.8807	59
	107	0.8490	59
	108	0.7038	57
	109	0.8443	60
	110	0.5866	57
	111	0.6482	57
	112	0.7800	57
	113	1.0186	59
	114	0.7692	57
	115	0.8407	57
	116	0.7135	57
	117	0.8890	59
	118	0.8061	57
	119	0.9935	59
	120	0.7237	57
	Median:	**0.826**	**58**
	Mode:		**57**
	Rep Mean:	**0.815**	**58.050**
	SEM:	**0.026**	**0.246**
	%CV:	**13.117**	**14.425**
	Median:	**0.843**	**59**
	Mode:		**59**
	Total Mean:	**0.830**	**58.250**
	SEM:	**0.019**	**0.159**
	%CV:	**13.399**	**13.186**

Gross Morphology

Comments:

TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00055 **Test No: 1**

Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 66.7 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	121	0.6020	60	
	122	0.7317	59	
	123	0.9243	59	
	124	0.8350	57	
	125	0.6316	59	
	126	1.0242	59	
	127	0.5894	59	
	128	0.7405	57	
	129	0.9432	59	
	130	0.8761	57	
	131	0.7626	57	
	132	1.0557	59	
	133	0.8837	59	
	134	0.6682	58	
	135	0.7678	57	
	136	1.0074	57	
	137	0.8193	59	
	138	0.8935	59	
	139	0.9436	59	
	140	0.7972	57	
	Median:	**0.827**	**59**	
	Mode:		**59**	
	Mean:	**0.825**	**58.300**	
	SEM:	**0.031**	**0.231**	
	%CV:	**15.281**	**13.504**	
D	141	0.7824	60	
	142	0.9850	59	
	143	0.8565	59	Kinked Tail
	144	0.8856	59	
	145	0.9478	59	
	146	0.9598	57	
	147	0.7600	57	
	148	0.6528	59	
	149	1.1115	59	
	150	0.8468	59	
	151	0.7597	59	
	152	0.7452	59	
	153	0.9101	59	
	154	0.9009	57	
	155	0.7944	59	
	156	0.7794	57	
	157	0.8077	57	
	158	0.8495	59	
	159	1.0204	59	
	160	0.9293	57	
	Median:	**0.853**	**59**	**Comments:**
	Mode:		**59**	
	Rep Mean:	**0.864**	**58.450**	
	SEM:	**0.024**	**0.223**	
	%CV:	**11.773**	**13.063**	
	Median:	**0.848**	**59**	
	Mode:		**59**	
	Total Mean:	**0.845**	**58.375**	
	SEM:	**0.020**	**0.159**	
	%CV:	**13.598**	**13.151**	

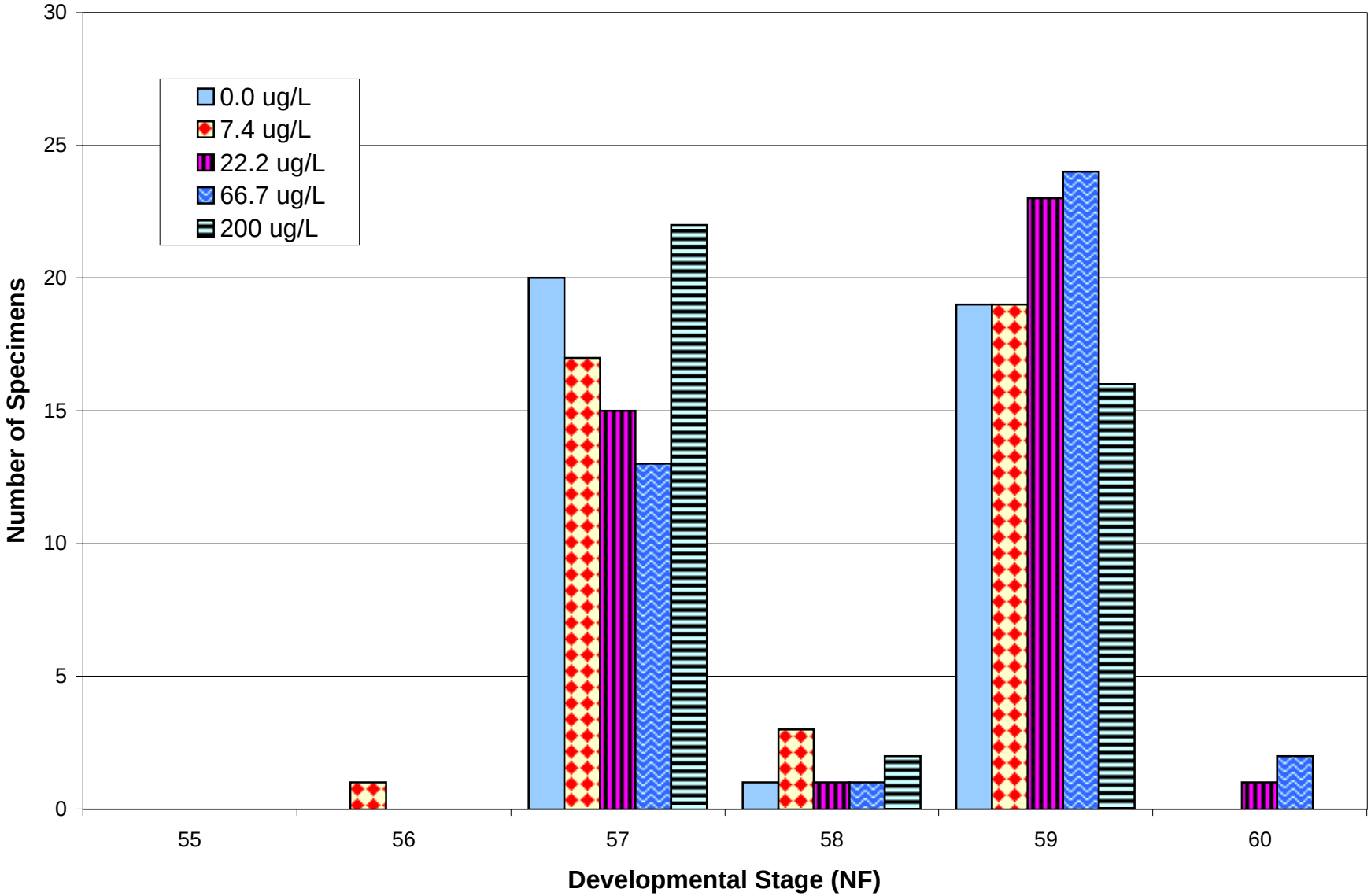
TEST TERMINATION DATA (Initial Stage 54)

Workorder No: 00055 **Test No: 1**

Test Chemical: Pregnenolone-16-a-carbonitrile (PCN) **Target Conc: 200 ug/L**

Stage 54 Replicate ID	Individual Specimen Number	Specimen Weight (g)	Growth Stage (NF)	Gross Morphology
C	161	0.8324	59	
	162	0.8822	59	
	163	0.9210	57	
	164	0.6188	57	
	165	0.8912	57	
	166	0.5902	57	
	167	0.8609	57	
	168	1.0098	57	
	169	0.8393	57	
	170	1.0475	58	
	171	0.9531	59	
	172	0.8012	59	
	173	0.5946	57	
	174	0.8116	57	
	175	1.0509	58	
	176	0.7225	57	
	177	0.8071	59	
	178	0.7619	57	
	179	1.1512	59	
	180	0.8892	59	
	Median:	**0.850**	**57**	
	Mode:		**57**	
	Mean:	**0.852**	**57.800**	
	SEM:	**0.034**	**0.213**	
	%CV:	**16.336**	**12.515**	
D	181	0.8063	57	
	182	1.0464	59	
	183	0.6572	57	
	184	0.6804	59	
	185	0.8191	59	
	186	0.8717	59	
	187	0.6327	57	
	188	0.8570	57	
	189	0.9678	57	
	190	0.8542	57	
	191	0.8037	57	
	192	1.4186	59	
	193	0.7828	57	
	194	1.0375	59	
	195	0.6735	57	
	196	0.6662	57	
	197	0.8486	59	
	198	0.7216	59	
	199	1.1525	57	
	200	0.7795	59	
	Median:	**0.813**	**57**	**Comments:**
	Mode:		**57**	
	Rep Mean:	**0.854**	**57.900**	
	SEM:	**0.043**	**0.228**	
	%CV:	**20.945**	**13.416**	
	Median:	**0.836**	**57**	
	Mode:		**57**	
	Total Mean:	**0.853**	**57.850**	
	SEM:	**0.027**	**0.154**	
	%CV:	**18.543**	**12.823**	

BATT02-00055
PCN
Stage Distribution for Initial Stage 54 Test



Pregnenolone-16-α-Carbonitrile (PCN) Metamorphosis Assay: Statistical Data – Stage and Growth

One Way Analysis of Variance

Data source: BATT02-00055_PCN_Stage 51_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0ug/L	40	0	56	56	57
7.4ug/L	39	0	57	57	59
22.2ug/L	40	0	57	57	58.5
66.7ug/L	40	0	57	57	57
200ug/L	40	0	57	57	57

H = 49.648 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

Multiple Comparisons versus Control Group (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05	
7.4ug/L vs 0.0ug/L	73.362	5.661	Yes	**LOEC**
66.7ug/L vs 0.0ug/L	59.512	4.621	Yes	
22.2ug/L vs 0.0ug/L	57.725	4.483	Yes	
200ug/L vs 0.0ug/L	42.075	3.267	Yes	

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance

Data source: BATT02-00055_PCN_Stage 51_Growth

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.083)

Group Name	N	Missing	Mean	Std Dev	SEM
0.0ug/L	40	0	0.481	0.143	0.0226
7.4ug/L	39	0	0.758	0.158	0.0254
22.2ug/L	40	0	0.68	0.13	0.0205
66.7ug/L	40	0	0.731	0.096	0.0152
200ug/L	40	0	0.65	0.102	0.0161

Source of Variation	DF	SS	MS	F	P
Between Groups	4	1.877	0.469	28.684	<0.001
Residual	194	3.174	0.0164		
Total	198	5.051			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Bonferroni t-test):

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050	
0.0ug/L vs. 7.4ug/L	0.277	9.616	<0.001	Yes	**LOEC**
0.0ug/L vs. 66.7ug/L	0.25	8.747	<0.001	Yes	
0.0ug/L vs. 22.2ug/L	0.199	6.955	<0.001	Yes	
0.0ug/L vs. 200ug/L	0.169	5.904	<0.001	Yes	

One Way Analysis of Variance

Data source: BATT02-00055_PCN_Stage 54_Stage

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
0.0ug/L	40	0	57.5	57	59
7.4ug/L	40	0	58	57	59
22.2ug/L	40	0	59	57	59
66.7ug/L	40	0	59	57	59
200ug/L	40	0	57	57	59

H = 7.374 with 4 degrees of freedom. (P = 0.117)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.117)

One Way Analysis of Variance

Data source: BATT02-00055_PCN_Stage 54_Growth

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.673)

Group Name	N	Missing	Mean	Std Dev	SEM
0.0ug/L	40	0	0.827	0.159	0.0251
7.4ug/L	40	0	0.783	0.148	0.0234
22.2ug/L	40	0	0.83	0.122	0.0193
66.7ug/L	40	0	0.845	0.125	0.0198
200ug/L	40	0	0.853	0.171	0.0271

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.116	0.029	1.355	0.251
Residual	195	4.168	0.0214		
Total	199	4.284			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.251).

Power of performed test with alpha = 0.050: 0.127

The power of the performed test (0.127) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

Pregnenolone-16-α-Carbonitrile (PCN)
Test Chemical Analytical Data from Battelle:
Purity / Stability Report

Pregnenolone-16-α-Carbonitrile (PCN)
Test Chemical Analytical Data from Battelle:
In-Life Test Chemical Analysis Report

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Walker
P.O. #:	

Fort Log No. (Lab Use Only): BATT02-00055

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested	Lab Use Only		
006	Dechlorinated Tap H_2O	10/14/04 1500	1	G	Blue Ice	PCN			
007	Dechlorinated Tap H_2O		1						
008	7.4 mg/L PCN		1						
009	22.2 mg/L PCN		1						
010	66.7 mg/L PCN		1						
011	200 mg/L PCN		2						
012	200 mg/L PCN Stock		1						

SPECIAL INSTRUCTIONS:
006 - (1L Dilution H_2O)
007 - 011 (Stage 5!, Rep. A)
012 (New stock prep.!)

For WA 3-11 Amphibian Study with Contract Questions to:
David Houchens (614-424-3564) or Michael Cobb (360-681-4580)
Please Return Ice Chest.

DATE:
INITIALS:

Andrea Walker	10/14/04 1700	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

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FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

COPY

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Walkers
P.O.#:	

BAT702-00055

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
013	200 µg/L PCN Stock	10/18/04 0800	1	G	Blue Ice	PCN
014	200 µg/L PCN Stock	↓ 1000	1	↓	↓	↓
015	Declin Tap H_2O	10/20/04 1000	1			
016	7.4 µg/L PCN		1			
017	22.2 µg/L PCN		1			
018	66.7 µg/L PCN		2			
019	200 µg/L PCN		1			
020	100 µg/L PCN Stock		1			
021	200 µg/L PCN Stock	↓ ↓	1	↓	↓	↓

SPECIAL INSTRUCTIONS:
013-(Old prep.1)
014-(New prep.2)
015-019 (Stage 5N, Rep C)
020-(Old prep.2)
021-(New prep.3)

For WA 3-11 AMPHIBIAN STUDY WITH CONTACT QUESTIONS TO: DAVID HOUCHENS (614-424-3564) OR MICHAEL COBB (360-697-4580)

Please return ice chest.

DATE:
INITIALS:

Andrea Walkers	10/20/04 1700	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

NOV 01 2004 16:13 FP BATTELLE - COLUMBUS 16144245721 TO 914053777888 P.02/02

** TOTAL PAGE.02 **

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FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Walker
P.O. #:	

BATT02-00055

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
022	200 µg/L PCN Stock	10/22/04 0930	1	G	Blue ice	PCN
023	200 µg/L PCN Stock	↓ ↓	1	↓	↓	↓
024	200 µg/L PCN Stock	10/25/04 0900	1			
025	200 µg/L PCN Stock	↓ ↓	1			
026	200 µg/L PCN Stock	10/27/04 0900	1			
027	200 µg/L PCN Stock	↓ ↓	1			
028	DeCl$_2$ Tap H$_2$O	10/28/04 1600	1			
029	7.4 µg/L PCN	↓ ↓	1			
030	22.2 µg/L PCN		1			
031	66.7 µg/L PCN		1			
032	200 µg/L PCN	↓ ↓	2	↓	↓	↓

SPECIAL INSTRUCTIONS:
022 (Old prep. 3) 025 (New prep. 5) 028-032 (Stage 51, Rep 3)
023 (New prep. 4) 026 (Old prep. 5)
024 (Old prep. 4) 027 (New prep 6)

For WA 3-11 Amphibian Study with contact questions to:
David Houchens (614-424-3564) or
Michael Cobb (360-681-4580)

Please return ice chest

DATE
INITIALS:

Andrea Walker	10/28/04 1700	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

COPY

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	Andrea Walker
P.O. #:	

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
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BATT01 - 00055

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested			
033	200 mg/L PCN Stock	10/29/04 0900	1	G	Blue ice	PCN			
034	200 mg/L PCN Stock	↓ ↓	1	↓	↓	↓			
035	200 mg/L PCN Stock	11/1/04 0900	1						
036	200 mg/L PCN Stock	↓ ↓	1						
037	200 mg/L PCN Stock	11/3/04 0800	1						
038	DeCl, Tap H_2O	↓ ↓	1						
039	7.4 mg/L PCN		1						
040	22.2 mg/L PCN		1						
041	66.7 mg/L PCN		2						
042	200 mg/L PCN	↓ ↓	1	↓	↓	↓			

SPECIAL INSTRUCTIONS:
033 (Old prep. 6) 036 (New prep 8)
034 (New prep. 7) 037 (Old prep. 8)
035 (Old prep. 7) 038-042 (Stage 54, Rep. D)

For WA 3-11 Amphibian Study with contact questions to:
David Houchens (614-424-3564) or Michael Cobb (360-681-4580)

PLEASE RETURN ICE CHEST.

DATE:
INITIALS:

Andrea Walker	11/3/04 1700	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

Pregnenolone-16-α-Carbonitrile (PCN)
Thyroid Histology Data from EPL:
Sample Identification Key
Thyroid Pathology Report

EPL Sample Key for PCN

FEL Sample Number	Individual Specimen Number[1]	Conc. Replicate Number	Initial Specimen Stage	Chemical Conc. (µg/L)
042	201, 203, 207, 213, 219 221, 229, 230, 235, 237	A B	51	0
043	243, 244, 247, 252, 258 264, 268, 271, 273, 279	A B	51	7.4
044	283, 286, 288, 292, 294 301, 306, 309, 311, 314	A B	51	22.2
045	320, 325, 327, 331, 339 341, 347, 353, 356, 358	A B	51	66.7
046	363, 366, 369, 376, 379 382, 384, 388, 393, 396	A B	51	200
047	004, 008, 010, 016, 018 026, 028, 030, 033, 039	C D	54	0
048	046, 047, 050, 052, 058 068, 070, 071, 075, 079	C D	54	7.4
049	081, 084, 088, 092, 097 103, 104, 106, 117, 119	C D	54	22.2
050	123, 125, 127, 137, 139 142, 144, 148, 155, 159	C D	54	66.7
051	163, 166, 169, 174, 178 181, 187, 190, 193, 195	C D	54	200

[1] Individual specimens for thyroid histology were randomly selected using *Principles and Procedures of Statistics, A Biometrical Approach*, 2nd Ed., by Robert G.D. Steel and James H. Torrie, McGraw-Hill Book Co., USA, 1980.

EPL®

EXPERIMENTAL PATHOLOGY LABORATORIES, INC.

BATTELLE
STUDY NUMBER BATT02-00055
EPL PROJECT NUMBER 237-011

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

DRAFT PATHOLOGY REPORT

Submitted by:

Experimental Pathology Laboratories, Inc.
P.O. Box 474
Herndon, VA 20172-0474
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Submitted to:

Battelle
Columbus, OH 43201

March 4, 2005

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DRAFT PATHOLOGY SUMMARY

BATTELLE
STUDY NUMBER BATT02-00055
EPL PROJECT NUMBER 237-011

AMPHIBIAN METAMORPHOSIS ASSAY DEMONSTRATION STUDY

DRAFT PATHOLOGY SUMMARY

A histomorphologic evaluation was performed on the thyroid glands of *Xenopus laevis* (South African clawed frog) that were exposed to various concentrations of pregnenolone-16-α-carbonitrile (PCN) to determine the differential sensitivity of exposing Stage 51 and Stage 54 larvae and to establish the necessary duration of exposure. Stage 51 larval frogs were exposed to PCN for 21 days and Stage 54 larval frogs were exposed to PCN for 14 days. The experimental designs were as follows:

Stage 51 Frog Larvae Exposed for 21 Days

Group/ Replicate	Pregnenolone-16-α-carbonitrile (PCN) (µg/L)	No. of Frogs/Group	Individual Frog Numbers
042A	0	5	201, 203, 207, 213, 219
042B	0	5	221, 229, 230, 235, 237
043A	7.4	5	243, 244, 247, 252, 258
043B	7.4	5	264, 268, 271, 273, 279
044A	22.2	5	283, 286, 288, 292, 294
044B	22.2	5	301, 306, 309, 311, 314
045A	66.7	5	320, 325, 327, 331, 339
045B	66.7	5	341, 347, 353, 356, 358
046A	200	5	363, 366, 369, 376, 379
046B	200	5	382, 384, 388, 393, 396

Stage 54 Frog Larvae Exposed for 14 Days

Group/ Replicate	Pregnenolone-16-α-carbonitrile (PCN) (µg/L)	No. of Frogs/Group	Individual Frog Numbers
047C	0	5	004, 008, 010, 016, 018
047D	0	5	026, 028, 030, 033, 039
048C	7.4	5	046, 047, 050, 052, 058
048D	7.4	5	068, 070, 071, 075, 079
049C	22.2	5	081, 084, 088, 092, 097
049D	22.2	5	103, 104, 106, 117, 119
050C	66.7	5	123, 125, 127, 137, 139
050D	66.7	5	142, 144, 148, 155, 159
051C	200	5	163, 166, 169, 174, 178
051D	200	5	181, 187, 190, 193, 195

MATERIALS AND METHODS

Slides were prepared by Experimental Laboratories, Inc. (EPL®). The heads of the frogs were cut from the body in a transverse plane just caudal to the eyes. During trimming, special care was taken to ensure that the cut was perpendicular to the long axis of the frog so that the right and left sides would be in the same plane. The heads were placed in cassettes with the cut side down to allow the heads to be sectioned in a transverse plane from the caudal to rostral direction. Each head was rough-cut until the thyroid gland was observed bilaterally, if possible, in the unstained section. When the thyroid glands were reached, five step sections were microtomed at approximately 30 micron intervals. The resulting step sections were mounted on one slide, whenever possible. During microtoming, a duplicate slide of these five step sections was also created. The slides were examined by a technician to assure that a sufficient amount of thyroid tissue was present bilaterally in the sections. If

necessary to meet these criteria, additional step sections were cut from the block. For each frog, one of the slides was stained with hematoxylin and eosin, and the other (duplicate) slide was left unstained and dipped in paraffin.

Microscopic findings for thyroid glands from each frog are listed in the Histopathology Incidence Tables. The changes were graded from 1 to 5 depending upon severity (1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high). Non-gradable changes were designated as present (P). All lesions are summarized by group on the Summary Incidence Tables. The sexes of the frogs were not reported. Representative photomicrographs are presented in Appendices A and B (Stages 51 and 54, respectively).

RESULTS

Stage 51 *Xenopus laevis* exposed to PCN for 21 days

Compared to the 0 µg/L group, there was a substantially higher incidence of thyroid follicular cell hyperplasia (minimal to mild) in groups exposed to 22.2, 66.7, and 200 µg/L PCN. The pattern of increase among the exposure groups also suggests that this effect was somewhat dose-dependent. Thyroid follicular cell hyperplasia was characterized by increased crowding and columnar elongation (hypertrophy) of follicular cells (which occasionally produced a pseudostratified appearance), a slight increase in cytoplasmic density, and/or focal invagination of the follicular basement membrane. In the vast majority of instances, this change was minimal rather than mild.

Compared to the 0 µg/L group, there was a substantially higher incidence of thyroid follicular cell karyomegaly (minimal) in groups exposed to 66.7 and 200 µg/L PCN. The pattern of increase among the exposure groups was not dose-dependent, as the incidence of this change was lower in the 200 µg/L PCN-

exposure group as compared to the 66.7µg/L PCN group. Karyomegaly was observed as multiple scattered individual thyroid follicular epithelial cells whose nuclei were enlarged when compared to those of other cells in the same follicle or other follicles of the same gland. To a small extent, affected follicles had a anisokaryotic appearance due to this focal distribution pattern.

Table 1
Prevalence with Replicates Combined of Selected Changes in Thyroid Glands of Stage 51 Frogs After Exposure to Various Concentrations of PCN for 21 Days

Group	42 A & B	43 A & B	44 A & B	45 A & B	46 A & B
Exposure Conc.	0 µg/L	7.4 µg/L	22.2 µg/L	66.7 µg/L	200 µg/L
Thyroid (No. Examined)	(10)	(10)	(10)	(10)	(10)
Hyperplasia, Follicular Cells	2	4	7	7	8
Karyomegaly, Follicular Cells	0	0	1	7	4
Diffuse Enlargement	0	1	0	0	2

Stage 54 *Xenopus laevis* exposed to PCN for 14 days

Compared to the 0 µg/L group, there was a substantially higher incidence of diffuse thyroid enlargement (minimal) in the group exposed to 66.7µg/L PCN. The incidence of this change in the 200 µg/L PCN group was not substantially higher than in controls, however. Diffuse enlargement was diagnosed when one or both thyroid glands appeared to occupy ¾ or more of the diameter of a 40x microscopic field.

Follicular cell hyperplasia was not substantially increased in PCN-exposed frogs compared to controls, and the severity of this diagnosis was minimal in all instances.

Table 2
Prevalence with Replicates Combined of Selected Changes in Thyroid Glands of Stage 54 Frogs After Exposure to Various Concentrations of PCN for 14 Days

Group	47 C & D	48 C & D	49 C & D	50 C & D	51 C & D
Exposure Conc.	0 µg/L	7.4 µg/L	22.2 µg/L	66.7 µg/L	200 µg/L
Thyroid (No. Examined)	(10)	(10)	(10)	(10)	(10)
Hyperplasia, Follicular Cells	5	3	7	7	7
Karyomegaly, Follicular Cells	0	0	1	0	0
Diffuse Enlargement	0	0	2	4	1
Colloid, Decreased	1	1	0	0	1

DISCUSSION

PCN is a microsomal enzyme inducer that has been shown to decrease circulating levels of thyroid hormones (e.g., thyroxine) when administered to rats (Barter and Klaassen, 1992; Vansell and Klaassen, 2002b). The primary mechanism for this appears to be enhanced biliary excretion of these hormones via the induction of hepatic UDP-glucuronosyltransferase activity (Hood and Klaassen, 2000; Vansell and Klaassen, 2002a). In rats, the decreased circulating thyroid hormone levels lead to an increase in thyroid stimulating hormone (TSH) (Japundzic et al., 1976; Vansell and Klaassen, 2002a), which in

turn, results in thyroid follicular cell proliferation (Hood et al., 2003; Hood et al., 1999a; Hood et al., 1999b).

In rats, the incidence of histologically-evident proliferative lesions, including "cystic and follicular hyperplasia, cystic and follicular adenoma, and follicular carcinoma" was increased significantly in rats following 19-weeks of treatment with PCN (Vansell et al., 2004). In another study in which rats were administered PCN for seven days, increased follicular cell proliferation in the treated rats was determined quantitatively via cell counts in PCNA-stained sections (Hood et al., 1999a). In the same study, the application of other morphometric techniques revealed that PCN-treated rats also had increased thyroid epithelial area, hypertrophy of follicular cells, and decreased colloid area when compared to controls. Despite the evidence of increased follicular cell proliferation in that study, the total number of follicular cells in PCN-treated rats was not comparatively increased. There are indications that this lack of overall increase in follicular cells may have been due to increased follicular cell apoptosis in rats that have been treated with PCN (Kolaja et al., 1999).

In the present study, the severity of PCN-induced (or spontaneous) thyroid follicular cell hyperplasia that could be discerned by visual inspection was generally very low, and a clear increase in the incidence of this lesion was only seen in the Stage 51 larvae that were exposed to PCN for 21 days. In Stage 54 larvae that were exposed for 14 days, there was essentially no demonstrable difference between control and PCN-treated frogs in either the incidence or severity of follicular hyperplasia. The overall subtlety of histologic changes in frogs when compared to rats may have been due to species differences; for example, PCN administration did not induce any histologic changes in the thyroids of mice according to one report (Viollon-Abadie et al., 1999).

In the Stage 54 / 14- day trial, the increased incidence of minimal diffuse thyroid enlargement that occurred in the 66.7µg/L PCN group was not duplicated in the 200 µg/L PCN group. Therefore, it is doubtful that this finding represents an exposure-related change.

The relative paucity of clear exposure-associated effects in the Stage 54 / 14-day trial suggests that this duration and stage combination is less sensitive than the Stage 51 / 21-day combination for the assessment of PCN exposure. In the Stage 54 / 14-day trial, the reason why follicular cell hyperplasia was diagnosed in five of ten control frogs was because similar diagnostic criteria were applied as for the Stage 51 / 21-day trial. It is possible that the baseline level of follicular cell proliferation was slightly higher in frogs of the Stage 54 / 14-day trial when compared to the frogs of the Stage 51 / 21-day trial, because the frogs in these two trials were of different ages (72 days compared to 68 days) at the time of sacrifice.

CONCLUSIONS

In the Stage 51 larvae that were exposed to PCN for 21 days there was a substantially higher incidence of minimal to mild thyroid follicular cell hyperplasia in groups exposed to 22.2, 66.7, and 200 µg/L PCN, and a substantially higher incidence of minimal thyroid follicular cell karyomegaly in groups exposed to 66.7, and 200 µg/L PCN, when compared to the 0 µg/L group. For follicular cell hyperplasia, the pattern of increase among the exposure groups also suggested that this effect was somewhat dose-dependent.

In the Stage 54 larvae that were exposed to PCN for 14 days, there were no effects that were clearly exposure-related.

JEFFREY C. WOLF, DVM, Diplomate, ACVP
Veterinary Pathologist

Date

JCW/skh

REFERENCES

Barter, R.A., Klaassen, C.D. (1992). UDP-glucuronosyltransferase inducers reduce thyroid hormone levels in rats by an extrathyroidal mechanism. *Toxicol Appl Pharmacol.* **113**(1):36-42.

Hood, A., Allen, M.L., Lui, Y., Liu, J., Klaassen, C.D. (2003). Induction of T(4) UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers. *Toxicol Appl Pharmacol.* **188**(1):6-13.

Hood, A., Hashmi, R., Klaassen, C.D. (1999a). Effects of microsomal enzyme inducers on thyroid-follicular cell proliferation, hyperplasia, and hypertrophy. *Toxicol Appl Pharmacol.* **160**(2):163-70.

Hood, A., Klaassen, C.D. (2000). Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation. *Toxicol Sci.* **55**(1):78-84.

Hood, A., Liu, J., Klaassen, C.D. (1999b). Effects of phenobarbital, pregnenolone-16alpha-carbonitrile, and propylthiouracil on thyroid follicular cell proliferation. *Toxicol Sci.* **50**(1):45-53.

Japundzic, M.M., Bastomsky, C.H., Japundzic, I.P. (1976). Enhanced biliary thyroxine excretion in rats treated with pregnenolone-16alpha-carbonitrile. *Acta Endocrinol (Copenh).* **81**(1):110-9.

Kolaja, K.L., Hood, A.M., Klaassen, C.D. (1999). The UDP-glucuronyltransferase inducers, phenobarbital and pregnenolone-16 alpha-carbonitrile, enhance thyroid-follicular cell apoptosis: Association with TGF-beta1 expression. *Toxicol Lett.* **106**(2-3):143-50.

Vansell, N.R., Klaassen, C.D. (2002a). Effect of microsomal enzyme inducers on the bilary excretion of triiodothyronine (T(3)) and its metabolites. *Toxicol Sci.* **65**(2):184-91.

Vansell, N.R., Klaassen, C.D. (2002b). Increase in rat liver UDP-glucuronosyltransferase mRNA by microsomal enzyme inducers that enhance thyroid hormone glucuronidation. *Drug Metab Dispos.* **30**(3):240-6.

Vansell, N.R., Muppidi, J.R., Habeebu, S.M., Klaassen, C.D. (2004). Promotion of thyroid tumors in rats by pregnenolone-16alpha-carbonitrile (PCN) and polychlorinated biphenyl (PCB). *Toxicol Sci.* **81**(1):50-9.

Viollon-Abadie, C., Lassere, D., Debruyne, E., Nicod, L., Carmichael, N., and Richert, L. (1999). Phenobarbital, α-Naphthoflavone, Clofibrate, and Pregnenolone-16α-Carbonitrile Do Not Affect Hepatic Thyroid Hormone UDP-Glucuronosyl Transferase Activity, and Thyroid Gland Function in Mice. *Toxicol Appl Pharmacol.* **155**(1):1-12.

COMPLIANCE STATEMENT

Client Name	Battelle	EPL Project Coordinator	Dr. Marilyn J. Wolfe
Client Study	BATT02-00055	EPL Pathologist	Dr. Jeffrey C. Wolf
Species	*Xenopus laevis*	EPL Project Number	237-011
Study Title	Amphibian Metamorphosis Assay Demonstration Study		
Test Article	Pregnenolone-16-α-carbonitrile		

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 160.

EPL Project Coordinator

Date

3/04/05 DRAFT

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Amphibian Metamorphosis Assay Demonstration Study

Client Study: BATT02-00055 EPL Project Coordinator: Marilyn J. Wolfe

EPL Project Number: 237-011 EPL Pathologist: 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 Project Coordinator and Management are indicated below.

Area Inspected	Dates	
	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management XXX

Date of last quarterly facility inspection 1/05

EPL Quality Assurance Unit Date

SUMMARY INCIDENCE TABLES

21 Days – Stage 51

SUMMARY INCIDENCE TABLE

BATT02-00055
Stage 51/21 Day Sacrifice
Xenopus laevis (South African Clawed frog)

	GROUP 042A	GROUP 042B	GROUP 043A	GROUP 043B	GROUP 044A	GROUP 044B
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Enlargement, Diffuse				1		
Hyperplasia, Follicular Cells	1	1	2	2	4	3
Karyomegaly, Follicular Cells					1	

SUMMARY INCIDENCE TABLE

BATT02-00055
Stage 51/21 Day Sacrifice
Xenopus laevis (South African Clawed frog)

	GROUP 045A	GROUP 045B	GROUP 046A	GROUP 046B		
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)		
Enlargement, Diffuse			2			
Hyperplasia, Follicular Cells	3	4	5	3		
Karyomegaly, Follicular Cells	5	2	3	1		

HISTOPATHOLOGY INCIDENCE TABLES

21 Days – Stage 51

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00055
Stage 51/21 Day Sacrifice
Xenopus laevis (South African Claw

	GROUP 042A						GROUP 042B						GROUP 043A						
ANIMAL	51-201	51-203	51-207	51-213	51-219		51-221	51-229	51-230	51-235	51-237		51-243	51-244	51-247	51-252	51-258		
THYROID	X	X	X	X			X	X		X	X				X	X	X		
Enlargement, Diffuse																			
Hyperplasia, Follicular Cells					1				1				1	1					
Karyomegaly, Follicular Cells																			

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00055
Stage 51/21 Day Sacrifice
Xenopus laevis (South African Claw

	GROUP 043B						GROUP 044A						GROUP 044B								
ANIMAL	51-2648	51-2681	51-2712	51-2733	51-2799		51-2833	51-2861	51-2888	51-2892	51-2944		51-3001	51-3036	51-3093	51-3111	51-3144				
THYROID	X			X	X								X			X					
Enlargement, Diffuse		1																			
Hyperplasia, Follicular Cells		1	1				1	1		1	1			1	1		2				
Karyomegaly, Follicular Cells									1												

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00055
Stage 51/21 Day Sacrifice
Xenopus laevis (South African Claw

	GROUP 045A						GROUP 045B						GROUP 046A							
ANIMAL	51-320	51-325	51-327	51-331	51-339		51-341	51-347	51-353	51-356	51-358		51-363	51-366	51-369	51-376	51-379			
THYROID									X											
Enlargement, Diffuse															1		1			
Hyperplasia, Follicular Cells		1		2	1		1	1		1	1		1	1	1	1	1			
Karyomegaly, Follicular Cells	1	1	1	1	1		1				1		1			1	1			

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00055
Stage 51/21 Day Sacrifice
Xenopus laevis (South African Claw

GROUP
046B

ANIMAL	51-382	51-384	51-388	51-393	51-396
THYROID	X				
Enlargement, Diffuse					
Hyperplasia, Follicular Cells		1	1	1	
Karyomegaly, Follicular Cells					1

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

SUMMARY INCIDENCE TABLES

14 Days – Stage 54

SUMMARY INCIDENCE TABLE

BATT02-00055
Stage 54/14 Day Sacrifice
Xenopus laevis (South African Clawed frog)

	GROUP 047C	GROUP 047D	GROUP 048C	GROUP 048D	GROUP 049C	GROUP 049D
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)	(5)	(5)
Colloid, Decreased		1	1			
Enlargement, Diffuse					1	1
Hyperplasia, Follicular Cells	1	4	1	2	3	4
Karyomegaly, Follicular Cells					1	

SUMMARY INCIDENCE TABLE

BATT02-00055
Stage 54/14 Day Sacrifice
Xenopus laevis (South African Clawed frog)

	GROUP 050C	GROUP 050D	GROUP 051C	GROUP 051D		
THYROID (NO. EXAMINED)	(5)	(5)	(5)	(5)		
Colloid, Decreased			1			
Enlargement, Diffuse	2	2		1		
Hyperplasia, Follicular Cells	3	4	4	3		
Karyomegaly, Follicular Cells						

HISTOPATHOLOGY INCIDENCE TABLES

14 Days – Stage 54

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00055
Stage 54/14 Day Sacrifice
Xenopus laevis (South African Claw

	GROUP 047C						GROUP 047D						GROUP 048C						
ANIMAL	54-004	54-008	54-010	54-016	54-018		54-026	54-028	54-030	54-033	54-039		54-046	54-047	54-050	54-052	54-058		
THYROID	X	X	X		X					X			X		X	X			
Colloid, Decreased								1						1					
Enlargement, Diffuse																			
Hyperplasia, Follicular Cells				1			1	1	1		1						1		
Karyomegaly, Follicular Cells																			

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00055
Stage 54/14 Day Sacrifice
Xenopus laevis (South African Claw

	GROUP 048D						GROUP 049C						GROUP 049D							
ANIMAL	54-0688	54-0700	54-0711	54-0755	54-0779		54-0810	54-0844	54-0888	54-0892	54-0907		54-1003	54-1044	54-1066	54-1117	54-1119			
THYROID	X	X			X					X							X			
Colloid, Decreased																				
Enlargement, Diffuse								1						1						
Hyperplasia, Follicular Cells			1	1			1		1		1		1	1	1	1				
Karyomegaly, Follicular Cells											1									

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00055
Stage 54/14 Day Sacrifice
Xenopus laevis (South African Claw

	GROUP 050C						GROUP 050D						GROUP 051C							
ANIMAL	54-123	54-125	54-127	54-137	54-139		54-142	54-144	54-148	54-155	54-159		54-163	54-166	54-169	54-174	54-178			
THYROID		X		X					X								X			
Colloid, Decreased														1						
Enlargement, Diffuse			1		1			1		1										
Hyperplasia, Follicular Cells	1		1		1		1	1		1	1		1	1	1	1				
Karyomegaly, Follicular Cells																				

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

BATT02-00055
Stage 54/14 Day Sacrifice
Xenopus laevis (South African Claw

	GROUP 051D				
	ANIMAL				
	54-1181	54-1187	54-1190	54-1193	54-1195
THYROID					X
Colloid, Decreased					
Enlargement, Diffuse			1		
Hyperplasia, Follicular Cells	1	1		1	
Karyomegaly, Follicular Cells					

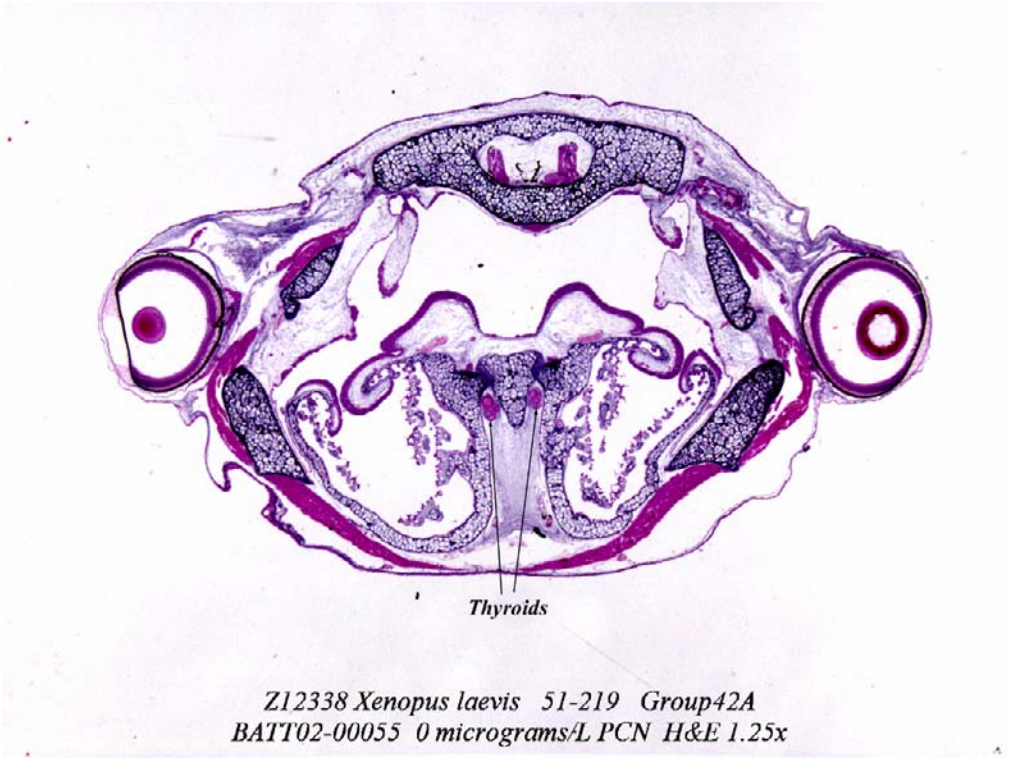
Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

APPENDIX A: PHOTOGRAPHS – STAGE 51





Z12340 and Z12341. Low (1.25x) and high (20x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* control.





Z12338 and Z12339. Low (1.25x) and high (20x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* control. Minimal thyroid follicular cell hyperplasia is characterized by crowding of columnar cells.





Z12342 and Z12343. Low (1.25x) and high (20x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* exposed to 66.7 µg/L PCN for 21 days. Slight/mild thyroid follicular cell hyperplasia. Note the increased folding of the follicular epithelium compared to Z12339.





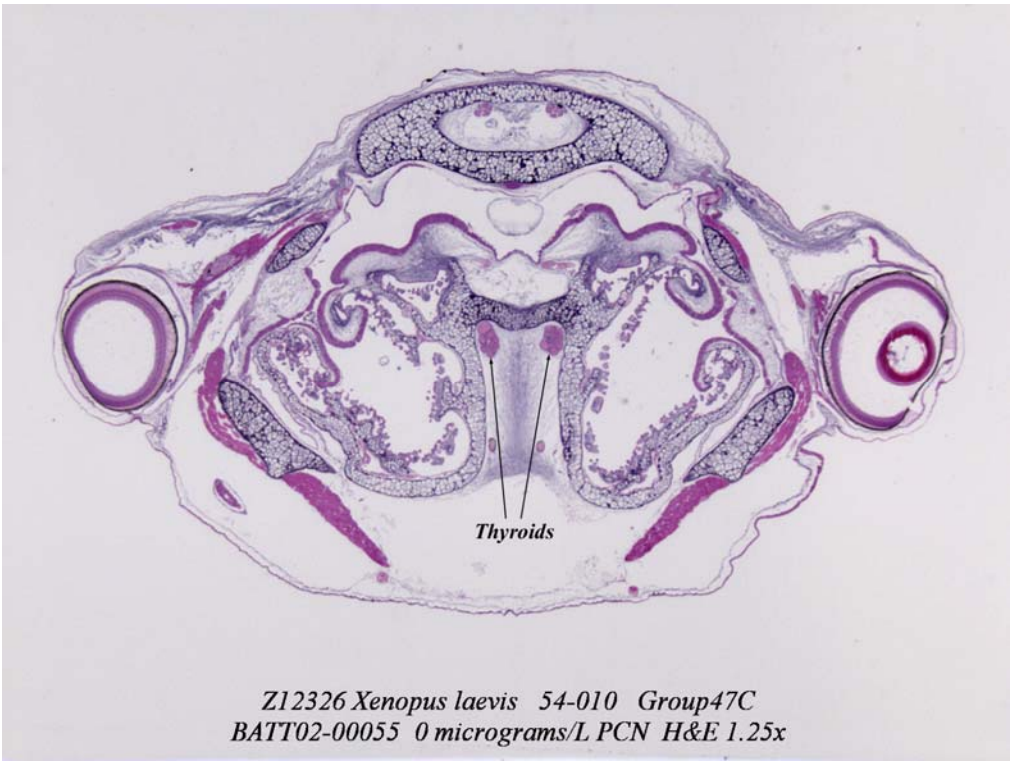
Z12344 and Z12345. Low (1.25x) and high (20x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* exposed to 200 µg/L PCN for 21 days. Minimal karyomegaly is characterized by occasional enlarged nuclei.





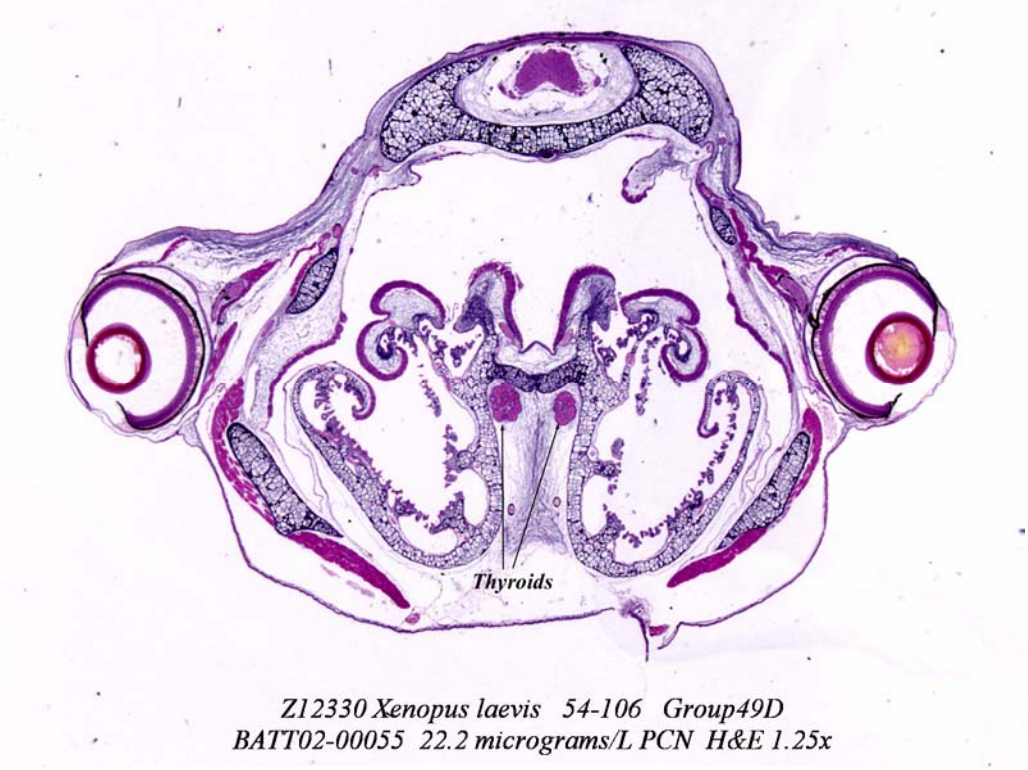
Z12346 and Z12347. Low (1.25x) and high (20x) magnification photomicrographs of thyroid glands from Stage 51 *Xenopus laevis* exposed to 7.4 µg/L PCN for 21 days. Minimal diffuse thyroid enlargement. Compare to size of glands in control.

APPENDIX B: PHOTOGRAPHS – STAGE 54





Z12326 and Z12327. Low (1.25x) and high (20x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* control.



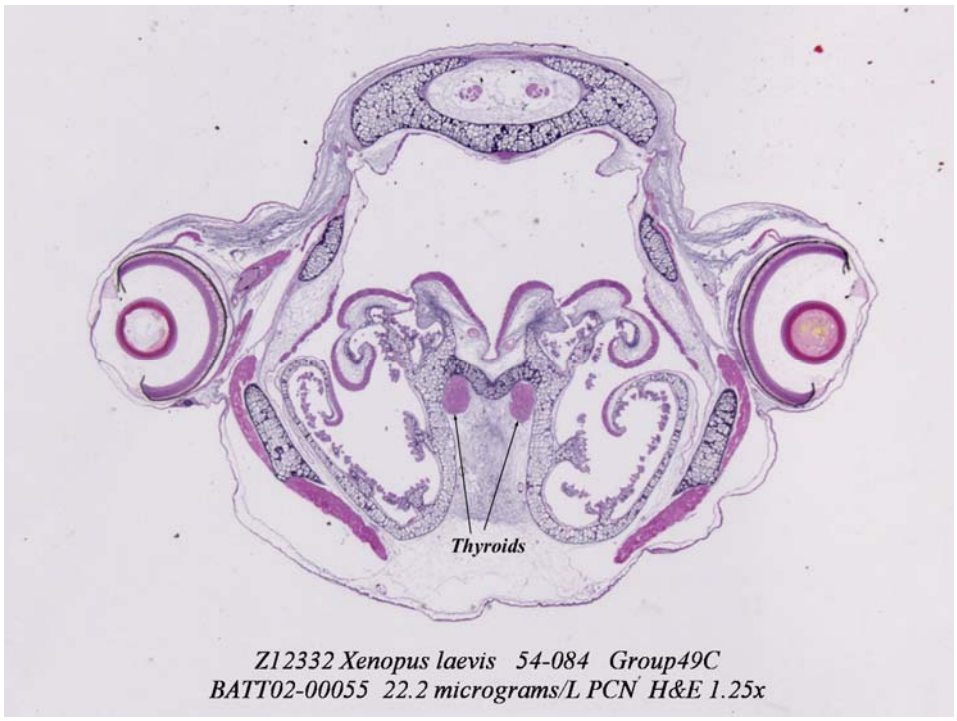


Z12330 and Z12331. Low (1.25x) and high (20x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* exposed to 22.2 µg/L PCN for 14 days. Minimal thyroid follicular cell hyperplasia is characterized by crowding of columnar cells.





Z12334 and Z12335. Low (1.25x) and high (20x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* exposed to 22.2 µg/L PCN for 14 days. Minimal karyomegaly is characterized by occasional enlarged nuclei.





Z12332 and Z12333. Low (1.25x) and high (20x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* exposed to 22.2 µg/L PCN for 14 days. Minimal diffuse thyroid enlargement. Compare to size of glands in control.





Z12328 and Z12329. Low (1.25x) and high (20x) magnification photomicrographs of thyroid glands from Stage 54 *Xenopus laevis* exposed to 7.4 µg/L PCN for 14 days. Minimal decreased colloid. On average, follicles are smaller and contain less colloid.

237-011

PHOTOGRAPH LOG

Verified Photocopy
Signature/Comment Date 3/2/05

		Species	Animal #	Histo #	Tissue/Lesion	Stain	35mm/ digital	Objective		Initial/ Date
		Frog	S4-162	T83492	Thyroid	HE	D	1.25x		kms 2-22-05
	Z12321		S4-162	T83492				10x		kms 2-22-05
	Z12322		S4-210	T83479				1.25x		kms 2-22-05
	Z12323		S4-210	T83479				10x		kms 2-22-05
	Z12324		S4-231	T83484				1.25x		kms 2-22-05
	Z12325		S4-231	T83484				10x		kms 2-22-05
237-011	Z12326		S4-010	T83139				1.25x		kms 2-22-05
	Z12327		S4-010	T83139				20x		kms 2-22-05
	Z12328		S4-047	T83178				1.25x		kms 2-22-05
	Z12329		S4-047	T83178				20x		kms 2-22-05
	Z12330		S4-106	T83174				1.25x		kms 2-22-05
	Z12331		S4-106	T83174				20x		kms 2-22-05
	Z12332		S4-084	T83166				1.25x		kms 2-23-05
	Z12333		S4-084	T83168				20x		kms 2-23-05
	Z12334		S4-047	T83171				1.25x		kms 2-23-05
	Z12335		S4-097	T83171				20x		kms 2-23-05
	Z12336		S1-314	T83126				1.25x		kms 2-23-05
	Z12337		S1-314	T83126				20x		kms 2-23-05
	Z12338		S1-219	T83091				1.25x		kms 2-23-05
	Z12339		S1-219	T83091				20x		kms 2-23-05
	Z12340		S1-221	T83092				1.25x		kms 2-23-05
	Z12341		S1-221	T83092				20x		kms 2-23-05
	Z12342		S1-331	T83110				1.25x		kms 2-23-05
	Z12343		S1-331	T83110				20x		kms 2-23-05
	Z12344		S1-396	T83106				1.25x		kms 2-23-05
	Z12345		S1-396	T83106				20x		kms 2-23-05
	Z12346		S1-268	T83133				1.25x		kms 2-23-05
	Z12347		S1-268	T83133				20x		kms 2-23-05

(a) RE kms 2-23-05
(b) RE kms 2-23-05

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

FORT ENVIRONMENTAL LABORATORIES
1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

Client:	Battelle
Submit Report To:	Dana Fort
Sampler's Signature:	Dana Fort
P.O. #:	

BAT02-00055

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
042	Specimen #s: 201, 203, 207,	11/30/04 1000	10	G	10% Formalin	Histological examination of Thyroid Gland ~~as per protocol # BAT02-1~~ ①
↓	213, 219, 221, 229, 230,	↓ ↓			↓	
↓	235, 237					
043	Specimen #s: 243, 244, 247,		10	G		
↓	252, 258, 264, 268,					
↓	271, 273, 279					
044	Specimen #s: 283, 286, 288,		10	G		
↓	292, 294, 301, 306, 309,					
↓	311, 314					
045	Specimen #s: 320, 325,		10	G		
↓	327, 331, 339, 341, 347,					
↓	353, 356, 358					
046	Specimen #s: 363, 366,		10	G		
↓	369, 376, 379, 382,					
↓	384, 388, 393, 396	↓ ↓			↓	

SPECIAL INSTRUCTIONS: Organisms exposed to PCN

DATE:
INITIALS:

Andrea Walker	12/2/04 1000	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C

① N/A - JT - 12/2/04

CHAIN OF CUSTODY RECORD

PLEASE COMPLETE THIS FORM AS THOROUGHLY AS POSSIBLE, USING BLACK INK

Client:	Battelle
Submit Report To:	Doug Fort
Sampler's Signature:	
P.O. #:	

FORT ENVIRONMENTAL LABORATORIES

1414 South Sangre Road
Stillwater, OK 74074
405-624-6771 Fax 405-377-3388
mail@fortlabs.com

BATT02-00055

Sample #	Sample Location	Sample Collected Date-Time	# of Cont.	Cont. Type P/G	Type of Preserv.	Analysis Requested
047	Specimen #s: 004, 008,	11/30/04 1000	10	G	10% Formalin	Histological examination of Thyroid Gland ~~as per Protocol # BATT02~~ (1)
↓	010, 016, 018, 026,	↓ ↓			↓	
↓	028, 030, 033, 039					
048	Specimen #s: 046, 047,		10	G		
↓	050, 052, 058, 068,					
↓	070, 071 075, 079					
049	Specimen #s: 081, 084,		10	G		
↓	088, 092, 097, 103,					
↓	104, 106, 117, 119					
050	Specimen #s: 123, 125,		10	G		
↓	127, 137, 139, 142,					
↓	144, 148, 155, 159					
051	Specimen #s: 163, 166,		10	G		
↓	169, 174, 178, 181,					
↓	187, 190, 193, 195	↓ ↓			↓	

DATE:
INITIALS:

SPECIAL INSTRUCTIONS: ORGANISMS EXPOSED TO PCN.

Anara Walker	12/2/04 1000	

*UNLESS OTHERWISE NOTED, ALL SAMPLES ARE STORED AT 4° C.

(1) N/A - JT - 12/2/04