FY2001 Final Report

Ecological Risk Assessment of Perchlorate In Avian Species, Rodents, Amphibians and Fish

SERDP Project ER-1235

April 2003

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FINAL REPORT FY2001
SERDP Project: ER-1235

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A FINAL REPORT

ENTITLED

LETHAL CONCENTRATION DETERMINATION OF AMMONIUM PERCHLORATE ON *RANA* SPECIES EMBRYOS

STUDY NUMBER:	AMPH-02-01
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	Department of Biological Sciences –**AP** Texas Tech University Box 4-3131 Lubbock, Texas 79409-3131
TEST SITE:	Department of Biological Sciences –**AP** Texas Tech University Box 4-3131 Lubbock, Texas 79409-3131
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	July 17, 2002
RESEARCH COMPLETION:	October 2, 2002

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GOOD LABORATORIES PRACTICES STATEMENT

Project AMPH-02-01, entitled "Lethal concentration determination of ammonium perchlorate on *Rana* species embryos", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

James A. Carr, Ph.D

3/27/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	7-18-02	7-19-02	7-19-02	7-25-02
Test Material Application	7-23-02	7-23-02	7-25-02	7-30-02
Test System Care and Application	7-23-02	7-23-02	7-25-02	7-30-02
Test Material mixing	7-23-02	7-23-02	7-25-02	7-30-02
Dosing	7-23-02	7-23-02	7-25-02	7-30-02
Final Report and Raw Data Review	2-24-03	3-07-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

Date 3/28/03

1.0 DESCRIPTIVE STUDY TITLE:

Lethal concentration determination of ammonium perchlorate on *Rana* species embryos.

2.0 STUDY NUMBER: AMPH-02-01

3.0 SPONSOR:

United States Air Force United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4.0 TESTING FACILITY NAME & ADDRESS:

Department of Biological Sciences –**AP**
Texas Tech University
Box 4-3131
Lubbock, Texas 79409-3131

5.0 EXPERIMENTAL START & TERMINATION DATES:

Start Date: July 17, 2002
Termination Date: October 2, 2002

6.0 KEY PERSONNEL:

James A. Carr, Co-Principle Investigator, Testing Facility Management
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist/ Assistant Director of Science
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principle Investigator

7.0 STUDY OBJECTIVES / PURPOSE:

To determine the lethal concentration of ammonium perchlorate (AP) on *Rana* species embryos.

8.0 STUDY SUMMARY

Embryonic *R. utriculata* were exposed to a range of nine concentrations of ammonium perchlorate or 0.5x magnesium Holtfreter's solution for 7 d beginning prior to hatching. Most mortality was observed within 4 days after exposure and was due in large part to reduced hatching success. The 4 d and 7 d LC_{50}s were 15.6 ppm and 3.12 ppm, respectively; extremely low compared to *Xenopus laevis* ($LC_{50} = 510 \pm 36$ ppm). No treatment-related incidences of edema bent tails, or abnormal swimming behaviors in viable hatchlings exposed to AP were observed..

9.0 TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate (AP)
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.
Source: Aldrich Chemical Company

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: 0.5x Magnesium Holtfreter's Solution, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations: NaCl, 1.75 g/L; $NaHCO_3$, 0.1 g/L; KCl, 0.025 g/L; $CaCl_2 \cdot 2\ H_2O$, 0.65 g/L; $MgSO_4 \cdot 7H_2O$, 0.1 g/L (http://www.indiana.edu/~axolotl).

10.0 JUSTIFICATION OF TEST SYSTEM

Perchlorate occurs in ground and surface waters in 44 states in the USA, primarily as a result of AP discharge from rocket fuel manufacturing facilities or from the demilitarization of missiles (Urbansky, 1998). AP is highly water-soluble and, as a result of the very slow reduction of the central chlorine atom, can persist in the environment for decades (Urbansky, 1998). Perchlorate is known to prevent intake of iodine from water or food and thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Ionic perchlorate also alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Calcium is a ubiquitous chemical messenger that is involved in the regulation of cellular function. Endocrine glands require calcium for the normal secretion of hormones and therefore contaminant-induced disruption of calcium balance can lead to systemic endocrine disruption. Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health.

We have previously examined aspects of growth and development and thyroid function in anuran larvae collected from AP-contaminated sites at the Longhorn Army Ammunition Plant (LHAAP) located in Karnack, Texas, and identified two possible cases of thyroid disruption. Bullfrog larvae collected from an AP-contaminated pond exhibited decreased hindlimb growth than larvae from a reference pond, even though the animals from both sites were of identical body length, and presumably, identical age class. Additionally, chorus frog larvae collected from another AP-contaminated site at LHAAP presented evidence of thyroid follicle hypertrophy and colloid depletion, both indicators of thyroid disruption.

In previous studies with *Xenopus laevis* we found 5-d and 70-d LC_{50}s for AP to be 510 $\pm$ 36 mg/L and 223 $\pm$ 13 mg/L, respectively (Goleman et al., 2002). While AP did not cause any concentration-related developmental abnormalities at concentrations below the 70-d LC_{50}, it did inhibit, in a concentration-dependent manner, several thyroid-hormone-dependent aspects of growth and metamorphosis in *X. laevis* larvae including hindlimb growth, forelimb emergence, and tail resorption. Although *Xenopus* is a widely used animal model in basic toxicological, developmental, and reproductive research, it is not native to North America. The effects of perchlorate on thyroid hormone-sensitive indices in native amphibian species throughout metamorphosis have yet to be determined.

11.0 TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: *Rana utriculata* (Southern leopard frog)
Strain: wild type
Age: embryos and larvae
Number: Approximately 2000
Source: Carolina Biological Supply and Charles Sullivan Company

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:
Each test beaker was labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:
Approximately fifty embryos/ larvae were exposed to ten conditions, nine concentrations of AP in 0.5x magnesium Holtfreter's solution (10^{-5}, 10^{-4}, 10^{-3}, $3x10^{-3}$, $5x10^{-3}$, 10^{-2}, $3x10^{-2}$, $5x10^{-2}$, 10^{-1} M) and 0.5x magnesium Holtfreter's solution alone for 7 d. Each treatment was performed in duplicate. Exposures were terminated on day 7. This gave 100 embryos/larvae per treatment, for a study total of 2000 animals.

14.0 METHODS:

14.1 Test System Acquisition, Quarantine, Acclimation
For each trial, *Rana* embryos were obtained from Carolina Biological Supply or Charles Sullivan Company. Embryos and larvae were maintained as stated in DBS SOP ET-1-02.

14.2 Test Condition Establishment
Naturally fertilized embryos were used. They were obtained from Carolina Biological Supply or Charles Sullivan Company. Embryos from 3 clutches were counted into 10 groups of approximately 50. Each group of 50 embryos was added to 250 mL glass beakers containing 50 mL of 0.5x magnesium Holtfreter's solution. Pre-mixed 2x test concentrations of AP or 0.5x magnesium Holtfreter's solution was then added to each beaker to bring the final volume to 100 mL of the appropriate concentration. Each beaker was labeled as indicated in section 5.5 of DBS SOP ET-1-02, which includes genus and species name, common name, project name, number, and start date, date embryos hatched, date of initial exposure, treatment, and the name of the person responsible for animal care.

14.3 Test Material Application
Test material was pre-mixed to 2x concentrations and added to the appropriate beaker containing 0.5x magnesium Holtfreter's solution. Beakers were labeled appropriately (see section 14.2). Embryos were added to all beakers prior to test material application.

Rates/concentrations: 0, 10^{-5}, 10^{-4}, 10^{-3}, $3x10^{-3}$, $5x10^{-3}$, 10^{-2}, $3x10^{-2}$, $5x10^{-2}$, 10^{-1} M.
0, 1, 12, 118, 353, 588, 1175, 3525, 5875, 11750 ppm.

Frequency: Constant exposure for 7 days.

Route/Method of Application: Embryos and larvae were exposed to AP in the beaker medium. Embryos/ larvae were maintained in 100 mL of the test solution in 250 mL beakers. Room temperature was maintained at an average of 21.5° C with a photoperiod of 12 h light: 12 h dark. All beakers were covered with clear plastic wrap and maintained in a water bath with an average temperature of 20.4° C. Method of application was immersion. Route of exposure was via dermal, oral, and respiratory exposure as the chemical was in the beaker medium.

Justification for Exposure Route: *R. utriculata* are fully aquatic as larvae and semi-aquatic as adults.

Exposure Verification: Samples of pre-mixed test and reference solutions were analyzed for perchlorate content according to the guidelines set forth in SOPs AC-2-11 and AC-1-01. At the end of the study all remaining larvae were euthanized and frozen for possible contaminant analysis at a later date.

14.4 Test System Observation

Beginning on the day of hatch, hatching success (# unhatched embryos/total # embryos), % deformities (# showing bent tails, asymmetric tails/total hatched), edema (% showing distention of body with fluid/total hatched), and abnormal swimming (% showing abnormal swimming/total) were noted daily for each test and reference solution. For free-swimming larvae, % mortality (#dead larvae/#hatched), percent showing deformities, and percent displaying abnormal swimming behavior were noted every day. Dead animals were removed daily and preserved in 10% neutral buffered formalin.

Water quality parameters (temperature, pH, specific conductivity, salinity, dissolved oxygen, and ammonia) were analyzed on day 0 and day7 of exposure. Ammonia levels were re-analyzed at a later date due to precipitate in original test concentrations greater than 1175 ppm AP (Table 1).

14.5 Animal Sacrifice and Sample Collections

The exposure was terminated after 7 d. Remaining larvae were euthanized by immersion in MS-222 (3-aminobenzoic acid ethyl ester, 0.1% solution) according to DBS AF-3-03 and frozen for possible analysis of perchlorate content at a later date.

14.6 Endpoint Analysis

Hatching success, deformities (bent tails, asymmetric tails), edema (distention of body with fluid), and abnormal swimming were noted for hatchlings. Percent mortality (#dead/#hatched), deformities, and abnormal swimming behavior were recorded for larvae.

15.0 STATISTICAL METHODS:

LC_{50}s were calculated by the Probit method with SoftTox™ (ChemSW® Software for Windows,

2001). Percent hatch, mortality, bent tails, edema, and abnormal swimming were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test.

16.0 PROTOCOL CHANGES / REVISIONS:

See attached change in study documentation forms.

17.0 RESULTS:

A total of two trials were conducted between July and October 2002. Because of high mortality in the initial trial, only data from the second trial is presented here. Exposure to AP resulted in a significant decrease in hatching compared to control animals, one-way ANOVA ($F = 73.993$, $p < 0.0001$) followed by Tukey-Kramer multiple comparisons test. As shown in Table 2, AP concentrations greater than 118 ppm were found to be 100% lethal to embryos, with the exception of 1 hatchling observed at 1175ppm. Post-hatch mortality was not found to be significantly different (one-way ANOVA, $F = 0.3628$, $p = 0.8263$) from controls. The 7 d mortality of embryos and hatchlings (Figure 1) ranged from 50% in 0.5x magnesium Holtfreter's solution to 87% in 118 ppm AP. The 4 d (hatching) and 7 d LC_{50}s for AP in *R. utriculata* were 15.6 ppm and 3.12 ppm, respectively, based on measured perchlorate concentrations. The incidences of bent tails, abnormal swimming behavior, and edema in larvae exposed to 1-118 ppm AP were not significantly different from controls with ranges of 71-90%, 55-81%, and 2%, respectively (Table 3).

Table 1. Water Quality Parameters Analyzed in Test Solutions on 09/25/02.

Nominal AP (ppm)	Temperature (° C)	pH	Specific Conductivity (µS/cm)	Salinity (ppt)	Dissolved Oxygen (mg/L)	Non-Ionized Ammonia* (mg/L)
11750	21.4	7.2	15270	8.9	6.82	6.0259
5875	21.4	7.4	9360	5.3	7.07	2.5837
3525	21.4	7.5	7200	4.0	6.52	1.9009
1175	21.4	7.8	4946	2.7	7.00	0.6999
588	21.4	7.9	4263	2.3	6.71	0.8676
353	21.4	8.0	4101	2.2	6.38	0.6558
118	21.3	8.0	3836	2.0	6.60	0.2533
12	21.3	8.1	3712	2.0	6.96	0.0407
1	21.5	8.0	3749	2.0	6.19	0.0115
0	21.5	7.6	3717	2.0	7.08	0.0000

Measured on exposure day 0.
*Like solutions re-analyzed on 01/04/03, with average temperature of 18.7° C and pH of 7.4.

Table 2. Percent Hatching and Mortality in Larval *R. utriculata* Exposed to Ammonium Perchlorate for 7-d during Trial 2.

Nominal (M)	Nominal (ppm)	Perchlorate[a] (ppm)	Ammonia[b] (ppm)	N	%Hatch[c]	%Mortality[d]
1E –1	11750	16379	6.0259	100	0.0	NA
5E –2	5875	821	2.5837	100	0.0	NA
3E –2	3525	5781	1.9009	100	0.0	NA
1E –2	1175	1915	0.6999	100	1.0	100
5E –3	588	563	0.6876	100	0.0	NA
3E –3	353	403	0.6558	100	0.0	NA
1E –3	118	159	0.2533	100	44.0	70.5
1E –4	12	32	0.0407	100	42.0	38.1
1E –5	1	3	0.0115	100	55.0	23.6
Holtfreter's[e]	0	0.1	0.0000	100	86.0	41.9

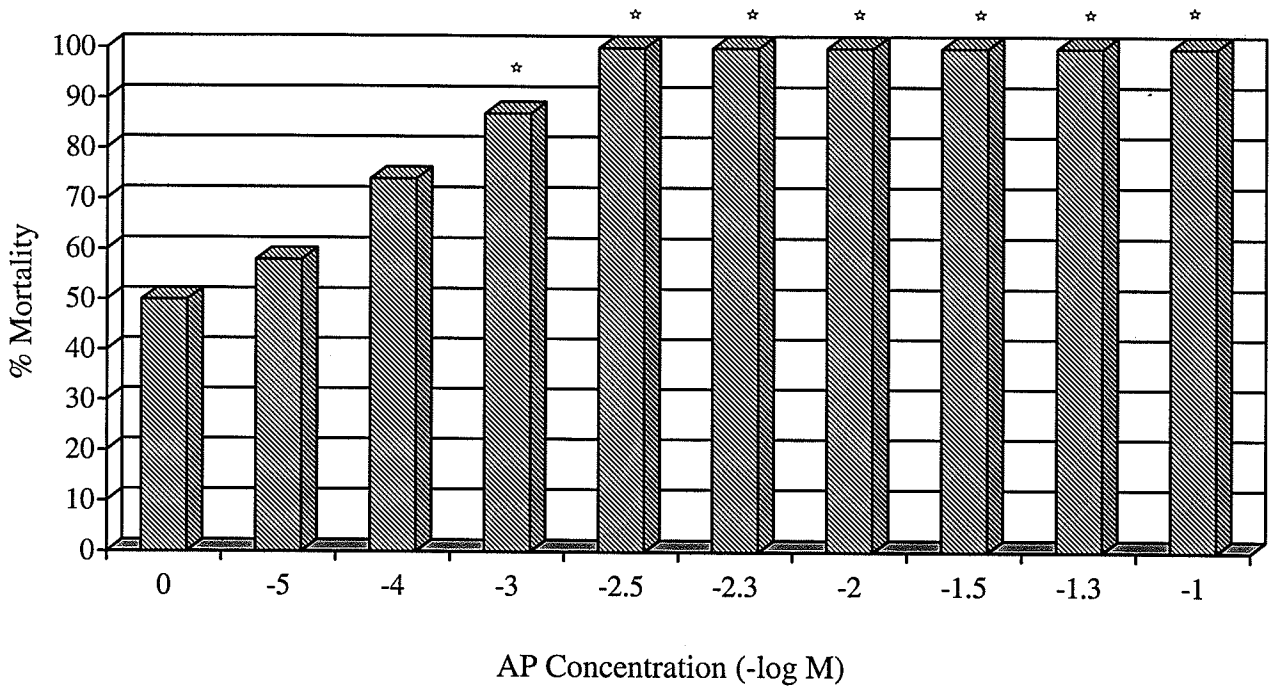
[a]Measured from 2x solutions used for initial exposures, diluted to 1x, on 9/25/02. [b]Measured as ammonia nitrogen on 9/25/02, corrected to un-ionized ammonia. [c]Calculated as a percent of total embryos. [d]Calculated as a percent of total hatchlings. [e]0.5x Magnesium Holtfreter's Solution.

Table 3. Developmental Abnormalities in Larval *R. utriculata* Exposed to Ammonium Perchlorate for 7-d during Trial 2.

Nominal (M)	Nominal (ppm)	N	Bent tails (%)	Edema (%)	Abnormal Swimming (%)
1E –1	11750	100	NA	NA	NA
5E –2	5875	100	NA	NA	NA
3E –2	3525	100	NA	NA	NA
1E –2	1175	100	NA	NA	NA
5E –3	588	100	NA	NA	NA
3E –3	353	100	NA	NA	NA
1E –3	118	100	84.1	0	54.6
1E –4	12	100	81.0	2.4	81.0
1E –5	1	100	70.9	0	67.3
Holtfreter's[a]	0	100	89.5	0	65.1

[a]0.5x Magnesium Holtfreter's solution

Figure 1. Total Mortality in *Rana utriculata* Embryos and Hatchlings After 7 Day AP Exposure.



* Significantly different from controls; one-way ANOVA ($F = 11.744$, $p = 0.0003$) followed by Tukey-Kramer multiple comparisons test.

18.0 DISCUSSION

Work in our laboratory (Goleman et al., 2002) determined a LC_{50} value of 510 ± 36 ppm in *Xenopus laevis* larvae exposed to AP for 5 d. In the current study the median lethal concentration was calculated for hatching success (4 d) and for total mortality (7 d). We found the 4 d (hatching) and 7 d (total) LC_{50}s for *Rana utriculata* to be 15.6 ppm and 3.12 ppm, respectively. Most mortality was observed within 4 days after exposure of embryos and was due to reduced hatching success. As of exposure day 3 there were no surviving embryos in concentrations greater than 118 ppm. A single animal did hatch at the 1175 ppm AP concentration on exposure day 2, however, this animal had already expired when daily observations were performed.

Our findings suggest a high incidence of bent tails and abnormal swimming, however, these findings may be, at least in part, due to the lack of experience with *R. utriculata* larvae. In *Xenopus*, the axial skeleton straightens very soon after hatching. Those that do not straighten exhibit abnormal swimming patterns, such as lying on the container floor, floating at the solution surface, or swimming in circles, and generally do not survive. A long-term study (AMPH-02-02) revealed that the apparent deformity in the axial skeleton resulting in bent tails may actually be a normal phase of larval development in *R. utriculata*. Likewise, the observer may have also over estimated abnormal swimming behavior, which generally follows the same trend as bent tails.

19.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20.0 REFERENCES:

Goleman, W.L., Urquidi, L.J, Anderson, T.A., Smith, E.E., Kendall, R.J., Carr, J.A. (2002). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environ. Toxicol. Chem. 21: 424-430.

Luttgau, H.C., Gottschalk, G., Kovacs, L., Fuxreiter, M. (1983). How perchlorate improves excitation-contraction coupling in skeletal muscle fibers Biophys. J. 43: 247-249.

Jong, D.S., Stroffekova, K., Heiny, J.A. (1997). A surface potential change in the membranes of frog skeletal muscle is associated with excitation-contraction coupling. J. Physiol.499: 787-808.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. Biocell 20: 147-153.

Thevenod, F. and Jones, S.W. (1992). Cadmium block of calcium current in frog sympathetic neurons. Biophys. J. 63: 162-168.

Urbansky, ET. (1998). Perchlorate chemistry: implications for analysis and remediation. Bioremediation J. 2: 81-95.

21.0 APPENDICES:

Study Protocol
Changes to Study Documentation
List of Standard Operating Procedures

A STUDY PROTOCOL

ENTITLED

LETHAL CONCENTRATION DETERMINATION OF AMMONIUM PERCHLORATE ON *RANA* SPECIES EMBRYOS

STUDY NUMBER: AMPH-02-01

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY

Name/Address: The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. Ronald J. Kendall

Study Director: Wanda L. Goleman

PROPOSED EXPERIMENTAL
START DATE JULY 17, 2002

1. **DESCRIPTIVE STUDY TITLE:** Lethal concentration determination of ammonium perchlorate on *Rana* species embryos.

2. **STUDY NUMBER:** AMPH-02-01

3. **SPONSOR:** United States Air Force United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: July 17, 2002
Termination Date: October 31, 2002

6. **KEY PERSONNEL:**
James A. Carr, Principle Investigator
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist/ Assistant Director of Science
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Testing Facility Management

7. DATED SIGNATURES:

Wanda L. Goleman	7/17/02	Ms. Wanda L. Goleman Study Director
James A. Carr	7/19/02	Dr. James A. Carr Principle Investigator
Ryan Bounds	7/18/02	Mr. Ryan Bounds Quality Assurance Manager
Todd Anderson	7-24-02	Dr. Todd Anderson Analytical Chemist/ Assistant Director of Science
Ronald J. Kendall	7-25-02	Dr. Ron Kendall Testing Facility Management

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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Lubbock, Texas 79409

9. STUDY OBJECTIVES / PURPOSE:

To determine the lethal concentration of ammonium perchlorate (AP) on *Rana* species embryos.

10. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number : 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.
Source: Aldrich Chemical Company

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Dawson and Bantle, 1987): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

11. JUSTIFICATION OF TEST SYSTEM

Perchlorate occurs in ground and surface waters in 44 states in the USA, primarily as a result of AP discharge from rocket fuel manufacturing facilities or from the demilitarization of missiles (Urbansky, 1998). AP is highly water-soluble and, as a result of the very slow reduction of the central chlorine atom, can persist in the environment for decades (Urbansky, 1998).

Perchlorate is known to prevent intake of iodine from water or food and thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Ionic perchlorate also alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Calcium is a ubiquitous chemical messenger that is involved in the regulation of cellular function. Endocrine glands require calcium for the normal secretion of hormones and therefore contaminant-induced disruption of calcium balance can lead to systemic endocrine disruption. Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health.

We have previously examined aspects of growth and development and thyroid function in anuran larvae collected from AP-contaminated sites at the Longhorn Army Ammunition Plant (LHAAP) located in Karnack, Texas, and identified two possible cases of thyroid disruption. Bullfrog larvae collected from an AP-contaminated pond exhibited decreased hindlimb growth than larvae from a reference pond, even though the animals from both sites were of identical body length, and presumably, identical age class. Additionally, chorus frog larvae collected from another AP-contaminated site at LHAAP

presented evidence of thyroid follicle hypertrophy and colloid depletion, both indicators of thyroid disruption.

In previous studies with *Xenopus laevis* we found 5-d and 70-d LC_{50}s for AP to be 510 ± 36 mg/L and 223 ± 13 mg/L, respectively (Goleman et al., 2002). While AP did not cause any concentration-related developmental abnormalities at concentrations below the 70-d LC_{50}, it did inhibit, in a concentration-dependent manner, several thyroid-hormone-dependent aspects of growth and metamorphosis in *X. laevis* larvae including hindlimb growth, forelimb emergence, and tail resorption. Although *Xenopus* is a widely used animal model in basic toxicological, developmental, and reproductive research, it is not native to North America. The effects of perchlorate on thyroid hormone-sensitive indices in native amphibian species throughout metamorphosis have yet to be determined.

12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: *Rana pipiens* (Northern leopard frog) or *R. utriculata* (Southern leopard frog)
Strain: wild type
Age: embryos and larvae
Number: Approximately 1000
Source: Carolina Biological Supply or Charles Sullivan Company

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:
Each test beaker will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:
Approximately fifty embryos/ larvae will be exposed to ten conditions, nine concentrations of AP in FETAX medium (10^{-5}, 10^{-4}, 10^{-3}, $3x10^{-3}$, $5x10^{-3}$, 10^{-2}, $3x10^{-2}$, $5x10^{-2}$, 10^{-1} M) and FETAX medium alone for 7 d. Each treatment will be performed in duplicate. Exposures will be terminated on day 7. This will give approximately 100 embryos/larvae per treatment, for a study total of approximately 1000 animals.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation
Rana embryos will be obtained from Carolina Biological Supply or Charles Sullivan Company. Embryos and larvae will be maintained as stated in SOP AQ-1-14.

15.2 Test condition establishment
Naturally fertilized embryos will be used. They will be obtained from Carolina Biological Supply or Charles Sullivan Company. Embryos will be counted into 10 groups of

approximately 50 (if more than one egg mass is obtained, embryos will be divided equally for a total of approximately 50 per beaker). Each group of 50 embryos will be added to 250 mL glass beakers containing 50 mL of FETAX solution. Pre-mixed 2x test concentrations of AP or 1x FETAX solution will then be added to each beaker to the final volume to 100 mL of the appropriate concentration. Each beaker will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

15.3 Test Material Application

Test material will be pre-mixed to appropriate 2x concentrations and added to the appropriate beaker. Beakers will be labeled appropriately (see section 15.2). Embryos will be added to all beakers prior to test material application.

Rates/concentrations: 0, 10^{-5}, 10^{-4}, 10^{-3}, $3x10^{-3}$, $5x10^{-3}$, 10^{-2}, $3x10^{-2}$, $5x10^{-2}$, 10^{-1} M.

Frequency: Constant exposure for 7 days.

Route/Method of Application: Embryos and larvae will be exposed to AP in the beaker medium. Embryos/ larvae will be maintained in 100 mL of the test solution in 250 mL beakers. Room temperature will be maintained at 22 ± 2° C with a photoperiod of 12 h light: 12 h dark. Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the tank medium.

Justification for Exposure Route: *R. pipiens* and *R. utriculata* are fully aquatic as larvae and semi-aquatic as adults.

Exposure Verification: Samples of pre-mixed test and reference solutions will be analyzed for perchlorate content. On day 7 all remaining larvae will be euthanized and frozen for possible contaminant analysis at a later date.

15.4 Test System Observation

Beginning on the day of hatch, hatching success (# unhatched embryos/total # embryos), % deformities (# showing bent tails, asymmetric tails/total hatched), edema (% showing distention of body with fluid/total hatched), and abnormal swimming (% showing abnormal swimming/total) will be noted daily for each test and reference solution. For free-swimming larvae, % mortality (#dead larvae/#hatched), percent showing deformities, and percent displaying abnormal swimming behavior will be noted every day. Dead animals will be removed daily and preserved in 10% formalin.

15.5 Animal Sacrifice and Sample Collections

The exposure will be terminated after 7 d. Remaining larvae will be euthanized by immersion in MS-222 (3-aminobenzoic acid ethyl ester, 0.1% solution) according to AQ-1-03 and frozen for possible analysis of perchlorate content at a later date.

15.6 Endpoint Analysis

Hatching success, deformities (bent tails, asymmetric tails), edema (distention of body with fluid), and abnormal swimming will be noted for hatchlings. Percent mortality (#dead/#hatched), deformities, and abnormal swimming behavior will be recorded for larvae.

16. PROPOSED STATISTICAL METHODS

Four-parameter logistic function will be used to determine the actual lethal concentration percentages.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include:

- Room and water temperature, dissolved oxygen, conductivity, salinity, pH, and ammonia will be collected.
- Number of expired larvae removed prior to termination of exposure will be recorded, including date and beaker.
- Deformities and abnormal swimming behavior will be recorded daily prior to termination of the experiment.

Report content will also include presentation of data, interpretation, and discussion of the following end-points:

- LC_{50} and concentration-response curve
- Discussion of the relevance of the findings
- List of all SOPs used.
- List of all personnel.

18. RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before December 31, 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be archived by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

Dawson, D.A. and Bantle, J. A. 1987. Development of a reconstituted water medium and preliminary validation of the frog embryo teratogenesis assay – *Xenopus* (FETAX). J. Appl. Toxicol.7: 237-244.

Goleman, W.L., Urquidi, L.J, Anderson, T.A., Smith, E.E., Kendall, R.J., Carr, J.A. (2002). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environ. Toxicol. Chem. 21: 424-430.

Luttgau, H.C., Gottschalk, G., Kovacs, L., Fuxreiter, M. (1983). How perchlorate improves excitation-contraction coupling in skeletal muscle fibers Biophys. J. 43: 247-249

Jong, D.S., Stroffekova, K., Heiny, J.A. (1997). A surface potential change in the membranes of frog skeletal muscle is associated with excitation-contraction coupling. J. Physiol.499: 787-808.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. Biocell 20: 147-153.

Thevenod, F. and Jones, S.W. (1992). Cadmium block of calcium current in frog sympathetic neurons. Biophys. J. 63: 162-168.

Urbansky, ET. (1998). Perchlorate chemistry: implications for analysis and remediation. Bioremediation J. 2: 81-95.

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Box 43131
Lubbock, TX 79409-3131
(806) 742-2715

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information

Check One: X Protocol ____ SOP ____ Other _____

Title: Lethal concentration determination of ammonium perchlorate on *Rana* species embryos.
Dated: July 17, 2002
Document # (if appropriate): AMPH-02-01
Page #(s): 2, 4, 5
Section #: 4, 6, 10, 14, 15.2

Text to reference:

Section 4. TESTING FACILITY NAME & ADDRESS:

The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Section 6. KEY PERSONNEL:

James A. Carr, Principle Investigator
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist/ Assistant Director of Science
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Testing Facility Management

Section 10. TEST MATERIALS:

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Dawson and Bantle, 1987): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

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$3x10^{-2}$, $5x10^{-2}$, 10^{-1} M) and FETAX medium alone for 7 d. Each treatment will be performed in duplicate. Exposures will be terminated on day 7. This will give approximately 100 embryos/larvae per treatment, for a study total of approximately 1000 animals.

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Naturally fertilized embryos will be used. They will be obtained from Carolina Biological Supply or Charles Sullivan Company. Embryos will be counted into 10 groups of approximately 50 (if more than one egg mass is obtained, embryos will be divided equally for a total of approximately 50 per beaker). Each group of 50 embryos will be added to 250 mL glass beakers containing 50 mL of FETAX solution. Pre-mixed 2x test concentrations of AP or 1x FETAX solution will then be added to each beaker to the final volume to 100 mL of the appropriate concentration. Each beaker will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

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Section 4. TESTING FACILITY NAME & ADDRESS:

Department of Biological Sciences –**AP**
Texas Tech University
Box 4-3131
Lubbock, Texas 79409-3131

Section 6. KEY PERSONNEL:

Ronald Kendall, Principle Investigator
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist/ Assistant Director of Science
Ryan Bounds, Quality Assurance Manager
James Carr, Testing Facility Management, Co-Principle Investigator

Section 10. TEST MATERIALS:

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: 0.5x Magnesium Holtfreter's Solution, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations: NaCl, 1.75 g/L; $NaHCO_3$, 0.1 g/L; KCl, 0.025 g/L; $CaCl_2 \cdot 2\ H_2O$, 0.65 g/L; $MgSO_4 \cdot 7H_2O$, 0.1 g/L (http://www.indiana.edu/~axolotl).

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Justification and Impact on Study:

Section 4. Study will be conducted in the Fisheries and Wildlife Research Building (Room 108) on the main Texas Tech University campus.

Section 6. Due to the location change Dr. James Carr will be the Testing Facility Manager/ Co-Principle Investigator with Dr. Ronald Kendall as Principle Investigator.

Section 10., 14., 15.2. 0.5x magnesium Holtfreter's solution will be the reference solution as this solution is may be more suited for raising *Rana* larvae.

Submitted by: Signature: Randal L. Coleman Date: 9/9/02

Authorized by: Study Director: Randal L. Coleman Date: 9/9/02

Received by: Quality Assurance Unit: Brian Bidwell Date: 9/12/02

* Sequentially numbered in order of the date that the change is effective

Dept. of Biological Sciences (DBS)
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Form No. 014 Rev 3. 06/00
Project No.: T9700.2
*Change No: 2
Page: 1 of 1

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Dated: July 17, 2002
Document # (if appropriate): AMPH-02-01
Page #(s): 7
Section #: 18

Text to reference:

Section 18. RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before December 31, 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be archived by the testing facility.

Change in Document:

Section 18. RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before February 28, 2003. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be archived by the testing facility.

Justification and Impact on Study:

Section 18. This change will allow more time for completion of the final report and will have no impact on the study itself.

Submitted by: Signature: Wanda L. Goleman Date: 1/29/03

Authorized by: Study Director: Wanda L. Goleman Date: 1/29/03

Received by: Quality Assurance Unit: Brian Budwell Date: 1-30-03

* Sequentially numbered in order of the date that the change is effective

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Lubbock, TX 79409-1163

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Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist/ Assistant Director of Science
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Testing Facility Management

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Reference Chemical name: deionized water
CAS number: not applicable
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James Carr, Testing Facility Management, Co-Principle Investigator

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Section 10., 14., 15.2. 0.5x magnesium Holtfreter's solution will be the reference solution as this solution is may be more suited for raising *Rana* larvae.

Submitted by: Signature: Randall L. Coleman Date: 9/9/02

Authorized by: Study Director: Randall L. Coleman Date: 9/9/02

Received by: Quality Assurance Unit: Brian Bidwell Date: 9/12/02

* Sequentially numbered in order of the date that the change is effective

Project No.: T9700
Study No.: AMPH-02-01

SOPs Referenced in the Protocol

1. AQ-1-17 Exposure of Amphibian Eggs/Larvae to Test Substance (s)
2. AQ-1-14 Care and Maintenance of Rana Larvae
3. AQ-1-03 Euthanasia of Small Mammals, Birds, Fish, and Reptiles

A FINAL REPORT

ENTITLED
AVIAN EXPOSURE TO PERCHLORATE---FIELD STUDIES

STUDY/PROTOCOL NUMBER: AFS-02-01

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY:

Name/Address:

The Institute of Environmental & Human Health
Texas Tech University / TTU Health Sciences Center
Box 41163
Lubbock, Texas 79409-1163

Test Facility Management:

Dr. Ronald J. Kendall
Director, TIEHH

Study Director:

Dr. Scott T. McMurry

RESEARCH INITIATION: October 2001

RESEARCH COMPLETION: December 2002

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Table 2. Perchlorate concentrations in potential wood duck forage items collected from the Longhorn Army Ammunition Plant (LHAAP) in Karnack, Texas in 2002.
Table 3. Concentrations (ppm) of perchlorate in kidney and liver samples collected from different avian species in 2002 near the Naval Weapons Industrial Reserve Plant in McGregor, Texas.
Table 4. Number of detectable concentrations of perchlorate in liver and kidney samples from five species of birds collected from two sites in the Las Vegas Wash in March, 2002.
Table 5. Concentrations of perchlorate residues in different environmental media collected from three sites in the Las Vegas Wash in March, 2002.
Table 6. Mean (±SE) concentrations of perchlorate in water and plant samples from collection sites at the Longhorn and Naval Weapons study areas in Texas.

GOOD LABORATORIES PRACTICES STATEMENT

Study Number AFS-02-01 titled "Avian exposure to perchlorate—field studies", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

Scott McMurry — 3/28/03
Scott McMurry — Date

Philip N. Smith — 3/28/03
Philip N. Smith — Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Final Report Review	02/25/03	03/03/03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

Date 3-28-03

1.0 DESCRIPTIVE STUDY TITLE:
Avian exposure to perchlorate—field studies

2.0 STUDY/PROTOCOL NUMBER:
AFS-02-01

3.0 SPONSOR:
United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4.0 CONTRACT ADMINISTRATOR:
The Institute of Environmental and Human Health
Texas Tech University / TTU Health Science Center
Box 41163
Lubbock, TX 79409

5.0 TESTING FACILITY NAME & ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, Texas 79409-1163

6.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: October 1, 2001
Termination Date: December 31, 2002

7.0 KEY PERSONNEL:
Dr. Scott T. McMurry, Project Manager
Dr. Philip N. Smith, Project Manager
Mr. Ryan Bounds, Quality Assurance Officer
Dr. Ronald J. Kendall, Principle Investigator / Testing Facility Management

8.0 STUDY OBJECTIVES /TEST SYSTEM JUSTIFICATION:

The Interagency Perchlorate Steering Committee (IPSC) identified avian exposure studies as a data gap in research efforts involving perchlorate. Given the lack of research on exposure and effects of perchlorate in avian species, several specific areas were identified as data gaps. These included assessing endpoints in birds such as levels of, and relationships between, perchlorate residues and hormone concentrations in adults. Also, the level of perchlorate transfer into eggs, reproductive success (e.g., number of clutches, clutch size, egg viability, hatching rates), and recruitment of viable chicks (e.g., survival, growth, development) were all identified as data gaps. Other data gaps included evaluating food items as sources of exposure in birds, determining safe perchlorate levels in birds using controlled laboratory studies, and developing PBPK models for avian species.

In this study, we proposed to study avian exposure to perchlorate using aquatic and terrestrial avian species. Recent analysis of perchlorate residues in various plant and animal matrices indicates that significant exposure can occur from ingestion of aquatic invertebrates and aquatic and terrestrial plants (Anderson et al., in preparation). For example, composites of damselfly larvae averaged 1.5 ppm ($\pm$0.3, n=3 composite samples) perchlorate in contaminated impoundments at the LHAAP. Bullrush samples (n=4) from the same site averaged 7.6$\pm$1.4 and 4.4$\pm$2.2 ppm in above and below waterline samples, respectively. Sediment samples from this site averaged 25.1$\pm$6.8ppm perchlorate. Terrestrial plant samples (n=1 for each sample type) collected near building 25C showed even higher concentrations of perchlorate, ranging from 6 ppm in stems of goldenrod to 5,557 ppm in blades of crabgrass. Seeds of these plants also contained significant amounts of perchlorate, with 1,880 ppm in crabgrass seeds and 184 ppm in goldenrod seeds. These data, although of limited sample size, indicate the potential for exposure through foodchains by omnivorous wildlife. Based on the data presented above, we believe that avian species that consume both animal and plant material are at risk of exposure to perchlorate at contaminanted sites. Avian species that use aquatic habitats are at risk of exposure to perchlorate through ingestion of aquatic invertebrates and aquatic plants. Likewise, terrestrial avian species are at similar risk of exposure.

9.0 TEST ANIMALS (number, weight, source, strain):

Species: Wood duck, Northern cardinal, Killdeer, American coot, Song sparrow, Abert's towhee, White-crowned sparrow, Eastern phoebe, Lincoln's sparrow, Mockingbird

Strain: Wild

Age: Adult

Number: 55 birds, 13 wood duck eggs

Source: Collected/captured on or near perchlorate contaminated sites within the Las Vegas Wash, Nevada, Longhorn Army Ammunition Plant, Texas, and the Naval Weapons Industrial Reserve Plant, Texas.

10.0 METHODS:

Sample Collection and Field Procedures

Longhorn Army Ammunition Plant (LHAAP), Texas

Wood duck nest boxes were erected at the LHAAP in late December 2001. Forty nine boxes were established for the duration of the study, including boxes distributed throughout Harrison Bayou, Goose Prairie Creek, Central Creek, Star Ranch Pond, and the INF pond. Boxes were checked from February 2002 through early May 2002. Nest box condition was monitored and recorded as to the presence of nesting material, eggs, adult birds, and chicks.

Eggs were collected from all boxes with wood duck nesting activity. Typically, a single egg was collected from each nest after laying was initiated (e.g., 6 or more eggs had been laid). All eggs that failed to hatched were collected after hatching. All eggs were placed in plastic bags, labeled (nest box number, date) and frozen at –20C until transport back to TIEHH for residue analysis.

Adult wood duck hens were captured with a net while incubating eggs and fitted with a radio-transmitter (necklace attachment). Hens were then placed back into the nest box and monitored with a hand-held telemetry receiver.

Naval Weapons Industrial Reserve Plant (NWIRP), Texas

A variety of passerine bird species was collected from two locations near the NWIRP site near McGregor, Texas in January, 2002. Mist nets were placed along a spring-fed creek adjacent to highway 84 west of McGregor, and along the North Branch of the South Bosque River along highway 317 south of McGregor, Texas. Nets were monitored continuously while open and all captured birds were euthanized and frozen at –20C in the field. Birds were placed in plastic bags, labeled with location, species, and date. Samples were transported back to TIEHH for analysis.

Las Vegas Wash, Nevada

Our research team traveled to the Las Vegas Wash in southern Nevada March 10-15, 2002. The Las Vegas Wash is located just to the southeast of Las Vegas, Nevada. The Wash has been identified as a water body heavily contaminated with perchlorate. Perchlorate moves through the wash into Lake Mead, which then empties into the Colorado River. Perchlorate in the Colorado River can be detected as far south as Yuma,

Arizona. Therefore, this site was determined to be an excellent location to study perchlorate exposure among wild small mammal populations.

We collected samples from three sites along the Wash, termed LVW1, LVW2, and LVW3. A total of four days was spent, at least in part, at both LVW1 and LVW2, while two days were spent at LVW3. LVW1 is the western-most sampling area located where the Henderson water treatment facility effluent enters into the Wash. The two other sites are respectively to the east, and downstream, of the first.

Mist nets were erected in the main channel of the wash and on sandy/gravel outcrops along and in the wash. Nets were monitored continuously while open and captured birds removed from nets immediately after capture, identified, and either released (non-target species) or euthanized and frozen in the field. Collected vegetation was classified into the following categories: terrestrial grass, terrestrial broadleaf, aquatic grass, aquatic broadleaf, seeding bush, terrestrial tree, tree debris, and detritus. All samples were labeled and stored for transport back to our laboratory.

Kidneys and livers from the collected birds, and soil, water, and vegetation samples were extracted and analyzed for total perchlorate concentrations to determine body burden, and potential uptake pathways.

All sites

Water samples were collected into clean vials from just below the water surface wherever possible. All water samples (5 mL) were filtered and either analyzed for perchlorate ion directly, or diluted with distilled, deionized water and then analyzed. Soil samples were taken from the top 5 cm of soil. Soil samples were weighed, placed in glass jars, and extracted (mechanical agitation) with distilled, deionized water (2:1 water:soil). Water extracts were filtered and either analyzed for perchlorate ion directly, or diluted with distilled, deionized water and then analyzed. Vegetation, invertebrate, and vertebrate diet samples were collected from areas adjacent to where animals were collected. Samples were removed from soil, sediment, or water and placed in plastic bags. Prior to extraction, samples were air-dried, and weighed.

Sample analysis

Livers, kidneys, and eggs were analyzed for perchlorate content using standard tissue extraction and analysis techniques developed in the analytical core of this project (Anderson and Wu, 2002; and unpublished data).

Statistical Methods

Data are typically presented as the mean and standard error. Detectable concentrations of perchlorate were scant and therefore we were unable to perform any significance tests.

11.0 RESULTS

LHAAP, Texas

Forty-nine wood duck nest boxes were monitored during the course of the breeding season in 2002 (Table 1). Of these, only 6 boxes showed any nesting activity by wood ducks. Other species seen in nest boxes included a hooded merganser, owls, and squirrels. However, these species were rare occurrences and most nest boxes had no activity. Of the 6 nest boxes with activity, one box was a single clutch, four boxes had double clutches, and one box had three clutches, for a total of 12 wood duck nests during the season. Mean clutch size was about 15 eggs with an average of about 4 eggs hatched from each clutch, and a mean hatching rate of almost 36%. Virtually all of the nesting activity occurred in boxes in the Star Ranch Pond. Exceptions included a double clutch nest along Goose Prairie Creek and single clutch nest along Central Creek.

Table 1. Summary statistics for nesting activity of wood ducks in 2002 at the Longhorn Army Ammunition Plant, Karnack, Texas.

Total number of nest boxes	49
Nest boxes with wood duck activity	6
No. of boxes with single clutches	1
No. of boxes with double clutches	4
No. of boxes with triple clutches	1
Mean (+SE) clutch size	14.9±1.3
Mean (+SE) number of eggs hatched	4.3±1.4
Mean (+SE) percent hatch rate	35.6±10.5

A total of 13 wood duck eggs, representing clutches from Star Ranch Pond, Central Creek, and Goose Prairie Creek, was analyzed for perchlorate. Two eggs, from the Central Creek and Goose Prairie Creek nests, had detectable concentrations of perchlorate, at 855 and 7,187 ppb (wet weight), respectively. All other eggs were non-detectable for perchlorate.

Potential diet items of wood ducks were collected and analyzed for perchlorate (Table 2). In general, perchlorate was not detected or in trace amounts in most samples, including invertebrates, fish, tadpoles, crustaceans, and plants. Exceptions included 514 ppb perchlorate in fish from Harrison Bayou, and a range from 379 to 2,442 ppb in plants from a variety of sites including Harrison Bayou, Star Ranch Pond, and Central Creek. Logistical difficulties precluded collection of diet items from Goose Prairie Creek, but previous data indicate similar residue patterns from that area (Smith et al., 2001). These authors noted perchlorate concentrations approximately 100 ppb in fish from Goose Prairie Creek.

Table 2. Perchlorate concentrations in potential wood duck forage items collected from the Longhorn Army Ammunition Plant (LHAAP) in Karnack, Texas in 2002.

		Concentration of perchlorate (ppb)					
Sample ID	Location	Invertebrates	Fish	Tadpoles	Crustaceans	Misc. plants	Aquatic plants
FA-1	SRP1	trace	trace	trace	--	ND	ND
FA-2	SRP	ND	ND	ND	ND	ND	trace
FA-3	HB 11/122	--	--	--	--	ND	--
FA-4	Boat ramp3	--	--	--	--	ND	--
FA-5	SRP	ND	trace	ND	--	--	trace
FA-6	CC4	ND	trace	trace	--	--	2442
FA-7	SRP	ND	trace	trace	--	ND	1292
FA-8	CC	trace	ND	trace	--	ND	722
FA-9	SRP creek5	ND	ND	--	trace	ND	ND
FA-10	HB6	trace	trace	ND	trace	trace	trace
FA-11	HB	ND	514	--	trace	390	ND
FA-12	HB	--	--	--	--	trace	379

1SRP = Star Ranch Pond

2HB 11/12 = Harrison Bayou nest box numbers 11 and 12

3Boat ramp = Boat ramp on Caddo Lake northeast of SRP on the LHAAP.

4CC = Central creek

5SRP creek = tributary connecting SRP with Caddo Lake

6HB = Harrison Bayou

NWIRP, Texas

Thirteen passerine birds were collected from two sites near the NWIRP site (Table 3). Species included insectivorous and granivorous species of birds. Twelve of the 13 birds were collected along a spring-fed creek with a history of perchorate contamination. All of these birds had detectable concentrations of perchlorate in kidney samples, but not in liver samples. Perchlorate concentrations in kidneys ranged from 10 to 86 ppm in a Northern cardinal and Eastern phoebe, respectively. Liver concentrations ranged from non detectable to 47 ppm in a Song sparrow. No perchlorate was detected in the liver or kidney of the single bird, a Northern cardinal, collected from the Bosque River site along highway 317.

Table 3. Concentrations (ppm)[1] of perchlorate in kidney and liver samples collected from different avian species in 2002 near the Naval Weapons Industrial Reserve Plant in McGregor, Texas.

Species	Scientific name	Kidney	Liver	Site[2]
Eastern Phoebe	*Sayornis phoebe*	86*	Trace	HCT
Eastern Phoebe	*Sayornis phoebe*	28*	ND	HCT
Eastern Phoebe	*Sayornis phoebe*	17*	9	HCT
Lincoln's Sparrow	*Melospiza lincolnii*	37*	ND	HCT
Mockingbird	*Mimus polyglottos*	18*	ND	HCT
Northern Cardinal	*Cardinalis cardinalis*	10*	7	HCT
Northern Cardinal	*Cardinalis cardinalis*	31*	ND	HCT
Northern Cardinal	*Cardinalis cardinalis*	17*	ND	HCT
Song Sparrow	*Melospiza melodia*	32	47	HCT
White Crowned Sparrow	*Zonotrichia leucophrys*	42*	ND	HCT
White Crowned Sparrow	*Zonotrichia leucophrys*	19*	7	HCT
Northern Cardinal	*Cardinalis cardinalis*	ND	ND	NB 317

[1]ND = not detected.
[2]HCT = Harris creek tributary at the Harris creek bridge on Hwy 84 west of McGregor, Texas. NB 317 = North branch of the South Bosque River on Hwy 317 south of McGregor, Texas.

* indicates solvent extraction with EtOH instead of water.

Las Vegas Wash, Nevada

Forty-three birds were collected from three sites along the Las Vegas Wash in Nevada (Table 4). Species included American coots, white-crowned sparrows, killdeer, song sparrows, and Abert's towhee. Perchlorate was not detected in any kidney or liver samples from these birds with the exception of two individuals, an Abert's towhee and white-crowned sparrow. In both cases, perchlorate was detected in trace quantities.

Perchlorate residues in environmental media showed a different pattern than birds (Table 5). Perchlorate was detected in all media types throughout the study area. Highest mean

concentrations were detected in water and terrestrial plant samples , followed by soil and aquatic plants.

Table 4. Number of detectable concentrations of perchlorate in liver and kidney samples from five species of birds collected from two sites in the Las Vegas Wash in March, 2002.

Species	n	No. of detects in liver	No. of detects in kidneys	Location[1]
Killdeer	2	0	0	LVW 2
American coot	17	0	0	LVW 1 and 2
Song sparrow	3	0	0	LVW 1 and 2
Abert's towhee	3	0	1 (trace)	LVW 1
White-crowned sparrow	8	0	0	LVW 1
White-crowned sparrow	10	1 (trace)	1 (trace)	LVW 2

[1]LVW = Las Vegas Wash, sites 1 and 2.

Table 5. Concentrations of perchlorate residues in different environmental media collected from three sites in the Las Vegas Wash in March, 2002.

Media	n	No. of detects	Concentration of detects (ppm)	Location[1]
Terrestrial plants	8	8	44.9 + 29.6	LVW 1
Aquatic plants	5	1	4.9	LVW 1
Soil/sediment	9	7	6.4 + 4.6	LVW 1
Water	7	7	88.8 + 22.2	LVW 1 (slo)
Water	5	4	0.1 + 0.02	LVW 1
Water	5	5	0.3 + 0.03	LVW 2
Water	5	5	1.1 + 0.05	LVW 3

[1]LVW = Las Vegas Wash, sites 1, 2 and 3. LVW 1 (slo) = a backwater area off the main channel of the Las Vegas Wash.

Table 6. Mean (+SE) concentrations[1] of perchlorate in water and plant samples from collection sites at the Longhorn and Naval Weapons study areas in Texas.

Location	Site	Media	n	Concentration	Time period
LHAAP, Texas	Harrison Bayou	Water	7	ND	March 2002
	Central Creek	Water	1	ND	March 2002
	Goose Prairie Creek	Water	6	7.7+2.5	March 2002
	Star Ranch Pond	Water	1	ND	March 2002
NWIRP, Texas	Spring at Hwy 84	Water	9	44.4+5.0	August 2001-02
	North Branch at Hwy 317	Water	16	270.8+38.8	March 2001-January 2003
	Spring at Hwy 84	Aquatic plants	25	8.3+2.0	August 2001 - October 2002
	North Branch at Hwy 317	Aquatic plants	7	17.4+9.3	October 2001 and August 2002

[1]concentration in ppb for water, ppm dry weight for plants.

Reporting limits were 1 ppm for bird tissues collected from the Las Vegas Wash, 400 ppb (estimated) for wood duck eggs, 8 ppm for kidney and 6 ppm for liver samples from birds collected from the NWIRP site. When perchlorate was detected but below the reporting limit based on the standard curve, the concentration was denoted as "Trace".

12.0 DISCUSSION

Birds represent a broad group of wildlife with a long history as sentinel species for assessing exposure to contaminants. We collected a variety of avian species in this study including two species of waterfowl and several species of passerines. These birds consume a variety of food items including plant material, invertebrates, and small vertebrates. This variation in diet facilitates a wide variety of exposure scenarios for individual species of birds. The results of this study indicate that different species of birds can accumulate perchlorate in different tissues, including kidney, liver, and eggs. Our results also elude to differential exposure depending on the level of perchlorate contamination in the immediate surroundings.

The three sites sampled in this study represent a significant gradient of perchlorate contamination. Although significant perchlorate contamination has been observed at the LHAAP in the past, current remediation efforts at this site have reduced the amount of perchlorate being released into the environment (Table 2 and 6). Perchlorate contamination in the surrounding areas of the NWIRP site is variable (Table 6). Birds collected in this study came from two areas at the NWIRP site, but primarily from the spring-fed tributary along Highway 84. This spring typically contains measurable levels of perchlorate whenever the spring is running, providing a consistent source of perchlorate via drinking water and through transport into plants (Table 6). Finally, the Las Vegas Wash contains significant amounts of perchlorate, with measurable residues several kilometers downstream of Las Vegas.

Based on these site-specific characteristics, we would predict greater levels of perchlorate exposure in birds collected from the NWIRP and Las Vegas Wash sites than the LHAAP. To a degree, the results support this hypothesis. The exception is the lack of residues in birds from the Las Vegas Wash, although this site contains the highest levels of perchlorate in environmental media of any site in this study. Two basic types of birds were collected at the Las Vegas site; waterfowl and passerines. American coots were expected to show a relatively high degree of exposure, given their life history requisites. They forage, nest, and loaf in the water and mudflats throughout the Wash. However, none of the coots sampled showed any detectable concentrations of perchlorate. This result is likely due to the relatively high background conductivity of those samples. Current plans are to run these samples again using a recently developed preconcentration/preelution method that is particularly useful for samples with high background conductivity (Tian et al., 2003).

The consistent detectable concentrations of perchlorate found in birds collected at the spring creek near the NWIRP site appear to show that perchlorate is being accumulated by birds foraging in that area. This sampling of birds includes granivores and insectivores, both of which show detectable concentrations of perchlorate. Exposure at this site could be due to accumulation through the food chain, although it is likely that most exposure is occurring via consumption of free-standing water.

This study was also designed to assess movement of perchlorate into eggs via transfer from females. Of 13 eggs from wood ducks, two had detectable concentrations of perchlorate. Although this does not represent a widespread observation of egg exposure, it does point to the fact that perchlorate is capable of moving into eggs under environmentally relevant exposure scenarios. Additional work on this question is forthcoming in a second year of study.

Overall, a variety of different species of birds are capable of accumulating perchlorate from the environment. The effects of these exposures are unclear at this time. Comparison of the residue levels observed in this study with controlled laboratory studies will be instrumental in estimating the potential risk of wild species of birds to perchlorate exposure.

13.0 REFERENCES

Anderson TA, TH Wu. 2002. Extraction, cleanup, and analysis of the perchlorate anion in tissue samples. *Bull Environ Contam and Toxicol.* 68:684-691.

Smith PN, Theodorakis CW, Anderson TA, Kendall RJ. 2001. Preliminary assessment of perchlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. *Ecotoxicology.* 10:305-313.

Tian, K., P. K. Dasgupta, and T. A. Anderson. 2003. Simple determination of trace perchlorate in high salinity water samples by ion chromatography with online preconcentration and preelution. Analytical Chemistry. (In press).

14.0 ACKNOWLEDGMENTS

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Project No. T9700

A STUDY PROTOCOL

ENTITLED

Assessment of Perchlorate Exposure and Effects in Aquatic and Terrestrial Avian Receptors

STUDY/PROTOCOL NUMBER: AFS-02-01

SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

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Study Director:

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Dr. Phil N. Smith

PROPOSED EXPERIMENTAL
START DATE: JANUARY 1, 2002

1. **DESCRIPTIVE STUDY TITLE:**
Assessment of perchlorate exposure and effects in aquatic and terrestiral avian receptors

2. **STUDY NUMBER: AFS-02-01**

3. **SPONSOR:**
United States Air Force
AFIERA/RSE
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4. **TESTING FACILITY NAME & ADDRESS:**

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5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: January 1, 2002
Termination Date: September 30, 2002

6. **KEY PERSONNEL:**
Dr. Scott T. McMurry, Study Director
Dr. Philip N. Smith, Co-Investigator
Dr. Todd Anderson, Analytical Chemist,
Robert Spenser, Technician
Mr. Ryan M. Bounds, Quality Assurance Manager
Dr. Ronald J. Kendall, Primary Investigator / Testing Facility Management

7. **DATED SIGNATURES:**

Scott McMurry	3/28/03	Dr. Scott T. McMurry Study Director
RJ Kendall	3/28/03	Dr. Ron Kendall Testing Facility Management
[illegible]	3/28/03	Mr. Ryan Bounds Quality Assurance Manager

_____ _____ Dr. Todd Anderson
Analytical Chemist

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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9. STUDY OBJECTIVES / PURPOSE:

The purpose of this study is to look at avian exposure and effects to perchlorate using aquatic and terrestrial avian species. Objectives of the field studies are to determine:

- levels of, and relationships between, perchlorate exposure and thyroid hormone profiles in birds collected foraging and nesting at the LHAAP,
- the reproductive success of wood ducks (aquatic avian model) nesting at the LHAAP and survival and growth of their chicks,
- the level of perchlorate in dietary items of wood ducks and terrestrial avian species, and
- the level of perchlorate in wood duck eggs as an index of maternal transfer of perchlorate.

10. JUSTIFICATION OF TEST SYSTEM

Wood ducks (Aix sponsa) are year-round residents throughout the southeastern U.S., occur in abundance throughout Caddo Lake and its backwaters associated with the LHAAP, and easily cultured in the field by erecting nestboxes in study areas. They

consume a variety of plant and animal foods including the seeds, fruits, and vegetative material of aquatic and terrestrial plants (Landers et al., 1977; Drobney and Fredrickson, 1979; Delnicki and Reinecke, 1986). In addition, wood ducks consume a diverse number of aquatic invertebrate species (Landers et al., 1977). Diet composition varies between males and females and breeding versus nonbreeding females (Hepp and Belrose, 1995). In general, plant material comprises 50 to 60% of the diet for males and females, with the balance consisting of animal material. The major exception is egg-laying females that consume nearly 80% animal material.

Water and food consumption rates for wood ducks are unclear, but estimates can be derived from similar waterfowl such as mallards and lesser scaup, that consume about 6% of their body mass in water, and 8% (scaup) of their body mass in food, each day. Given the variability in perchlorate concentrations in water, plant, and invertebrates at the LHAAP, clear estimates of exposure are difficult to determine. However, water concentrations have been documented at 500ppb in Harrison Bayou-fed ponds. Concentrations of perchlorate for plant and animal samples were provided above. Based on these concentrations, a 600 g wood duck could consume as much as 18ug of perchlorate per day from water consumption, and 46ug to 231mg of perchlorate per day from food consumption.

Similar to wood ducks, terrestrial avian species have the potential for significant exposure, especially those species that consume seeds (e.g., finches) and invertebrates from the soil (e.g., American robins). Given that these species are typically small birds, their overall consumption of perchlorate (total mass) would be less than for wood ducks. However, food and water ingestion rates will typically be at or greater than for the larger bodied wood ducks. Because of the diversity of avian species that will occur in upland terrestrial sites at the LHAAP, we propose monitoring the terrestrial avian community, focusing on granivores and vermivores such as pigeons, and perching birds such as American robins, various sparrows and other finches, European starlings, and others.

11. TEST ANIMALS:

Species: Wood Ducks (*Aix sponsa*), Finches (*Fringillidae* spp.), Sparrows (*Emberizinae* spp.), and American Robins (*Turdus migratorius*)

Strain: Wild

Age: All ages, as encountered/trapped

Number: Approximately 50 wood ducks, 50 finches, 50 sparrows, and 50 robins

Source: Collected/captured on or near LHAAP, Karnack, Texas

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

All captured birds will be marked (as appropriate) with a uniquely numbered legband to ensure proper identification. In addition, hen wood ducks will be fitted with harness-type radio transmitters so that home range data and fledging survival may be monitored. Non-viable and early incubation wood duck eggs will be collected and placed in uniquely identified containers.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Field studies will include monitoring of wood ducks and selected species of terrestrial birds on the LHAAP. Wood duck studies will involve establishing artificial nesting structures throughout Harrison Bayou, extending from the INF pond toward Caddo Lake. Fifty to 100 nest boxes will be established according to established methods (Bellrose and Holm, 1994). Spacing of nest boxes will be determined after site visits and identification of appropriate habitat. Because wood ducks do not actively defend territories, they are amendable to nesting in high densities when provided artificial nest structures in clumped arrangements (Haramis, 1990). Nest boxes will be monitored throughout the nesting season, approximately January through July for southern latitudes. Exposure and effects assessments will be monitored via blood residues, thyroid hormone profiles, nesting success, survival and growth of fledglings, perchlorate residues in eggs, and perchlorate residues in diet items.

Terrestrial birds will be studied by collecting adult birds in mist nets along established transects radiating out from the INF pond/Harrison Bayou area. Ten mist nets (3mx10m) will be erected along four transects radiating in the four cardinal directions. Nets will be placed at approximately 50 m intervals. Nets will be monitored in the early morning hours (ca. 0600-1000hr) when stress to captured birds can be minimized. Exposure and effects assessments will be monitored via blood and tissue residues and thyroid hormone profiles. Effects on reproduction in terrestrial birds will not be assessed in this study given the difficulty in locating and monitoring natural nests.

14. METHODS:

Trapping

Wood duck studies will involve establishing artificial nesting structures throughout Harrison Bayou, extending from the INF pond toward Caddo Lake. A maximum of 50 nest boxes will be established according to established methods (Bellrose and Holm,

1994). Spacing of nest boxes will be determined after site visits and identification of appropriate habitat. Because wood ducks do not actively defend territories, they are amendable to nesting in high densities when provided artificial nest structures in clumped arrangements (Haramis, 1990). Nest boxes will be monitored throughout the nesting season, approximately January through July for southern latitudes.

All non-viable eggs (those that do not hatch) of wood ducks will be collected from nests in the field for attempted residue analysis. In addition, a maximum of two eggs will be collected early in incubation for residue analysis.

Terrestrial birds will be studied by collecting adult birds in mist nets along established transects radiating out from the INF pond/Harrison Bayou area. Ten mist nets (3mx10m) will be erected along four transects radiating in the four cardinal directions. Nets will be placed at approximately 50 m intervals. Nets will be monitored in the early morning hours (ca. 0600-1000hr) when stress to captured birds can be minimized.

Sample Collection

Blood (approximately 1 to 5ml) will be collected from each bird (TIEHH SOP IN 3-08-01) if possible. Serum and plasma samples will be kept frozen until analysis.

GI tract contents will be taken during necropsy and contents identified to the extent possible and analyzed for perchlorate. Samples collected during necropsy (e.g., thyroid, liver, kidney, stomach, etc) may be used for residue analysis and/or histology. Necropsies will be performed as described in TIEHH SOP IN 3-01-01 and Smith (2000).

Residue analysis will be attempted on eggs, but given the high level of organic constituents in eggs, extraction procedures are expected to be difficult. Currently, we are using ion exchange methods to clean biologic samples, and significant amounts of organics (as found in eggs) may foul ion membranes. We will attempt to analyze perchlorate by extracting homogenates of yolk, albumin, and yolk and albumin combined.

Sample Analysis

Portions of whole blood and eggs will be transferred to the analytical lab (Todd Anderson) for detecting perchlorate in blood, egg, and analysis.

15. PROPOSED STATISTICAL METHODS

Linear and/or logistic regression and Chi-square analysis will be used to examine the relationship between perchlorate exposure (as indicated through serum perchlorate concentrations) and thyroid hormone concentrations. Analysis of variance techniques may

be used to evaluate differences in hormone (and possibly perchlorate concentrations) concentrations, embryonic development, and reproductive success among areas considered contaminated and those designated as clean.

16. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include field capture data, sample collection and handling logs, GPS coordinates of all captures and radio-telemetry data, analytical data, embryonic growth and development, and hormone analysis data.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

- Capture success
- Serum perchlorate concentrations
- Thyroid hormone concentrations
- Developmental anomalies
- Fledging success
- Embryonic growth and development
- Spatial distribution (maps) of perchlorate exposure and thyroid hormone alterations.

List individual endpoints and analyses.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

17. RECORDS TO BE MAINTAINED / LOCATION:

A final report will be delivered to the Sponsor on or before March 31, 2003. Copies of all data, documentation, records, protocol information, and the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility.

18. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention

of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

19. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

20. REFERENCES:

Delnicki and Reinecke, 1986
Drobney and Fredrickson, 1979
Hepp and Belrose, 1995
Landers et al., 1977
Haramis, 1990
Smith, 2000

A FINAL REPORT

ENTITLED

THE INFLUENCE OF IODIDE ON THE RESPONSE OF LARVAL *XENOPUS LAEVIS* TO AMMONIUM PERCHLORATE

STUDY NUMBER:	XEN-02-03
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Texas Tech University Health Sciences Center Box 41163 Lubbock, TX 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	10/06/02
RESEARCH COMPLETION:	12/15/02

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GOOD LABORATORIES PRACTICES STATEMENT

Project XEN-02-3, entitled " THE INFLUENCE OF IODIDE ON THE RESPONSE OF LARVAL *XENOPUS LAEVIS* TO AMMONIUM PERCHLORATE ", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

James A. Carr, Ph.D

3/27/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	1-15-03	1-22-03	1-22-03	1-22-03
Collection and Euthanasia	11-22-02	11-22-02	12-3-02	12-17-02
Final Report and Raw Data Review	3-4-03	3-27-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/03
Date

1. DESCRIPTIVE STUDY TITLE:
The Influence of Iodide on the Response of Larval *Xenopus Laevis* to Ammonium Perchlorate.

2. STUDY NUMBER: XEN-02-03

3. SPONSOR:
United States Air Force United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. TESTING FACILITY NAME & ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: 10/06/02
Termination Date: 12/15/02

6. KEY PERSONNEL:
James A. Carr, Co-Principle Investigator
Dr. Angela Gentles, Study Director
Fang Hu, Graduate Research Assistant
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ken Dixon, Statistical/Modeling support
Ron Kendall, Principal Investigator/ Testing Facility Manager

7. STUDY SUMMARY
Larval *Xenopus laevis* were exposed to FETAX medium, ammonium perchlorate (AP, 14 ppm), or AP in the presence or absence of 14 ppm iodide beginning at Nieuwkoop-Faber stages 1-10 and continuing for a period of 70-d past fertilization. As expected, exposure to AP significantly reduced the percentage of animals initiating and completing metamorphosis and reduced hindlimb growth during the exposure period. Simultaneous exposure to iodide and AP completely reversed the antimetamorphic effects of AP. These data clearly indicate that perchlorate-inhibition of metamorphosis is dependent upon the amount of iodide available in the tadpoles environment.

8. STUDY OBJECTIVES / PURPOSE:
To determine the response of larval *Xenopus laevis* to ammonium perchlorate and sodium iodide when they are exposed at NF developmental stages 1-10 for 70 d.

9. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.
Source: Aldrich Chemical Company
Reference Chemical name: deionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- Xenopus) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: Steam plant condensate water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Sunderman et al., 1991): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

10. JUSTIFICATION OF TEST SYSTEM

Perchlorate prevents intake of iodide from water or food and is goitrogenic in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Thyroid hormones (TH) play a significant role in animal development and reproduction, thus, disruption of thyroid function is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health. Since perchlorate is a reversible competitive inhibitor of iodide transport, we determined if simultaneous exposure to perchlorate and iodide will nullify the deleterious effects of perchlorate on development in *Xenopus laevis*.

Xenopus laevis are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their aquatic environment. They also are easily and economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of AP on aquatic fauna.

11. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: eggs, larvae, and metamorphosed subadults
Number: Approximately 800
Source: Laboratory colony.

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each tank was be labeled as indicated in AQ-1-17, which includes genus and species name, common name, project name and number, date eggs were laid/hatched (if applicable), the group number and the name of the person responsible for animal care.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Approximately 800 *Xenopus laevis* embryos were raised in FETAX medium alone, 14 ppm AP in FETAX medium, NaI (14 ppm) in FETAX medium, or a combination of NaI (14 ppm) and AP (14 ppm) in FETAX medium for 70 d. Treatments began at NF developmental stages 1-10 and continued for 70 d. Each treatment was performed in quadruplicate. Each treatment group contained 200 animals, for a study total of approximately 800 (See Table 1). The selected 14 ppm AP concentration is within levels reported in AP contaminated surface waters at LHAAP (Smith et al., 2001). The concentration of NaI was selected in order to determine the effective concentration of NaI that will inhibit the effects of perchlorate.

Table 1. Design for XEN-02-03

Treatments	Treatment initiation	N per tank	Replicates tanks	Total per treatment
FETAX medium	NF 1-10	50	4	200
AP 14 ppm	NF 1-10	50	4	200
NaI 14 ppm	NF 1-10	50	4	200
AP 14 ppm + NaI 14 ppm	NF 1-10	50	4	200

14. METHODS:

14.1 Test System acquisition, quarantine, acclimation

Five adult male and five female *Xenopus laevis* were obtained from our lab colony for each experiment. Refer to SOP AQ-1-06 for details on routine *Xenopus* husbandry. Adults were maintained in dechlorinated tap water in flow-through 160 L tanks at 19° C at a density of 20 frogs per tank on a 12L: 12D light regimen. [[NOTE:THE FLOW THROUGH SYSTEM IS RELATIVELY NEW AND REPRESENTS A DEVIATION FROM THE STANDARD SOP. WILL TRY TO MODIFY SOP PRIOR TO SUBMISSION]]. Male and female *Xenopus* were maintained separately for at least 7 d before breeding. Please refer to SOP AQ-1-04 for details on *Xenopus* breeding.

14.2 Test condition establishment

Embryos were obtained from five pairs of adults that had been artificially induced to spawn (see SOPAQ-1-04). A representative sample of embryos from each breeding pair was collected and examined under a microscope for viability (SOP AQ-1-17). Equal numbers of embryos were taken from three or more pairs of frogs for a total of approximately 50 embryos per tank. Embryos were held in 21-L glass tanks containing 8 L of FETAX or AP and/or NaI dissolved in FETAX. Each tank was labeled as indicated in section 5.5 of SOP AQ-1-17, which includes species name, common name, project name and number, date eggs were

laid/hatched (if applicable), treatment group, and the name of the person responsible for animal care. AP or control solution was added to each tank at NF stages 1-10. Exposures continued for 70-d at 22 ± 2 °C and 12:12 L: D

14.2a Adults were induced to spawn according to SOP AQ-1-04.

14.2b At the initiation of the experiment a total of 50 stage 1-10 embryos were added to 4L of FETAX medium in each tank. FETAX and 2x test solutions (as listed in Table 1 above) were then used to bring each tank to 8L. This was done in order to prevent cross contamination while setting up the tanks. Here after test solutions were prepared as indicated in Table 2.

Fifty-percent of the test and reference solution was changed every Sunday, Tuesday, and Thursday as stated in the SOP for *Xenopus laevis* care (SOP AQ-1-06) and was added back to each aquarium daily as needed to maintain test conditions.

Table 2. Preparation of Tank Solutions

Nominal Concentration		**STOCK SOLUTIONS**				
NaI (ppm)	AP (ppm)	Water (mL)	(10X) FETAX (mL)	100X AP (mL)	100X NaI (mL)	Total Volume (mL)
0	0	16,200	1,800	-	-	18,000
14	0	16,020	1,800	-	180	18,000
0	14	16,020	1,800	180	-	18,000
14	14	15,840	1,800	180	180	18,000

14.3 Test Material Application

Rates/concentrations: ammonium perchlorate-14 ppm; sodium iodide- 14 ppm

Frequency: Constant exposure from NF stage 1-10 to day 70.

Route/Method of Application: Embryos and larvae were exposed to FETAX, AP, NaI or a combination of AP and NaI. Stock solutions of AP (100x) and NaI (100x) were prepared as follows and stored in 4L amber bottles. 5.616 g of each reagent was dissolved in 4 L ultrapure water, and stored in 4 L amber bottle. FETAX stock solution (10x) was prepared on demand. See SOP AQ-1-13 for FETAX preparation. AP, NaI, and FETAX were added to the appropriate tanks according to Table1. Tank solutions were prepared according to Table 2.

Method of application was immersion. Route of exposure was via dermal, oral, and respiratory exposure as the chemical was be in the aquaria medium.

Justification for Exposure Route: *Xenopus* are fully aquatic as larvae and as adults.

Exposure Verification: Samples of test and reference solutions were analyzed for perchlorate content (AC-2-11).

14.4 Test System Observation

Water quality, including salinity, conductivity, pH, dissolved O_2, and ammonia was performed once a week. Temperature, by means of a surrogate tank, was monitored daily. Water samples from each tank and diluted stock solution will be removed every 3 d for perchlorate analysis. Tank water samples will be collected after the medium has been renewed. Dead animals were removed and preserved in 10% neutral buffered formalin (NBF).

14.5 Animal Sacrifice and Sample Collections

Sample collections were performed at day 70. All larvae from each tank were euthanized by immersion in MS-222 (1g/L, AQ-1-03), staged, measured for snout-vent length, and weighed. Five larvae were frozen for subsequent determination of whole body thyroid hormone content, which is currently in progress. The remaining larvae were fixed in Bouin's fixative for subsequent histological assessment of the thyroid gland and gonad.

14.6 Endpoint Analysis

Assays for whole body tetraiodothyronine (T_4) are currently being performed by RIA (DBS SOP IN-2-04). Gonads of all fixed animals sampled were examined by visual inspection to determine sex (DBS SOP IN-2-03). Gonads and thyroid glands were examined using routine histological methods (Bouin's fixative, paraffin sections) currently operational in Dr. Carr's laboratory. Sections were stained with hematoxylin and eosin. See DBS SOP IN-2-08 for thyroid gland histopathology.

15. STATISTICAL METHODS

Time to forelimb emergence, thyroid follicle cell height and colloid depletion, whole body T_3 and T_4, hindlimb length, snout-vent length, and body weight were analyzed by one-way ANOVA. Gonadal sex ratios will be analyzed with the chi-square test to determine deviations from expected ratios of 1:1. Statistics were performed using GraphPad InStat v. 3.05 and SPSS v. 11 for Windows.

16. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

17. RESULTS

Mortality in this experiment was low (approximately 5%) in all treatments except for the NaI treatment, which exhibited a mortality of 12% (Table 3). There were no treatment-related effects on hatching (Table 3). Approximately 90% of all animals that hatched completed metamorphosis within the 70-d exposure period except for the 14 ppm AP treatment, in which none of the animals initiated or completed metamorphosis. There was a significant treatment effect on body weight, as body weight was greater in the 14 ppm AP treatment than in the other treatments. However, this was most likely due to the fact that these animals still possessed their tails, which contributed to their greater weight, as none of the animals in this treatment completed tail absorption during the 70-d exposure. There were no obvious treatment-related effects on snout-vent length (Table 4). However, as predicted, the 14 ppm treatment resulted in significantly reduced hindlimb growth compared to the other treatments (Table 4). Interestingly, several animals in the AP treatment exhibited noticeable goiters that resulted from enlargement of the thyroid gland (Fig. 1). These goiters were never observed in any of the other treatments. Histopathological analysis is currently ongoing, but given the clear-cut picture presented by the metamorphosis data, and previous studies from our laboratory showing that AP effects on metamorphosis always are associated with dramatic histological changes in the thyroid gland (Goleman et al., 2002a; Goleman et al., 2002b; Carr et al., 2003a, Carr etal., 2003b), it is likely that the conclusion based on the histological analysis will be the identical to that obtained from the metamorphosis data. Likewise whole-body T4 analysis is still underway, although this endpoint is not as sensitive a marker of AP exposure as thyroid histopathology. Regardless, the data from these additional analyses are unlikely to alter the conclusions that iodide reverses the antimetamorphic effects of perchlorate.

Between the test dates of 10/8/02 and 11/26/02, perchlorate concentrations in the 14 ppm AP and 14 ppm AP/NaI stock solutions prior to addition to the test tanks averaged 11.4 $\pm$ 0.12 ppm (14 ppm nominal). Perchlorate was not detected in the FETAX medium control stock solution or the 14 ppm NaI stock solution. Unionized ammonia levels in the 14 ppm AP and 14 ppm AP/NaI treatment tanks averaged approximately double that observed in the FETAX medium control and NaI treatment tanks. Average unionized ammonia concentrations for all treatments averaged 0.016 $\pm$ 0.003 mg/L (n=34, FETAX medium controls), 0.028 $\pm$ 0.004 mg/L (n=36, 14 ppm AP), 0.03 $\pm$ 0.003 mg/L (n=34, 14 ppm AP/NaI), and 0.017 $\pm$ 0.003 mg/L (n=36, NaI). Unionized ammonia concentrations were significantly greater ($p<0.05$) in the AP/NaI treatment than in the FETAX medium controls.

Table 3. Mean (± S.E.M.) incidence (%) of Mortality, Hatching, and Metamorphosis in *Xenopus laevis* Tadpoles Exposed to FETAX[a] Medium or Ammonium Perchlorate (AP, 14 ppm) in the Presence or Absence of Sodium Iodide (NaI, 14 ppm).

Treatment	Mortality	Hatching	FLE[b]	Tail Absorption
FETAX	5.24 ± 1.73	94.5 ± 1.26	91.6 ± 1.83	91.1 ± 2.44
AP	5.08 ± 1.66	87.5 ± 2.06	0.00 ± 0.00*	0.00 ± 0.00*
AP, NaI	5.29 ± 0.73	95.5 ± 2.63	94.3 ± 1.21	92.1 ± 0.66
NaI	12.3 ± 3.50	92.0 ± 2.00	88.3 ± 3.61	84.5 ± 5.68

[a] FETAX, Frog Embryo Teratogenesis Assay-*Xenopus*
[b] Forelimb emergence, # number with forelimbs divided by # hatching
Asterisks indicate significantly different from FETAX medium control, ($p < 0.05$).

Table 4. Mean (+ S.E.M.) Body Weights, Snout-vent Lengths, and Hindlimb Lengths in *Xenopus laevis* Tadpoles Exposed to FETAX[a] Medium or Ammonium Perchlorate (AP, 14 ppm) in the Presence or Absence of Sodium Iodide (NaI, 14 ppm).

Treatment	Body Weight (g)	Snout-vent Length (mm)	Hindlimb Length (mm)
FETAX	0.68 + 0.01 (n=174)	17.8 ± 0.13 (n=158)	19.1 ± 0.19 (n=158)
AP	1.48 ± 0.05* (n=167)	21.0 ± 0.23 (n=167)	2.45 ± 0.07* (n=167)
AP, NaI	0.64 ± 0.02 (n=177)	17.6 ± 0.15 (n=162)	19.0 ± 0.19 (n=162)
NaI	0.70 ± 0.02 (n=159)	18.0 ± 0.14 (n=147)	20.1 ± 0.22 (n=147)

[a] FETAX, Frog Embryo Teratogenesis Assay-*Xenopus*
Asterisks indicate significantly greater than control.

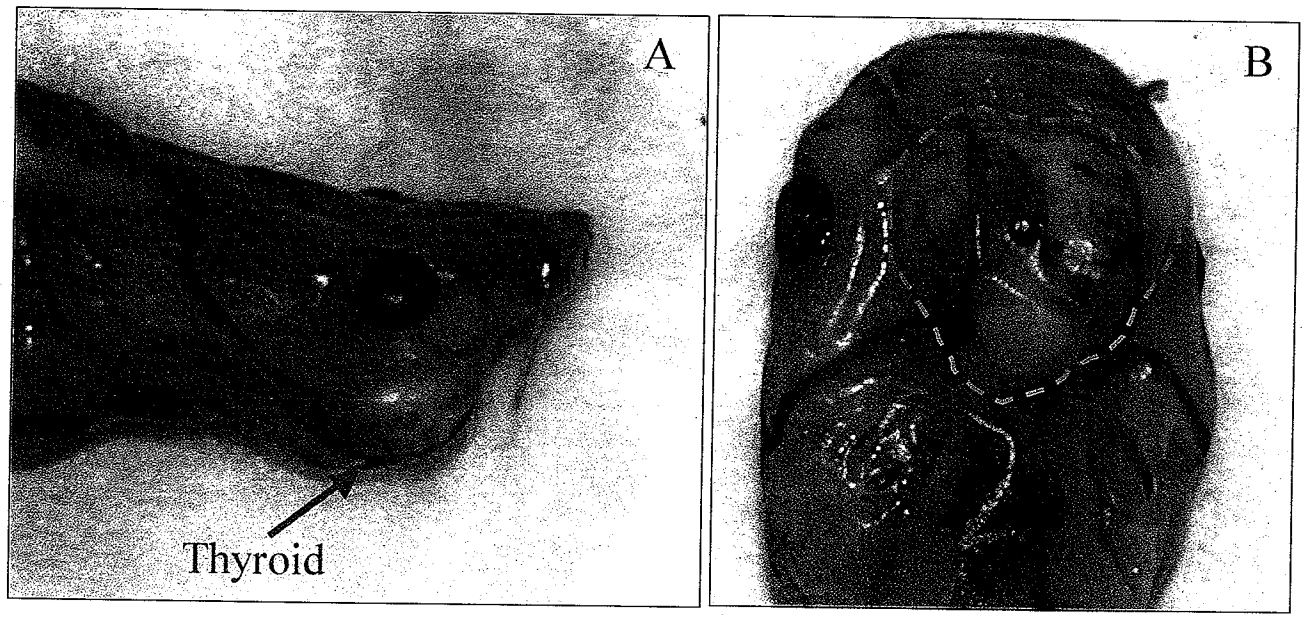

Figure 1. Goiter (enlarged thyroid gland) that was formed in a tadpole exposed to 14 ppm AP. Goiters were never observed in any of the other treatments, including the combination 14 ppm aP and 14 ppm NaI treatments. Fig. 1A. Lateral view. Fig. 1B. Ventral view. The enlarged thyroid gland lies in the ventral midline, just rostral to the heart, and appears to be heavily vascularized. The thyroid gland is outlined by the hatched line

19. DISCUSSION

Our data clearly indicate that the antimetamorphic effects of AP can be fully reversed by simultaneous exposure to iodide in the environment. This is the first report of such an effect in *X. laevis*. Exposure to iodide did not appear to affect the rate of metamorphosis on its own, but dramatically reversed every effect of AP including reversal of AP inhibition of FLE, tail resorption (Table 3) and hindlimb growth (Table 4). Also, we did not detect any animals with goiters in the 14 ppm/NaI treatment, although several were identified in the 14 ppm AP treatment group. These findings lend support to the current hypothesis regarding the mechanism of perchlorate inhibition of thyroid function in frogs, which is based on the large volume of literature indicating that perchlorate anions competitively inhibit iodide transport into thyrocytes by binding reversibly to the sodium/iodide symporter (NIS). If iodide and perchlorate are presented simultaneously, the degree of iodide uptake (and conversely the degree of perchlorate inhibition of iodide transport) will be determined by the relative affinity of the NIS to iodide and perchlorate, as well as the concentration of each anion. At present, the xNIS has not been sequenced or purified and there are no data on the affinity of this protein for iodide, perchlorate, or other anions. However, data in mammals suggests that the NIS has a greater affinity for perchlorate than iodide. Full characterization of the xNIS is in progress, but specificity and affinity of the transporter awaits the sequencing of this protein.

20. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

21. REFERENCES:

Carr, J.A., Urquidi, L.J., Goleman, W. L., Hu, F., Smith, P. N., and Theodorakis, C.W., (2003a). Ammonium perchlorate disruption of thyroid function in natural amphibian populations: Assessment and potential impact, " ***Multiple Stressor Effects in Relation to Declining Amphibian Populations,*** ASTM STP 1443, G. Linder, Ed., ASTM International, West Conshohocken, PA. In press.

Carr, J.A., Hu, F., and Gentles, A. (2003b). Response of larval *Xenopus laevis* to ammonium perchlorate at different stages of development. Draft Final Report for SERDP/TIEHH {NEED CORRECT ATTRIBUTION HERE}}Study Number XEN-02-2, submitted.

Goleman,W.L., Urquidi, L.J., Anderson, T.A., Kendall, R.J., Smith E.E., and Carr, J.A. (2002a). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis.* Environ. Toxicol. Chem. 21: 424-430.

Goleman, W.L., Carr, J.A., and Anderson, T.A. (2002b). Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. Environ. Toxicol. Chem. 21:590-597.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). *Gen. Comp. Endocrinol.* 106: 211-220.

Nieuwkoop, P.D. and Faber, J. (1967). Normal table of *Xenopus laevis (Daudin),* North Holland, Amsterdam.

Shi, Y-B. (2000). Amphibian Metamorphosis. John Wiley & Sons, Inc. N.Y.

Smith, P.N., Theodorakis, C.W., Anderson, T.A., Kendall, R.J. 2001. Preliminary assessment of perchlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. Ecotoxicology 10:305-313.

Sunderman, F.W., Plowman, M.C. and Hopfer, S.M. (1991). Embryotoxicity and teratogenicity of cadmium chloride in *Xenopus laevis*, assayed by the FETAX procedure. Ann. Clin. Lab. Sci. 21: 381-391.

U.S. Army Corps of Engineers. 1999. Results of a chemical test of water samples, groundwater treatment plant, Longhorn Army Ammunition Plant-Tulsa District. SWF-EV-D report No. 16967-1.

22. APPENDICES

Study Protocol
List of Key SOPs
Change in Study Documentation

A STUDY PROTOCOL

ENTITLED

THE INFLUENCE OF IODIDE ON THE RESPONSE OF LARVAL *XENOPUS LAEVIS* TO AMMONIUM PERCHLORATE

STUDY/PROTOCOL NUMBER: XEN-02-03

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY

Name/Address: The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Angella Gentles

PROPOSED EXPERIMENTAL START DATE **OCTOBER 4, 2002**

1. **DESCRIPTIVE STUDY TITLE:** The Influence of Iodide on the Response of Larval *Xenopus laevis* to Ammonium Perchlorate

2. **STUDY NUMBER:** XEN-02-03

3. **SPONSOR:**
United States Air Force United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: October 4, 2002
Termination Date: December 31, 2002

6. **KEY PERSONNEL:**
James Carr, Co-Principle Investigator
Angella Gentles, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ken Dixon, Statistical/Modeling support
Ron Kendall, Principal Investigator/ Testing Facility Manager

7. DATED SIGNATURES:

_____	_____	Dr. Angella Gentles Study Director
_____	_____	Dr. James Carr Principle Investigator
_____	3/28/03	Ryan Bounds Quality Assurance Manager
Todd Anderson	3-28-03	Dr. Todd Anderson Analytical Chemist/Asst. Dir. Of Science
_____	_____	Dr. Ken Dixon Statistical/Modeling Support
_____	_____	Dr. Ron Kendall Principal Investigator/ Testing Facility Management

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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Dr. James A. Carr
Department of Biological Sciences
Texas Tech University
Box 4-3131
Lubbock, Texas 79409

9. STUDY OBJECTIVES / PURPOSE:

To determine the response of larval *Xenopus laevis* to ammonium perchlorate and sodium

iodide when they are exposed at NF developmental stages 1-10 for 70 days.

10. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate (AP)
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 d.
Source: Aldrich Chemical Company

Test Chemical name: Sodium Iodide (NaI)
CAS number: 7681-82-5
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 d.
Source: Aldrich Chemical Company

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: Steam plant condensate water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Dawson and Bantle, 1987): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

11. JUSTIFICATION OF TEST SYSTEM

Perchlorate prevents intake of iodide from water or food and is goitrogenic in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Thyroid hormones (TH) play a significant role in animal development and reproduction, thus, disruption of thyroid function is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health. Since perchlorate is a reversible competitive inhibitor of iodide transport, we propose to determine if simultaneous exposure to perchlorate and iodide will nullify the deleterious effects of perchlorate on development in *Xenopus laevis*.

Xenopus laevis are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their aquatic environment. They also are easily and economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of AP on aquatic fauna.

12. **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: embryos
Number: Approximately 800
Source: *Xenopus* Express

13. **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**
Each tank will be labeled as indicated in AQ-1-17, which includes genus and species name, common name, project name and number, date embryos were laid/hatched (if applicable), the date of exposure, and the name of the person responsible for animal care.

14. **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**
Approximately 800 *Xenopus laevis* embryos will be raised in FETAX medium alone, 14 ppm AP in FETAX medium, NaI (14 ppm) in FETAX medium, or a combination of NaI 14 (ppm) and AP (14 ppm) in FETAX medium for 70 d. Treatments will begin at NF developmental stages 1-10 and continue for 70 d. Each treatment will be performed in quadruplicate. Each treatment group will contain 200 animals, for a study total of approximately 800 (See Table 1). The selected 14 ppm AP concentration is within levels reported in AP contaminated surface waters at LHAAP and referred in Goleman et al. (2002). The concentration of NaI was selected in order to determine the effective concentration of NaI that will inhibit the effects of perchlorate.

Table 1. Design for XEN-02-03

Treatments	Treatment initiation	N per tank	Replicates tanks	Total per treatment
FETAX medium	NF 1-10	50	4	200
14 ppm AP	NF 1-10	50	4	200
14 ppm AP + 14 ppm NaI	NF 1-10	50	4	200
14 ppm NaI	NF 1-10	50	4	200

15. **METHODS:**

15.1 **Test System acquisition, quarantine, acclimation**
Five adult male and five female *Xenopus laevis* will be obtained from our lab colony. Refer to SOP AQ-1-06 for details on routine *Xenopus* husbandry. They will be maintained in 45-L glass tanks containing 18 L of ultrapure reverse osmosis water for 1-2 d at approximately 22 $\pm$ 2° C on a 12L: 12D light regimen. Male and female *Xenopus* will

be maintained separately for at least 7 d before breeding. Please refer to SOP AQ-1-04 for details on *Xenopus* breeding.

15.2a Test condition establishment

Embryos will be obtained from 3-5 pairs of adults that have been artificially induced to spawn (see SOPAQ-1-04). A representative sample of embryos from each breeding pair will be collected and examined under a microscope for viability (SOP AQ-1-17). Equal number of embryos will be taken from three or more pairs of frogs for a total of approximately 50 embryos per tank. Embryos will be held 21-L glass tanks containing 8 L of FETAX or AP and/or NaI dissolved in FETAX. Each tank will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes species name, common name, project name and number, date eggs were laid/hatched (if applicable), treatment group, and the name of the person responsible for animal care. AP or control solution will be added to each tank at NF stages 1-10. Exposures will continue for 70-d at 22 ± 2 °C and 12:12 L: D

15.2b At the initiation of the experiment a total of 50 stage 1-10 embryos will be added to 4L of FETAX medium in each tank. FETAX and 2X test solutions (as listed in Table 1 above) will then be used to bring each tank to 8L. This will be done in order to prevent cross contamination while setting up the tanks. Here after test solutions will be prepared as indicated in Table 2.

Fifty-percent of the test and reference solution will be changed every Sunday, Tuesday, and Thursday as stated in the SOP for *Xenopus laevis* care (SOP AQ-1-06) and will be added back to each aquarium daily as needed to maintain test conditions.

15.3 Test Material Application

Rates/concentrations: ammonium perchlorate-14 ppm; sodium iodide- 14 ppm

Frequency: Constant exposure from NF stage 1-10 to day 70.

Route/Method of Application: Embryos and larvae will be exposed to FETAX, AP, NaI or a combination of AP and NaI. Stock solutions of AP (100X) and NaI (100X) will be prepared as follow and stored in 4L amber bottles. 5.616 g of each reagent will be dissolved in 4 L ultrapure water, and stored in 4 L amber bottle. FETAX stock solution (10X) will be a prepared on demand. See SOP AQ-1-13 for FETAX preparation. AP, NaI, and FETAX will be added to the appropriate tanks according to Table1. Tank solutions will be prepared according to Table 2.

Table 2. Preparation of Tank Solutions

Nominal Concentration		STOCK SOLUTIONS				
NaI (ppm)	AP (ppm)	Water (mL)	(10X) FETAX (mL)	100X AP (mL)	100X NaI (mL)	Total Volume (mL)
0	0	16,200	1,800	-	-	18,000
14	0	16,020	1,800	-	180	18,000
0	14	16,020	1,800	180	-	18,000
14	14	15,840	1,800	180	180	18,000

Route of exposure will be via dermal, oral, and respiratory exposure as the chemicals will be in the aquaria medium.

Justification for Exposure Route: *Xenopus* are fully aquatic as larvae and as adults.

Exposure Verification: Samples of test and reference solutions will be analyzed for perchlorate content (SOP AC-2-11).

15.4 Test System Observation

Water quality, including salinity, conductivity, pH, dissolved O_2, and ammonia will be performed at least once a week. Temperature, by means of a surrogate tank, will be monitored daily. Water samples from each tank and diluted stock solution will be removed every Sunday and Thursday for perchlorate analysis. Tank water samples will be collected after the medium has been renewed. Dead animals will be removed and preserved in 10% neutral buffered formalin (NBF).

15.5 Animal Sacrifice and Sample Collections

Sample collections will be performed at day 70. All larvae from each tank will be euthanized by immersion in MS-222 (1g/L, AQ-1-03), staged, measured for snout-vent length, and weighed. Five larvae will be frozen on dry ice for subsequent determination of whole body thyroid hormone content. The remaining larvae will be fixed in Bouin's fixative for subsequent histological assessment of the thyroid gland and gonad. About 10 animals will be frozen for perchlorate analysis.

15.6 Endpoint Analysis

Assays for whole body tetraiodothyronine (T_4) and triiodothyronine (T_3) will be performed by RIA (DBS SOP IN-2-04). Gonads of all fixed animals sampled will be examined by visual inspection to determine sex (DBS SOP IN-2-03). Gonads and thyroid glands will be examined using routine histological methods (Bouin's fixative, paraffin

sections) currently operational in Dr. Carr's laboratory. Sections will be stained with hematoxylin and eosin. See DBS SOP IN-2-08 for thyroid gland histopathology.

16. PROPOSED STATISTICAL METHODS

Time to forelimb emergence will be analyzed by one-way ANOVA. Thyroid follicle cell height, whole body T_3 and T_4, hindlimb length, snout-vent length, and body weight will be analyzed by two-way ANOVA (Treatment x Time). Chi-square analysis will be used to determine significant differences among percentages. Gonadal sex ratios will be analyzed with the chi-square test to determine deviations from expected ratios of 1:1.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include:

- Room temperature and water temperature, salinity, pH, dissolved oxygen content, and ammonia will be collected.
- Date, time, frequencies and amount of feedings per tank will be recorded. Number of expired larvae removed prior to termination of exposure will be recorded, including each date and tank.
- Deformities, abnormal swimming behavior and percent metamorphosed animals (complete tail resorption) will be recorded daily prior to termination of the experiment.

Report content will also include presentation of data, interpretation, and discussion of the following end-points:

- Whole body thyroid hormone content
- Gonadal sex differentiation
- Gonadal and thyroid histopathology
- Discussion of the relevance of the findings
- List of all SOPs used.
- List of all personel.

18. RECORDS TO BE MAINTAINED / LOCATION:

A draft of the final report will be delivered to the Sponsor on or before Dec. 31, 2002. The final report will be delivered to the Sponsor on or before Feb 31, 2003. All data, documentation, records, protocol information, specimens shall be sent to the Sponsor, or designated delivery point, for final archive within six months of study completion. A copy of all data, the protocol and the final report shall be maintained by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection,

findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

Goleman, W. L., Carr, J. A. and Anderson, T. A (2002). Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. Environ. Toxicol. Chem. 21 (3): 590-597.

Hamli, N. S., Stuelke, R. G., Schnell, M. D. 1956. Radioiodide in the thyroid and in other organs of rats treated with large doses of perchlorate. Endocrinol. 58:634-650.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. *Biocell* 20: 147-153.

Nieuwkoop, P.D. and Faber, J. (1967). Normal table of *Xenopus laevis (Daudin),* North Holland, Amsterdam.

Shi, Y-B. (2000). Amphibian Metamorphosis. John Wiley & Sons, Inc. N.Y.

Sunderman, F.W., Plowman, M.C. and Hopfer, S.M. (1991). Embryotoxicity and teratogenicity of cadmium chloride in *Xenopus laevis*, assayed by the FETAX procedure. Ann. Clin. Lab. Sci. 21: 381-391.

U.S. Army Corps of Engineers. 1999. Results of a chemical test of water samples, groundwater treatment plant, Longhorn Army Ammunition Plant-Tulsa District. SWF-EV-D report No. 16967-1.

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Form No. 014 Rev. 3.06/00
Project No.: *T9700*___
*Change No: __1_____
Page: ___1__ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: __X __ Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: ____ Protocol _X___ SOP ____ Other _____
Title: The Influence Of Iodide On The Response Of Larval *Xenopus Laevis* To Ammonium Perchlorate
Dated: _____10/04/02_____
Document # (if appropriate): XEN-02-03___
Page #(s): ____7-8_____
Section #: __15.6_____
Text to reference:. Assays for whole body tetraiodothyronine (T_4) and triiodothyronine (T_3) will be performed by RIA (DBS SOP IN-2-04). Gonads of all fixed animals sampled will be examined by visual inspection to determine sex (DBS SOP IN-2-03). Gonads and thyroid glands will be examined using routine histological methods (Bouin's fixative, paraffin sections) currently operational in Dr. Carr's laboratory. Sections will be stained with hematoxylin and eosin. See DBS SOP IN-2-08 for thyroid gland histopathology

Change in Document: Assays for whole body tetraiodothyronine (T_4) are currently being performed by RIA (DBS SOP IN-2-04). Assays for T3 will not be peformed because of the low T3 concentrations that we have previously found in *X. laevis* tadpoles. Gonads of all fixed animals sampled were examined by visual inspection to determine sex (DBS SOP IN-2-03). Gonads and thyroid glands were examined using routine histological methods (Bouin's fixative, paraffin sections) currently operational in Dr. Carr's laboratory. Sections were stained with hematoxylin and eosin. See DBS SOP IN-2-08 for thyroid gland histopathology.

Justification and Impact on Study: The study protocol indicates that whole body T_3 levels would be determined, however assay of whole body T3 is still in the method development stage, thus, currently, reliable results for T_3 levels cannot be obtained from this method.

Submitted by: Signature: BASlentle Date: 3/27/03

Authorized by: Study Director: BASlentle Date: 3/27/03

Received by: Quality Assurance Unit: [signature] Date: 3/27/03

*** Sequentially numbered in order of the date that the change is effective**

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Form No. 014 Rev. 3.06/00
Project No.: *T9700*____
*Change No: __2_____
Page: ___1__ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ___ Amendment ____ Deviation __X__ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: The Influence Of Iodide On The Response Of Larval *Xenopus Laevis* To Ammonium Perchlorate
Dated: __10/04/02_____
Document # (if appropriate): _XEN-02-03_____
Page #(s): ____8_____
Section #: __16_____
Text to reference:. Time to forelimb emergence will be analyzed by one-way ANOVA. Thyroid follicle cell height, whole body T_3 and T_4, hindlimb length, snout-vent length, and body weight will be analyzed by two-way ANOVA (Treatment x Time).

Change in Document: Time to forelimb emergence, thyroid follicle cell height and colloid depletion, whole body T_4, hindlimb length, snout-vent length, and body weight were analyzed by one-way ANOVA.

Justification and Impact on Study: Colloid depletion was analyzed because it is an indicator of abnormality in the thyroid gland.

Submitted by: Signature: BAKyentes **Date:** 3/27/03

Authorized by: Study Director: BAKyentes **Date:** 3/27/03

Received by: Quality Assurance Unit: [signature] **Date:** 3-27-03

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Form No. 014 Rev. 3.06/00
Project No.: *T9700*____
*Change No: __3_____
Page: ___1__ of _____

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X___ Amendment ____ Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: The Influence Of Iodide On The Response Of Larval *Xenopus Laevis* To Ammonium Perchlorate
Dated: __10/04/02_____
Document # (if appropriate): ___XEN-02-03_____
Page #(s): ____7_____
Section #: __15.5_____
Text to reference: Sample collections will be performed at day 70. All larvae from each tank will be euthanized by immersion in MS-222 (1g/L, AQ-1-03), staged, measured for snout-vent length, and weighed. Five larvae will be frozen on dry ice for subsequent determination of whole body thyroid hormone content. The remaining larvae will be fixed in Bouin's fixative for subsequent histological assessment of the thyroid gland and gonad. **About 10 animals will be frozen for perchlorate analysis.**

Change in Document: No animal was collected for perchlorate analysis.

Justification and Impact on Study: No animal was collected for perchlorate analysis in this study because no valuable information was obtained from such analysis in previous studies. The animals were thus used for determination of sex ratios.

Submitted by: Signature: BABGentes **Date:** 3/28/03

Authorized by: Study Director: BABGentes **Date:** 3/28/03

Received by: Quality Assurance Unit: [signature] **Date:** 3/27/03

*** Sequentially numbered in order of the date that the change is effective**

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Form No. 014 Rev. 3.06/00
Project No.: *T9700*
*Change No: 4
Page: 1 of 1

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: X Protocol ____ SOP ____ Other _____
Title: The Influence Of Iodide On The Response Of Larval *Xenopus Laevis* To Ammonium Perchlorate
Dated: 10/04/02
Document # (if appropriate): XEN-02-03
Page #(s): 5
Section #: 15.1
Text to reference:. They will be maintained in 45-L glass tanks containing 18 L of ultrapure reverse osmosis water for 1-2 d at approximately 22 ± 2° C on a 12L: 12D light regimen.

Change in Document: Adults were maintained in dechlorinated tap water in flow-through 160 L tanks at 19° C at a density of 20 frogs per tank on a 12L: 12D light regimen.

Justification and Impact on Study: The flow through system, which was recently acquired, was used to house the animals instead of the glass aquaria because it has an elaborate filtering system which keeps the water clean and helps to maintain water quality. Use of the flow through system reduces the time and manpower that is normally required for water changes in the regular glass aquaria.

Submitted by: Signature: BASgentle Date: 3/27/03

Authorized by: Study Director: BASgentle Date: 3/27/03

Received by: Quality Assurance Unit: [signature] Date: 3/27/03

*** Sequentially numbered in order of the date that the change is effective**

A FINAL REPORT

ENTITLED

RESPONSE OF ADULT FEMALE *XENOPUS LAEVIS* TO AMMONIUM PERCHLORATE: ASSESSMENT OF THYROID AND GONADAL PARAMETERS

STUDY NUMBER:	XEN-02-01
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Texas Tech University Health Sciences Center Box 41163 Lubbock, TX 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	2/22/02
RESEARCH COMPLETION:	2/21/03

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GOOD LABORATORIES PRACTICES STATEMENT

Project XEN-02-02, entitled " RESPONSE OF LARVAL *XENOPUS LAEVIS* TO AMMONIUM PERCHLORATE AT DIFFERENT STAGES OF DEVELOPMENT ", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

James A. Carr, Ph.D

3/27/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	2-5-03	2-5-03	2-5-03	2-11-03
Facility Inspection	3-21-02	3-21-02	3-25-02	3-25-02
Test Material Application	3-22-02	3-22-02	3-28-02	4-1-02
Test System Care and Application	3-22-02	3-22-02	3-28-02	4-1-02
Test Material mixing	3-21-02	3-21-02	3-25-02	3-26-02
Dosing	12-13-02	12-13-02	1-22-03	2-13-03
Final Report and Raw Data Review	2-25-03	3-10-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/03
Date

1. **DESCRIPTIVE STUDY TITLE:**
Response of adult female *Xenopus laevis* to ammonium perchlorate: assessment of thyroid and gonadal parameters.

2. **STUDY NUMBER:** XEN-02-01

3. **SPONSOR:**
United States Air Force United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: 2/22/02
Termination Date: 2/21/03

6. **KEY PERSONNEL:**
James A. Carr, Co-Principle Investigator/ DBS Testing Facility Management
Angella Gentles, Study Director
Wanda L. Goleman, Graduate Research Associate
Todd Anderson, Analytical Chemist/ Asst. Director for Science
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principle Investigator/ TIEHH Testing Facility Management

7. STUDY SUMMARY

Adult female *Xenopus laevis* were exposed to environmentally-relevant concentrations of AP for 10-14 wk and various aspects of thyroid and reproductive function determined. There was no effect of either 38 ppb or 14 ppm AP on thyroid histopathological endpoints. There were no effects of either AP concentration on any of the reproductive endpoints examined. Our data suggest that environmentally-relevant concentrations of AP do not significantly affect thyroid function in adult female X. laevis.

8. STUDY OBJECTIVES / PURPOSE:

To determine the sublethal effects of ammonium perchlorate (AP) on thyroid and reproductive function in adult female *Xenopus laevis* when exposed for 70 d and allowed to recover for a 28-d nontreatment period to assess AP effects on maternally deposited thyroid hormone (TH) in eggs

9. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 d
Source: Sigma-Aldrich Chemical Company

Reference Chemical name: aged tap water
CAS number: Not Applicable
Characterization: The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been aged 2-3 d to facilitate dechlorination.

10. JUSTIFICATION OF TEST SYSTEM

Ionic perchlorate alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Perchlorate is known to prevent intake of iodine from water or food and thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife stability as well as human health. Although the effects of AP on thyroid function of larval frogs has already been examined (Goleman et al., 2002a, b) the effects in adult frogs remain unknown. An important indirect effect of inhibiting thyroid function in female frogs is the possibility that disruption of thyroid activity may impact the deposition of thyroid hormone into the eggs. Maternally inherited TH can be detected in developing anuran embryos within 24 hr after fertilization, well prior to thyroid differentiation (Weber et al., 1994). Likewise, maternally inherited thyroid hormone receptor alpha (TRα) is detectable at embryonic stage 35, at a time when TRα mRNA expression is not detectable (Eliceiri and Brown, 1994). Both of these findings suggest that maternally inherited TH may be important for early embryonic development. We propose to

investigate the effects of AP exposure on adult female reproductive and thyroid parameters and on maternally deposited TH in eggs of *X. laevis*.

X. laevis are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their environment. They are also easily and economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of contaminants such as AP on aquatic fauna.

11. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: adult females
Number: approximately 186
Source: *Xenopus* Express (Homosassa, FL)

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each container will be labeled as indicated in SOPs TIEHH AQ-1-24/ DBS ET-1-03, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), test solution name, concentration and date of initial exposure, and the name of the person primarily responsible for animal care.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

In experiment 1 (XEN-02-1-1), adult female *X. laevis* were exposed to one of two concentrations of AP (38 ppb, 14040 ppb) or untreated water for 10 wk. In experiment 2 (XEN-02-1-2), adult female *X. laevis* were exposed to one of two concentrations of AP (38 ppb, 14040 ppb) or untreated water for 10 wk. After the 10 wk exposure, frogs were moved to treatment-matched aquaria containing untreated water for a 28 d non-exposure period. For both experiments females were injected with 750 IU of human chorionic gonadotropin via the dorsal lymph sac, weighed, and their nails clipped for identification prior to initiation of exposure. Blood and thyroid tissue was collected upon exposure termination. Sample size for experiments 1 and 2 was 9 replicates with 3 animals per replicate for a total of 27 animals per treatment. In experiment 3 (XEN-02-1-3), 24 adult female *X. laevis* (n=12) were exposed to either untreated water or 14040 ppb AP for 10 wk to assess AP effects on maternally deposited TH in eggs during oogenesis. Females were weighed and cryobranded (DBS AF-1-07) for identification prior to exposure. On day 69 of exposure, females were injected with 750 IU of human chorionic gonadotropin via the dorsal lymph sac and placed into individual 10 L glass tanks. Egg masses were then collected on day 70. Blood and thyroid tissue was collected from adults upon exposure termination. Sample size for experiment 3 was 4 replicates with 3 animals per replicate for a total of 12 animals per treatment. Approximately 78 animals were used for the entire study.

14. METHODS:

14.1 Test System acquisition, quarantine, acclimation

Adult female *X. laevis* were obtained from *Xenopus* Express (Homosassa, FL) or from our *Xenopus* colony maintained in Biology 504. Refer to TIEHH AQ-1-06/ DBS AF-1-01 for details on routine *X. laevis* husbandry. They were maintained in 45-L glass tanks containing 18 L of dechlorinated tap water at 22 ± 2 °C on a 12L: 12D light regimen for at least 7 d prior to initiation of exposure.

14.2 Test condition establishment

Adult female frogs were weighed and then added to 45 L glass aquaria containing 18 L of test substance or reference solution at a stocking density of 3 per tank. Each tank was labeled as indicated in SOPs TIEHH AQ-1-24/ DBS ET-1-03, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), test solution name, concentration and date of initial exposure, and the name of the person primarily responsible for animal care.

14.3 Test Material Application

Rates/concentrations: 0, 38 ppb, 14040 ppb

Frequency: Constant exposure for 10 wk

Route/Method of Application: Frogs were exposed to AP or untreated water in the glass tanks. Test and reference solutions were changed every 96 hr. Water containing the identical concentration of test substance was added back to each tank as needed to maintain test conditions. The stock solutions were made in 100 fold concentrations (Table 1), stored in 4 L amber bottles, and added to the appropriate containers according to Table 2.

Table 1. Preparation of Test Solutions and Initial Solutions

Chemical	Initial Stock g/L	Dilution Factor	Final Concentration (ppb*)
AP	0.0038	1:100	38
AP	1.4040	1:100	14040

*ppb = µg/L

Table 2. Solutions for 50% Change

	38 ppb	14040 ppb	Control
Aged Tap Water	0	0	9,000 mL
100x (0.0038 g/L)	90 mL	0 mL	8,910 mL
100x (1.4040 g/L)	0 mL	90 mL	8,910 mL

*ppb = µg/L

Fifty percent of the test and reference solutions was changed every 4 d. Room temperature will be maintained at 22 ± 2° C with a photoperiod of 12 h light: 12 h

dark. Method of application was immersion. Route of exposure were via dermal, oral, and respiratory exposure as the chemical will be in the tank medium. **Justification for Exposure Route**: *X. laevis* are fully aquatic as larvae and as adults.

Exposure Verification: Samples of stock and reference solutions were collected for perchlorate content prior to initiation of the experiment. Additional samples from freshly prepared test solutions were taken every 96 hr.

14.4 Test System Observation

once per week. Temperature of a surrogate tank containing the same volume of water were noted every day. Dead animals were removed and preserved in 10% neutral buffered formalin (NBF).Water quality, including salinity, conductivity, pH, and ammonia was performed at least

14.5 Animal Sacrifice and Sample Collections

Animals were sacrificed at 10 and 14 wk as appropriate. Animals were quickly weighed and then euthanized in 3-aminobenzoic acid ethyl ester (MS-222, 1g/L, TIEHH AQ-1-03/ DBS AF-3-03) and rinsed in distilled water. The body cavity was opened and blood collected by cardiac puncture (TIEHH XXX/ DBS AQ-3-01) and plasma frozen for subsequent T4 radioimmunoassay (RIA; DBS IN-2-04). The carcasses were then placed in Bouin's fixative (TIEHH MT-2-13/ DBS IN-1-05). The lower jaw and gonadal tissue was then dissected out for subsequent histological analysis of adult tissues. Egg masses were collected and frozen for subsequent TH RIA (DBS SOPs IN-2-01, IN-2-04, IN-2-05).

14.6 Endpoint Analysis

For adult frogs, we attempted to measure plasma T_3, T_4 by RIA (DBS IN-2-04). Plasma estradiol and progesterone will also be determined if there is sufficient plasma samples remaining. Thyroid glands will be prepared using routine histological methods (TIEHH SOPs MT-4-09, MT-4-10, MT-3-03, MT-4-03, MT-4-08/ DBS/TCFWRU SOPs IN-1-05, IN-1-06, IN-4-06, IN-4-07, DBS SOPs IN-1-02, IN-1-01, IN-1-04). Sections will be stained with hematoxylin and eosin (TIEHH MT-3-03/ DBS IN-1-04). Thyroid gland activity will be assessed for evidence of follicular hypertrophy (DBS IN-2-09). The concentration of T_3, T_4, deposited into eggs will be determined by RIA (DBS SOPs IN-2-01, IN-2-04, IN-2-05).

15. STATISTICAL METHODS

Treatment differences were analyzed by analysis of variance. Statistics were performed using GraphPad InStat v. 3.05 and SPSS v. 11 for Windows.

16. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, were documented,

signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

17. RESULTS

A total of three 10-14 week experiments were conducted between March of 2002 and February 2003. In XEN-02-1-1, adult female frogs were injected with HCG to induce ovulation 24 h prior to initiating exposure to 38 ppb or 14 ppm AP or remained in untreated water for 10 wk. In XEN-02-1-2, adult female frogs were injected with HCG to induce ovulation 24 h prior to initiating exposure to 38 ppb or 14 ppm AP or untreated water for 10 wk. After 10 wk, animals were transferred to treatment-matched aquaria containing untreated water for a 28-d non-treatment recovery period. In XEN-02-1-3, adult female frogs were exposed to 14 ppm AP or untreated water for 10 wk. After 10 wk, frogs were transferred to treatment-matched aquaria containing untreated water and injected with HCG to induce ovulation. After 24 h, ovulated eggs were collected for weighing and immediate freezing for subsequent T4 extraction. Weight of the ovulated eggs was used an indirect measure of clutch size. Mortality in XEN-02-1-2 was extraordinarily high (greater than 50%), and only data from XEN-02-1-1 and XEN-02-1-3 are reported here.

Frogs were size-matched to treatments in XEN-02-1-1, and there were no significant differences in body weight between treatments. Although there was a trend for decreasing body weight during the course of the experiment, there were no treatment-related effects on body weight at the end of the experiment. There were no treatment effects on ovarian weight or gonadosomatic index (Table 3). Initial histological analysis revealed no significant treatment related effects on hypertrophy or hyperplasia (Table 4). Artifacts of histological processing prevented accurate assessment of colloid depletion as described by Carr et al. (2003a). Nonetheless, these data were surprising, given the large amount of data from our lab and others indicating that larval frogs exposed to these concentrations of AP developed marker histopathology changes in the thyroid gland (Goleman et al., 2002a,b; Carr et al., 2003a,b). Our data indicated that this was not the case for the adults. Although not initially proposed in the study protocol, we believed that it was important to confirm the lack of perchlorate effect on adult thyroid. In order to more precisely determine even small changes in thyroid gland size and colloid depletion, serially-sectioned thyroid glands were digitally photographed and then thyroid gland cross-sectional area and follicle lumen area were determined using image analysis as described by Carr et al. (2003c). This involved photographing 10-20 sections per animal and performing over 2000 digitized measurements of lumen area to gauge colloid depletion and hypertrophy. As shown in Table 5 the quantification of thyroid gland cross-sectional area and follicle lumen area confirmed the initial semi-quantitative analysis of thyroid histopathology. There were no significant differences in thyroid gland cross-sectional area or lumen area between any of the treatments, although there was a trend to reduced lumen area with increasing AP concentration, but this difference was not statistically significant.

An endpoint that was initially proposed was to measure plasma T4 and T3 in the adult frogs after AP exposure. Initial attempts to measure plasma T4 by RIA in adult *Xenopus laevis* plasma were unsuccessful, as the values for T4 were nondetectable. As a

result, we attempted to extract T4 from plasma as described by Darras and Kuhn (1982) and Kowalczyk and Sotowska-Brochocka (2000) for other frog species. These extractions were performed using varying amounts of adult *X. l*aevis plasma ranging from 800-1600 µL and concentrating them 4-8-fold. Even after extraction and concentration by 8-fold, plasma T4 was barely detectable. Moreover, dilution of *X. l*aevis plasma extracts did not exhibit parallelism with authentic T4 standards (Fig. 2). The inability to detect T4 in *X. laevis* plasma was not due to a problem with the RIA, as plasma T4 was easily detectable in unextracted samples from deer mice and duck (Fig. 1).

Perchlorate analysis of tank solutions in XEN-02-1-1 revealed that the control tanks contained an average perchlorate concentration of 50.9 ± 25.0 µg perchlorate/L. This resulted from tanks that initially contained perchlorate during the first part of the trial, as perchlorate in the control tanks averaged 94 ppb on 3/22/02, 54 ppb on 5/5/02, and 0 ppb on 5/31/02. Thus the perchlorate in the control tanks may have arisen from a single event early in the experiment and the perchlorate was diluted out as the experiment progressed. Perchlorate in the 38 ppb and 14-ppm nominal treatments averaged 128 ± 27 ppb and 14,835 ± 2300 ppb over the course of the trial. Perchlorate in the 14-ppm test tanks for XEN-02-1-3 averaged 11,971 ± 463 ppb. No perchlorate was detected in the control tanks during XEN-02-1-3.

Table 3. Mean (± S.E.M.) Initial Body Weight, Final Body Weight, Fixed Ovary Weight, and Gonadosomatic Index (GSI) in adult Female *Xenopus laevis* Exposed to Ammonium Perchlorate (AP) for 10 Weeks in Experiment XEN-02-1-1.

Treatment	Initial Body Weight (g)	Final Body Weight (g)	Fixed Ovary Weight (g)	GSI
Control	101 ± 3.84	96.4 ± 1.26	4.72 ± 0.39	0.050 ± 0.006
38 ppb AP	107 ± 3.66	107 ± 2.83	6.18 ± 0.49	0.057 ± 0.005
14 ppm AP	111 ± 4.41	105 ± 4.57	5.77 ± 0.63	0.054 ± 0.005

Table 4. Thyroid Histopathology of Adult Female *Xenopus laevis* Exposed to Ammonium Perchlorate (AP) for 10 Weeks in Experiment XEN-02-1-1

Treatment	Mean Colloid Depletion	Mean Follicle Cell Hypertrophy	Mean Hyperplasia
reference	ND	0.37 ± 0.11	0.003 ± 0.003
38 ppb AP	ND	0.36 ± 0.15	0.000 ± 0 000
14 ppm AP	ND	0.69 ± 0.17	0.000 ± 0.000

Table 5. Mean (± S.E.M.) Thyroid Gland Cross-sectional Area[a] and Follicle Lumen Area[b] in Adult Female *Xenopus laevis* Exposed to Ammonium Perchlorate (AP) for 10 Weeks in Experiment XEN-02-1-1.

Treatment	Thyroid Gland Area (μm^2)	Follicle Lumen Area (μm^2)
FETAX	595,726 + 177,732 (n=8)	32,159 ± 8631 (n=8)
38 ppb AP	391,055 ± 69,373 (n=6)	31,097 ± 3645 (n=6)
14 ppm AP	433,867 ± 25,957 (n=7)	26,300 ± 3496 (n=7)

[a]Largest cross-sectional area through the right thyroid gland.

[b]Determined from an average of 70-100 follicles per animal.

Table 6. Mean (± S.E.M.) Initial Body Weight, Final Body Weight, and Egg Clutch Weight in adult Female *Xenopus laevis* Exposed to Ammonium Perchlorate (AP) for 10 Weeks in Experiment XEN-02-1-3.

Treatment	Initial Body Weight (g)	Final Body Weight (g)	Clutch Weight (g)
Control	125 ± 7.35	113 ± 4.81	16.2 ± 4.93
14 ppm AP	126 ± 7.05	115 ± 6.32	16.9 ± 2.24

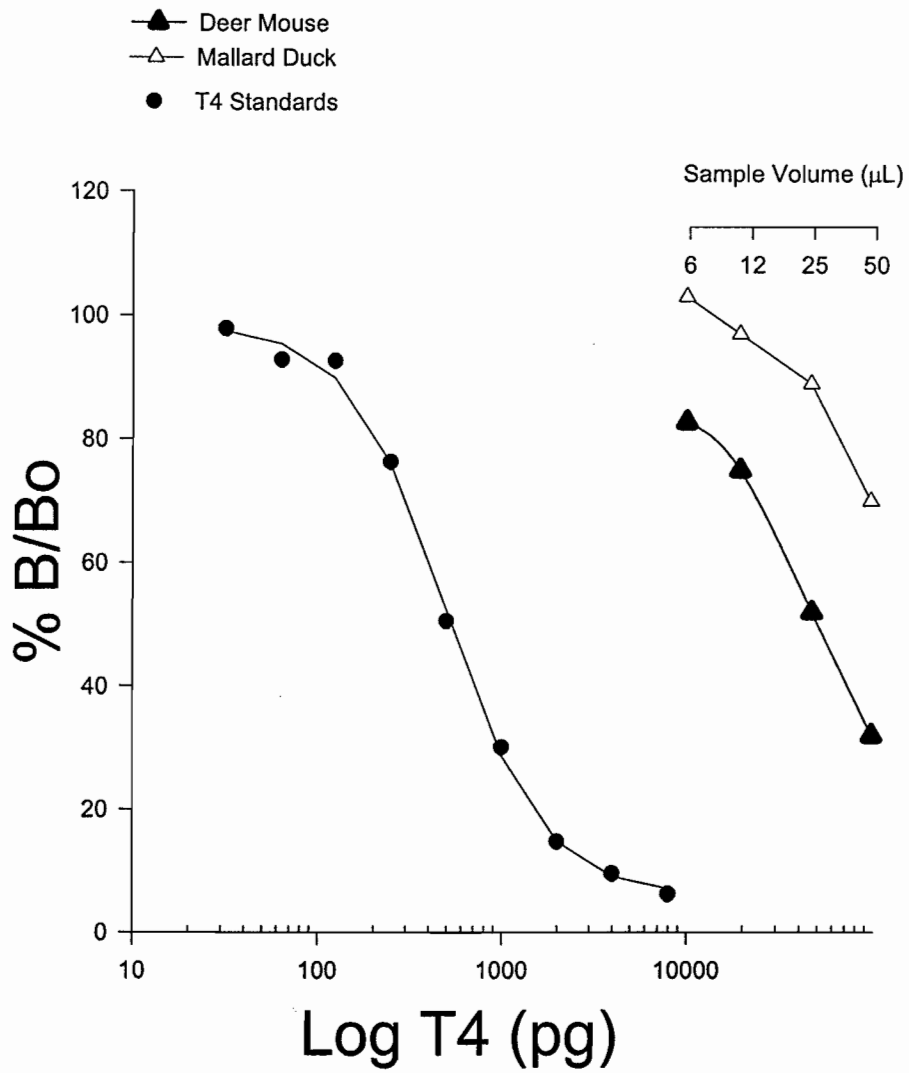

Figure 1. Measurement of T4 in serial dilutions of plasma from deer mouse and mallard duck showing parallelism with authentic T4 standards. This graph shows that the T4 assay used in this report was functioning properly and was sensitive enough to detect circulating T4 in mammals and birds.

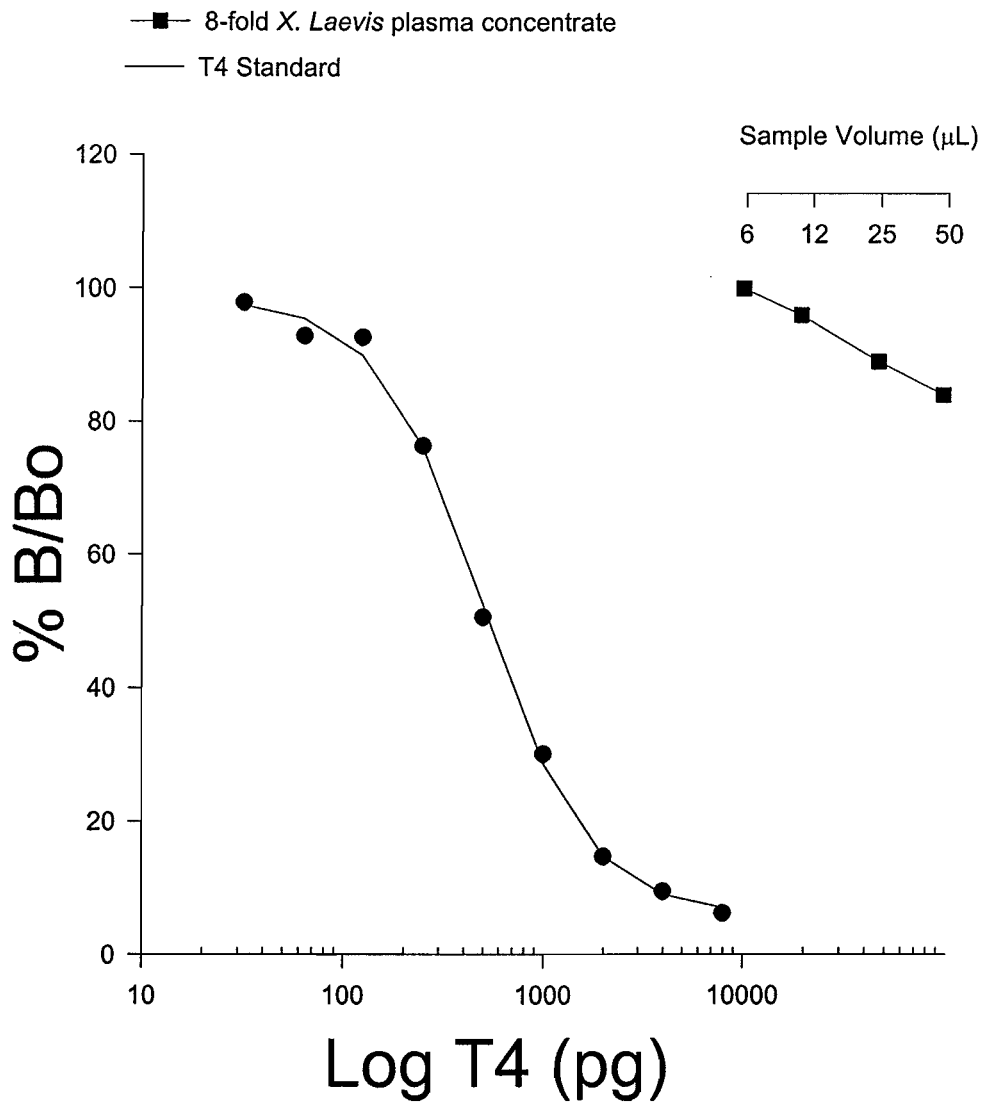

Figure 2. Measurement of T4 in serial dilutions of plasma extracts from adult female *X. laevis* showing that plasma dilutions do not demonstrate parallelism with authentic T4 standards. Plasma samples were incubated with ANS and plasma T4 extracted with four volumes of methanol as described by Darras and Kuhn (1982) and Kowalczyk and Sotowska-Brochocka (2000). The data above represent an 8-fold concentration of T4 from plasma samples.

18. DISCUSSION

The data collected from XEN-02-1-1 indicate that adult female *X. laevis* do not respond to environmentally relevant AP concentrations with any measurable change in thyroid function. There was no evidence of hypertrophy or colloid depletion (based on mean lumen area) in the 14 ppm treatment. Although we were unable to reliably measure plasma T4 in the adult frogs, this in itself is important and indicates that plasma T4 levels are extraordinarily low in adult *X. laevis*, especially when compared to other vertebrate groups. In addition, we observed no measurable effect of T4 on GSI or ovulated clutch size, suggesting that AP does not appear to influence reproductive status in adult frogs. Taken together, these data indicate that adult *X. laevis* do not appear to respond to environmentally relevant concentrations of AP. This is dramatically different than the situation for larval frogs, as the AP concentrations used in the present study dramatically influence thyroid function in developing frogs as determined by reduction in whole-body T4 and dramatic changes in thyroid gland histopathology, including the formation of goiters (XEN-02-2 draft final report). These data suggest that AP represents a greater potential risk to developing rather than adult frogs.

The present report includes significant additional analyses that were not planned, including the digital analysis of over 2000 individual thyroid follicles from the frogs in XEN-02-1-1. As a result, the analysis of egg thyroid hormone content is still underway, although we do not expect to have a great deal of success in measuring egg T4 content given the extraordinarily low T4 concentrations in the adult female frogs. Another initial goal of the present work was to determine if any perchlorate effects on thyroid function were reversible. However, experiment XEN-02-1-2 experienced a great deal of mortality and we were not able to directly test this hypothesis. Furthermore, given the lack of AP effects on thyroid function observed in the present study it is unlikely that we would have been able to detect any reversible effects.

19. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20. REFERENCES:

Carr, J.A., Urquidi, L.J., Goleman, W. L., Hu, F., Smith, P. N., and Theodorakis, C.W., (2003a). Ammonium perchlorate disruption of thyroid function in natural amphibian populations: Assessment and potential impact, " ***Multiple Stressor Effects in Relation to Declining Amphibian Populations,*** ASTM STP 1443, G. Linder, Ed., ASTM International, West Conshohocken, PA. In press.

Carr, J.A., Hu, F., and Gentles, A. (2003b). Response of larval *Xenopus laevis* to ammonium perchlorate at different stages of development. Draft Final Report for SERDP/TIEHH {NEED CORRECT ATTRIBUTION HERE}}Study Number XEN-02-2, submitted.

Carr, J.A., Gentles, A., Smith, E.E., Goleman, W.L., Urquidi, L.J., Thuett, K., Kendall, R.J., Giesy, J.P., Gross, T.S., Solomon, K.R., Van Der Kraak, G. (2003c). Response of larval *Xenopus laevis* to atrazine: Assessment of growth, metamorphosis, and gonadal and laryngeal morphology. Environ. Toxicol. Chem., 22: 396-405.

Goleman,W.L., Urquidi, L.J., Anderson, T.A., Kendall, R.J., Smith E.E., and Carr, J.A. (2002a). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis.* Environ. Toxicol. Chem. 21: 424-430.

Goleman, W.L., Carr, J.A., and Anderson, T.A. (2002b). Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. Environ. Toxicol. Chem. 21:590-597.

Kowalczyk, P., Sotowska-Brochocka, J. (2000). Changes in thyroid hormones in the serum and the thyroid gland of hibernating frogs, *Rana temporaria* L. Gen. Comp. Endocrinol. 119:172-180.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). *Gen. Comp. Endocrinol.* 106: 211-220.

Weber, G.M., Farrar, E.S., Tom, C.K., Grau, E.G. (1994). Changes in whole-body thyroxine and triiodothyronine concentrations and total content during early development and metamorphosis of the toad *Bufo marinus*. Gen. Comp. Endocrinol. 94: 62-71.

21. APPENDICES

Study Protocol
Changes to Study Documentation
List of Key Standard Operating Procedures

Project No. T9700.2

A STUDY PROTOCOL

ENTITLED

RESPONSE OF ADULT FEMALE *XENOPUS LAEVIS* TO AMMONIUM PERCHLORATE: ASSESSMENT OF THYROID AND GONADAL PARAMETERS

STUDY NUMBER: XEN-02-01

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY:

Name/Address: The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. Ronald J. Kendall

Study Director: Dr. Angella Gentles

PROPOSED EXPERIMENTAL
START DATE: February 22, 2002

1. **DESCRIPTIVE STUDY TITLE:** Response of Adult Female *Xenopus laevis* to Ammonium Perchlorate: Assessment of Thyroid and Gonadal Parameters.

2. **STUDY NUMBER:** XEN-02-01

3. **SPONSOR:** United States Air Force
   AFIERA/RSE
   2513 Kennedy Circle
   Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
   The Institute of Environmental and Human Health
   Texas Tech University
   Texas Tech University Health Sciences Center
   Box 41163
   Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
   Start Date: February 15, 2002
   Termination Date: February 20, 2003

6. **KEY PERSONNEL:**
   James A. Carr, Co-Principle Investigator/ DBS Testing Facility Management
   Angella Gentles, Study Director
   Wanda L. Goleman, Graduate Research Associate
   Todd Anderson, Analytical Chemist/ Asst. Director for Science
   Ryan Bounds, Quality Assurance Manager
   Ronald Kendall, Principle Investigator/ TIEHH Testing Facility Management

7. DATED SIGNATURES:

_____	1/30/03	Dr. Angella Gentles Study Director
_____	1/30/03	Dr. James Carr Co-Principle Investigator/ DBS Testing Facility Management
_____	1/29/03	Wanda L. Goleman Graduate Research Associate
_____	2-11-03	Dr. Todd Anderson Analytical Chemist/ Asst. Director for Science
_____	2-5-03	Ryan Bounds Quality Assurance Manager
_____	2-13-03	Dr. Ronald Kendall Principle Investigator/ TIEHH Testing Facility Management

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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Dr. James Carr
Department of Biological Sciences
Texas Tech University
Box 4-3131
Lubbock, Texas 79409

9. STUDY OBJECTIVES / PURPOSE:

To determine the sublethal effects of ammonium perchlorate (AP) on thyroid and reproductive function in adult female *Xenopus laevis* when exposed for 70 d and allowed to recover for a 28-d nontreatment period to assess AP effects on maternally deposited thyroid hormone (TH) in eggs.

10. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 d
Source: Sigma-Aldrich Chemical Company

Reference Chemical name: aged tap water
CAS number: Not Applicable
Characterization: The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been aged 2-3 d to facilitate dechlorination.

11. JUSTIFICATION OF TEST SYSTEM

Ionic perchlorate alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Perchlorate is known to prevent intake of iodine from water or food and thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife stability as well as human health. Although the effects of AP on thyroid function of larval frogs has already been examined (Goleman et al., 2002a, b) the effects in adult frogs remain unknown. An important indirect effect of inhibiting thyroid function in female frogs is the possibility that disruption of thyroid activity may impact the deposition of thyroid hormone into the eggs. Maternally inherited TH can be detected in developing anuran embryos within 24 hr after fertilization, well prior to thyroid differentiation (Weber et al., 1994). Likewise, maternally inherited thyroid hormone receptor alpha (TRα) is detectable at embryonic stage 35, at a time when TRα mRNA expression is not detectable (Eliceiri and Brown, 1994). Both of these findings suggest that maternally inherited TH may be important for early embryonic development. We propose to investigate the effects of AP exposure on adult female reproductive parameters and on maternally deposited TH in eggs of *X. laevis*.

X. laevis are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their environment. They are also easily and economically

maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of contaminants such as AP on aquatic fauna.

12. TEST ANIMALS:

Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: adult females
Number: approximately 186
Source: *Xenopus* Express (Homosassa, FL)

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each container will be labeled as indicated in SOPs TIEHH AQ-1-24/ DBS ET-1-03, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), test solution name, concentration and date of initial exposure, and the name of the person primarily responsible for animal care.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

In experiment 1, adult female *X. laevis* (n=27) will be exposed to one of 2 concentrations of AP (38 ppb, 14040 ppb) or untreated water for 10 wk. In experiment 2, adult female *X. laevis* (n=27) will be exposed to one of two concentrations of AP (38 ppb, 14040 ppb) or untreated water for 10 wk. After the 10 wk exposure, frogs will be moved to treatment-matched aquaria containing untreated water for a 28 d non-exposure period. For both experiments 1 and 2, females will be injected with 750 IU of human chorionic gonadotropin via the dorsal lymph sac, weighed, and their nails will be clipped for identification prior to initiation of exposure. Blood and thyroid tissue will be collected upon exposure termination. Sample size for experiments 1 and 2 will be 9 replicates with 3 animals per replicate for a total of 27 animals per treatment. In experiment 3, 24 adult female *X. laevis* (n=12) will be exposed to either untreated water or 14040 ppb AP for 10 wk to assess AP effects on maternally deposited TH in eggs during oogenesis. Females will be weighed and cryobranded (DBS AF-1-07) for identification prior to exposure. On day 69 of exposure females will be injected with 750 IU of human chorionic gonadotropin via the dorsal lymph sac and placed into individual 10 L glass tanks. Egg masses will then be collected on day 70. Blood and thyroid tissue will be collected from adults upon exposure termination. Sample size for experiment 3 will be 4 replicates with 3 animals per replicate for a total of 12 animals per treatment. Approximately 186 animals will be used for the entire study.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation

Adult female *X. laevis* will be obtained from *Xenopus* Express (Homosassa, FL) or from our *Xenopus* colony maintained in Biology 504. Refer to TIEHH AQ-1-06/ DBS AF-1-01 for details on routine *X. laevis* husbandry. They will be maintained in 45-L glass tanks

containing 18 L of dechlorinated tap water at 22 ± 2 °C on a 12L: 12D light regimen for at least 7 d prior to initiation of exposure.

15.2 Test condition establishment

Adult female frogs will be weighed and then added to 45 L glass aquaria containing 18 L of test substance or reference solution at a stocking density of 3 per tank. Each tank will be labeled as indicated in SOPs TIEHH AQ-1-24/ DBS ET-1-03, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), test solution name, concentration and date of initial exposure, and the name of the person primarily responsible for animal care.

15.3 Test Material Application

Rates/concentrations: 0, 38 ppb, 14040 ppb

Frequency: Constant exposure for 10 wk

Route/Method of Application: Frogs will be exposed to AP or untreated water in the glass tanks. Test and reference solutions will be changed every 96 hr. Water containing the identical concentration of test substance will be added back to each tank as needed to maintain test conditions. The stock solutions will be made in 100 fold concentrations (Table 1), stored in 4 L amber bottles, and added to the appropriate containers according to Table 2.

Table 1. Preparation of Test Solutions and Initial Solutions

Chemical	Initial Stock g/L	Dilution Factor	Final Concentration (ppb*)
AP	0.0038	1:100	38
AP	1.4040	1:100	14040

*ppb = µg/L

Table 2. Solutions for 50% Change

	38 ppb	14040 ppb	Control
Aged Tap Water	0	0	9,000 mL
100x (0.0038 g/L)	90 mL	0 mL	8,910 mL
100x (1.4040 g/L)	0 mL	90 mL	8,910 mL

*ppb = µg/L

Fifty percent of the test and reference solutions will be changed every 4 d. Room temperature will be maintained at 22 ± 2° C with a photoperiod of 12 h light: 12 h dark. Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the tank medium.

Justification for Exposure Route: *X. laevis* are fully aquatic as larvae and as adults.

Exposure Verification: Samples of stock and reference solutions will be collected for perchlorate content prior to initiation of the experiment. Additional samples from freshly prepared test solutions will be taken every 96 hr.

15.4 Test System Observation

Water quality, including salinity, conductivity, pH, and ammonia will be performed at least once per week. Temperature of a surrogate tank containing the same volume of water will be noted every day. Dead animals will be removed and preserved in 10% neutral buffered formalin (NBF).

15.5 Animal Sacrifice and Sample Collections

Animals will be sacrificed at 10 and 14 wk as appropriate. Animals will be quickly weighed and then euthanized in 3-aminobenzoic acid ethyl ester (MS-222, 1g/L, TIEHH AQ-1-03/ DBS AF-3-03) and rinsed in distilled water. The body cavity will opened and blood collected by cardiac puncture (TIEHH XXX/ DBS AQ-3-01) and frozen for subsequent TH radioimmunoassay (RIA; DBS IN-2-04). The carcass will be placed in Bouin's fixative (TIEHH MT-2-13/ DBS IN-1-05). The lower jaw and gonadal tissue will then be dissected out for subsequent histological analysis of adult tissues. Egg masses will be collected and frozen for subsequent TH RIA (DBS SOPs IN-2-01, IN-2-04, IN-2-05).

15.6 Endpoint Analysis

For adult frogs, plasma T_3, T_4, will be determined by RIA (DBS IN-2-04). Plasma estradiol and progesterone will also be determined if there is sufficient plasma samples remaining. Thyroid glands will be prepared using routine histological methods (TIEHH SOPs MT-4-09, MT-4-10, MT-3-03, MT-4-03, MT-4-08/ DBS/TCFWRU SOPs IN-1-05, IN-1-06, IN-4-06, IN-4-07, DBS SOPs IN-1-02, IN-1-01, IN-1-04). Sections will be stained with hematoxylin and eosin (TIEHH MT-3-03/ DBS IN-1-04). Thyroid gland activity will be assessed for evidence of follicular hypertrophy (DBS IN-2-09). The concentration of T_3, T_4, deposited into eggs will be determined by RIA (DBS SOPs IN-2-01, IN-2-04, IN-2-05).

16. PROPOSED STATISTICAL METHODS

Treatment differences will be analyzed by two-way analysis of variance for treatment length and concentration level.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include.

- Water temperature, salinity, pH, ammonia, and conductivity will be collected.

- Date, time and amount of feedings per tank will be recorded. Number of dead animals removed prior to termination of exposure will be recorded including each date and tank.

Report content will also include presentation of data, interpretation, and discussion of the following endpoints:

List individual endpoints and analyses.
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before February 28, 2003. Copies of all data, documentation, records, protocol information, and the specimens shall be sent to the Sponsor, or designated delivery point, upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

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Food Item Transfer of Perchlorate into Food: Laboratory Dosing with Perchlorate Contaminated Foods

STUDY NUMBER:	FIT-02-01
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	Texas Tech University Human Sciences Building Box 42002 Lubbock, TX 79409-2002
RESEARCH INITIATION:	4/28/02
RESEARCH COMPLETION:	12/31/02

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GOOD LABORATORIES PRACTICES STATEMENT

Project FIT-02-01, entitled, "Food Item Transfer of Perchlorate into Food: Laboratory Dosing with Perchlorate Contaminated Foods", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

Submitted By:

Ernest E. Smith, Ph.D.

3/28/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Audits were performed on making dosing solution, chemical application to rodent chow, and deer mouse euthanization. The Quality Assurance Officer notified the Study Director of all findings in writing following each audit.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	5-7-02	5-13-02	5-13-02	6-6-02
Test Material Application	11-1-02	11-4-02	11-5-02	11-5-02
Euthanasia	9-27-02	9-27-02	9-30-02	10-1-02
Final Report and Raw Data Review	1-6-03	1-19-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

3-28-03
Date

1.0 DESCRIPTIVE STUDY TITLE:

Response of Deer Mice and Voles to Perchlorate Contaminated Food and Water Following Oral Exposure

2.0 STUDY NUMBER: FIT-02-01

3.0 SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Cir
Brooks AFB, TX 7235-5123

4.0 TESTING FACILITY NAME & ADDRESS:

The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start Date: April 28, 2002
Termination Date: December 31, 2002

6.0 KEY PERSONNEL:

Ernest E. Smith, Co-Principal Investigator
Philip N. Smith, Co-Principal Investigator
Anna Herboldsheimer, Student Assistant
Lisa Perlmutter, Study Director
Lance Williams, Histopathologist
Angella Gentles, RIA/Histopathologist
Ryan Bounds, Quality Assurance Manager
James Surles, Statistical Support
Ron Kendall, Principal Investigator and Testing Facility Management

7.0 STUDY OBJECTIVES / PURPOSE:

To determine the response of deer mice and prairie voles to perchlorate by assessing general toxicity and histological end points of selected organs.

8.0 STUDY SUMMARY:

In this study, a comparison of the effect of exposure of rodents to perchlorate via food, water, or a combination of both was investigated. This experiment provides a realistic assessment of exposures that are likely to occur where environmental contamination with perchlorate occurs. Overall the toxicity of exposure to perchlorate was evident in alteration of thyroid histology. The vole's thyroid presented an interesting morphology that requires further evaluation and characterization. Although the data generated from exposure to perchlorate for 30 days or less resulted in limited general toxicological changes, we believe that perchlorate would induce significant alterations in general toxicity over a longer period of exposure to deer mice and voles. These changes would be associated with direct effect of perchlorate and indirectly through secondary effects of altered thyroid gland and thyroid hormone synthesis.

9.0 TEST MATERIALS:

Test Chemical:	Ammonium Perchlorate 99.999% pure
Source:	Sigma-Aldrich
Characterization:	Oxidizer; explodes when heated
Test Medium:	Deionized Water

10.0 JUSTIFICATION OF TEST SYSTEM

This project is intended to evaluate risks of perchlorate exposure among organisms consuming perchlorate-contaminated food. Rodents were used as surrogates to livestock (the most likely exposed organisms) because they are easily handled, permit large sample sizes, and are more economically feasible in a study of this nature. There are no other suitable models for assessing exposure risks in an ecological environmental setting.

This study represents the second phase of an ongoing project and requires live animals for each experiment and cannot be substituted with culture or computer generated models. Culture and computer models cannot simulate changes in general homeostasis and thyroid hormone alteration. In addition it would not provide pertinent scientific data for future use in risk assessment.

11.0 TEST ANIMALS:

Species:	Deer Mice and Prairie Voles
Strain:	Wild type
Age:	Juvenile (Post natal day PND 22) and Adults; respectively
Sex:	Males and Females
Number:	Deer mice = 249 and Prairie Voles = 48

Source: In house breeding colony and The University of Maryland, respectively.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each cage was labeled as indicated in TIEHH SOP IN-3-06, which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. Each cage was labeled to include sex of the individuals (if appropriate), date of birth, date of exposure, the name of the test substance and its concentration. Rodents were ear marked with unique identification numbers according to SOP ET-3-18.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Mice were dosed for 30 days. Voles were dosed for 21 days.

Perchlorate Treated Water Dosing Only:
Eleven male and 6 female deer mice were treated with 100nM (1.1749×10^{-2} ppm) ammonium perchlorate laden water. 8 male and 7 female deer mice were treated with 100µM (11.749 ppm) ammonium perchlorate laden water. Twelve male and 6 female deer mice were treated with 1mM (117.49 ppm) ammonium perchlorate laden water.
Nine male and 10 female prairie voles were treated with 700 ppb sodium perchlorate laden water.

Perchlorate Treated Food Dosing Only:
Ten male and 18 female deer mice were treated with 100nM ammonium perchlorate laden food. 14 male and 20 female deer mice were treated with 100µM ammonium perchlorate laden food. 18 male and 16 female deer mice were treated with 1mM ammonium perchlorate laden food.
8 male and 9 female prairie voles were treated with 2.1 ppm sodium perchlorate laden food.

Perchlorate Treated Food and Water Combination Dosing for 30 Day Study:
Eleven male and 10 female deer mice were treated with 100nM (1.1749×10^{-2} ppm) ammonium perchlorate laden food. 10 male and 9 female deer mice were treated with 100µM (11.749 ppm) ammonium perchlorate laden food. 10 male and 12 female deer mice were treated with 1mM (117.49 ppm) ammonium perchlorate laden food.
No prairie voles were used for this portion of the study.

Control:
There was only one group of controls for each species. 26 male and 15 female deer mice were used for the control group. 5 male and 5 female prairie voles were used for the control group.

Table 1 Number of rodents for experimental exposure.

Treatment	Deer mouse Female	Deer mouse Male	Vole Female	Vole Male
Water x4tmt	19	31	10	9
Food x 4tmt	54	42	9	8
Water/Food x 4tmt	31	31	0	0
Control	15	26	5	5
Total	119	130	24	22

Grand total for experimental exposure = 295

14.0 METHODS:

All personnel were required to read their appropriate material safety data sheets including, but not limited to ammonium perchlorate and sodium perchlorate. All laboratory personnel were required to wear gloves, lab coat, and hair restraints at all times when working with rodents and/or dosing solutions.

A pilot study was used to acclimatize the animal technicians and the study director to the study protocol. This initial study enabled the study director to work out the details of the full experiment as well as tissue collection for method validation following exposure to perchlorate contaminated food and/or water. All pilot studies and research involving rodents were conducted in accordance with Standard Operating Procedures (SOP's) described in the protocol.

When pilot studies were completed, prairie voles and deer mice were randomly assigned to control, perchlorate contaminated food (PCF), perchlorate contaminated drinking water (PCW), or a combination of PCF & PCW (with the exception of the prairie voles for PCF & PCW. Initial body weights were recorded for all rodents prior to initiation of the study. Rodents were caged individually because quantity of treated food and/or water consumption is an important variable that can not be obtained if rodents are housed together. The deer mice were housed in standard rodent cages (11 1/2 x 7 x 5 1/2) lined with aspen sani-chips and allowed to acclimate to test facility conditions of 72°F and 16:8 hours of light: dark for 24 to 48 hours prior to initiation of dosing. The prairie voles were housed in standard rodent cages (11 1/2 x 7 x 5 1/2) without lining because of our collection of consumption data. The prairie voles would mix their food with their bedding in turn making the collection of consumption data very difficult. They were allowed to acclimate to test conditions for five months, while the project details were being finalized. Since this is a toxicological study that is reasonably short, animals were not provided environmental enrichment because they would chew and consume these items and this would add an unknown variable to our assessment.

Water and food were available *ad libitum* throughout the experiments. Daily observations were made on all rodents. Animals showing signs of distress or disease were treated according to the instructions of the University Veterinarian.

All rodent handling was done according to Standard Operating Procedures.

14.1 Test System acquisition, quarantine, and acclimation.

PND 22 deer mice were obtained from our breeding colony at Texas Tech University and voles were purchased through The University of Maryland. The deer mice were maintained in standard cages lined with sani-chip bedding and kept on a 16L: 8D light regimen. The prairie voles were maintained in standard cages without lining and kept on a 12L: 12D light regimen.

14.2 Assignment of Animals to Study Group and Identification

Deer mice were ear punched on day 21 and were arbitrarily assigned to treatment groups. Voles also were arbitrarily assigned to treatment groups.

14.3 Test Material Application

Deer mice in the PCF group were fed a standard rodent chow in pellet form purchased from Harlan Teklad. Ammonium perchlorate was sprayed on the feed for the deer mice (SOP AF-1-07-01) at concentrations of control (0 ppm), 100 nanomolar (1.1749×10^{-2} ppm), 100 micromolar (11.749 ppm), and 1 millimolar (117.49 ppm). The feed for the prairie voles was custom made. Plant material was grown in soil and watered with sodium perchlorate (100 ppm) laden water resulting in an average plant concentration of 7840 ppm. Once there was a sufficient amount of plant material, it was sent to Purina's special blends laboratory in Richmond, Indiana and made into rodent chow. So, 10 grams of plant material was incorporated into 50 kg of Prolab Rabbit Diet 5P26, resulting in an average dose of 600ng of perchlorate per gram per day. This chow was then analyzed at The Institute of Environmental and Human Health (TIEHH) to give the resulting concentration of 2.1 ppm. Total dosage per animal was calculated by determining the mass of food consumed daily multiplied by the concentration of perchlorate in the food divided by mass of the rodent. Ammonium perchlorate was dissolved in the drinking water of deer mice and sodium perchlorate was dissolved in the drinking water of the prairie voles (SOP IN 3-05) in the PCW group Rodents in the control group were given deionized water and food not treated with perchlorate. Deer mice weights were recorded every 5 days (Thuett et al., 2002) and voles weights were recorded daily to evaluate weight gain or loss.

Rates/concentrations: Deer mice: ammonium perchlorate for food and water is 1.1749×10^{-2} ppm, 11.749 ppm, 117.49 ppm, or no treatment. Prairie voles: sodium perchlorate for food was 2.1 ppm and for water was 700 ppb.

Frequency: Test substances were supplied *ad libitum* and renewed as needed.

Route/Method of Application: PND25 deer mice were exposed to water and/or food contaminated with ammonium perchlorate for 30 days. Adult prairie voles were exposed to water or food contaminated with sodium perchlorate for 21 days. Route of exposure was oral.

14.4 Daily Observations

Animals were monitored daily for changes in general health. If any changes were observed, animals were treated according to the University Veterinarian.

14.5 Animal Euthanasia and Sample Collections

Treatment in the above manner was maintained for 30 days for deer mice and 21 days for prairie voles. At dosing days 31 and 22 respectively, all animals were weighed using an aluminum animal weighing pan with an even distribution of holes and a lid. After the rodents were weighed, they were rendered unconscious by carbon dioxide, bled, and euthanized by exsanguination and the following tissues were collected for the deer mice: livers, kidneys, hearts, brains, testes, ovaries, adrenal glands and thyroid glands and the following tissues were collected for the prairie voles: Livers, intestinal tracts, kidneys, hearts, muscle and thyroid glands. Blood samples were placed in EDTA tubes, centrifuged, and the resulting plasma was used in steroid and thyroid hormone assays.

14.6 Evaluations

Selected organs and blood samples (SOP ET-3-19) were collected according to Thuett, et al, (2002) for chemical and histological analyses.

15.0 RESULTS-DEER MICE

In this study adult male and female deer mice were exposed to perchlorate in food, water and food and water (food-water) simultaneously. Several one-way analyses of variance were used to determine overall levels of significance and Tukey's LSD was used to separate means. A one-way ANOVA revealed that sex did not influence the response of body weight or other somatic indices to AP treatment, therefore, data for male and female mice were treated as one set, except for the gonads (as expected between males and females) which were statistically significantly different ($p= 0.0001$).

AP (117 ppm) in food alone caused a significant reduction in body weight at PND 25 (Figure 1). AP in water alone did not significantly affect body weight of treated mice. These representative means are listed in Tables 1 and 2 below.

AP at 117 ppm (1mM) resulted in a decrease in body weight when compared to that of control. A separate one-way ANOVA revealed an overall significant difference for body weight at PND 25 ($p = 0.009$) (Figure 2), PND30 ($p = 0.002$)

(Figure 3), PND35 (p = 0.036) (Figure 4), PND40 (p = 0.032) (Figure 5), PND50 (p = 0.027) (Figure 6), and PND55 (p = .03) (Figure 7) for mice treated with AP in food –water.

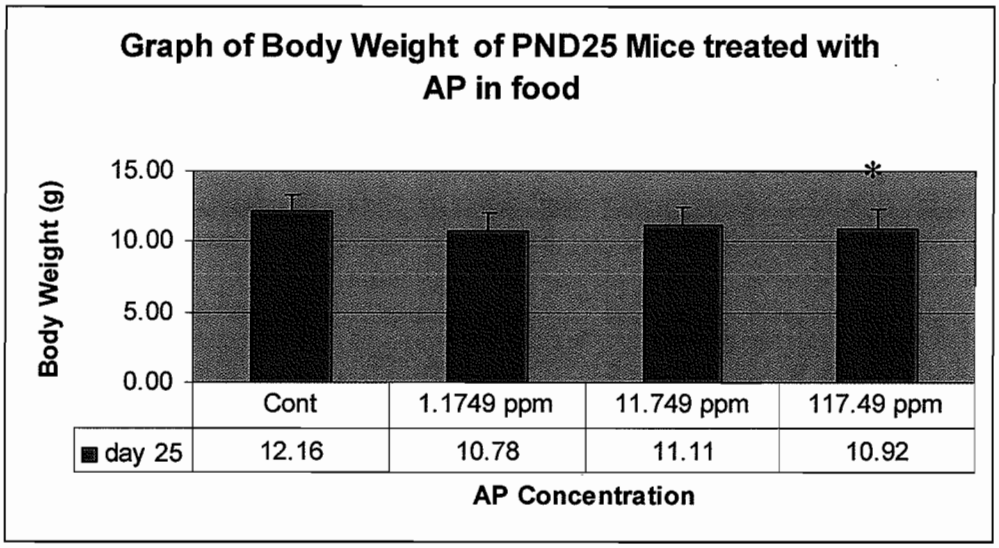

Figure 1 The effect of ammonium perchlorate in food on body weight of PND 25 mice.

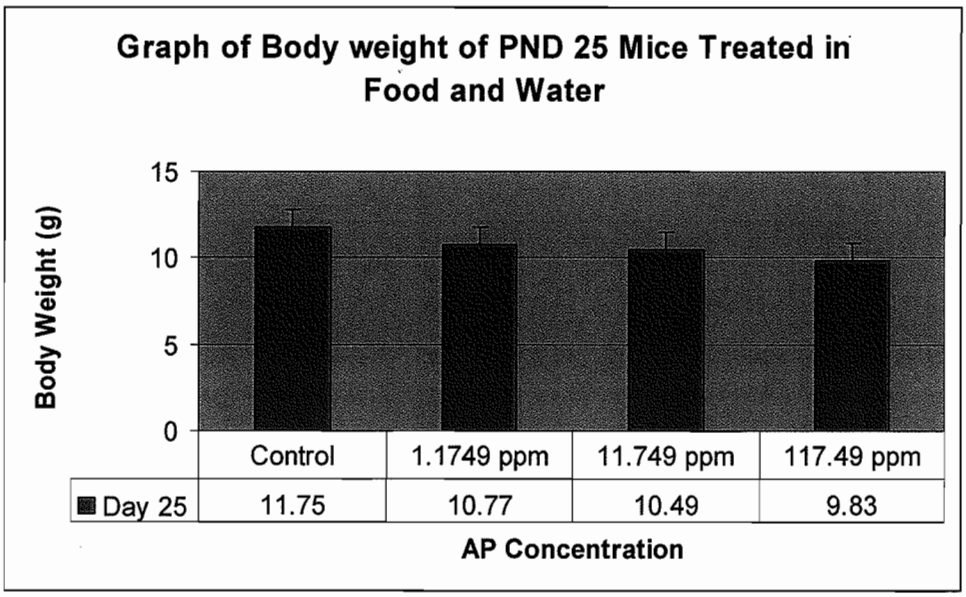

Figure 2 The effect of ammonium perchlorate in food and water on body weight of PND 25 mice.

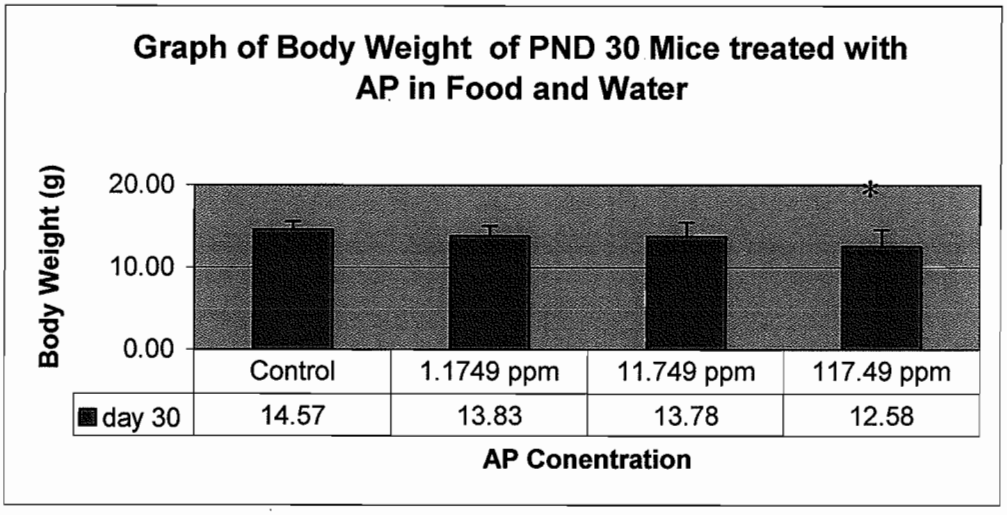

Figure 3 The effect of ammonium perchlorate in food and water on body weight of PND 30 mice.

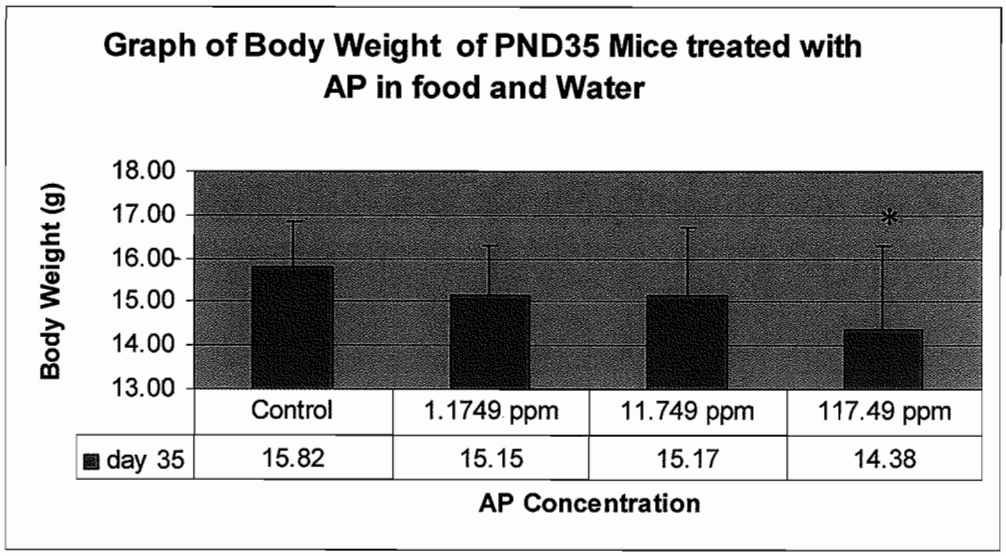

Figure 4 The effect of ammonium perchlorate in food and water on body weight of PND 35 mice

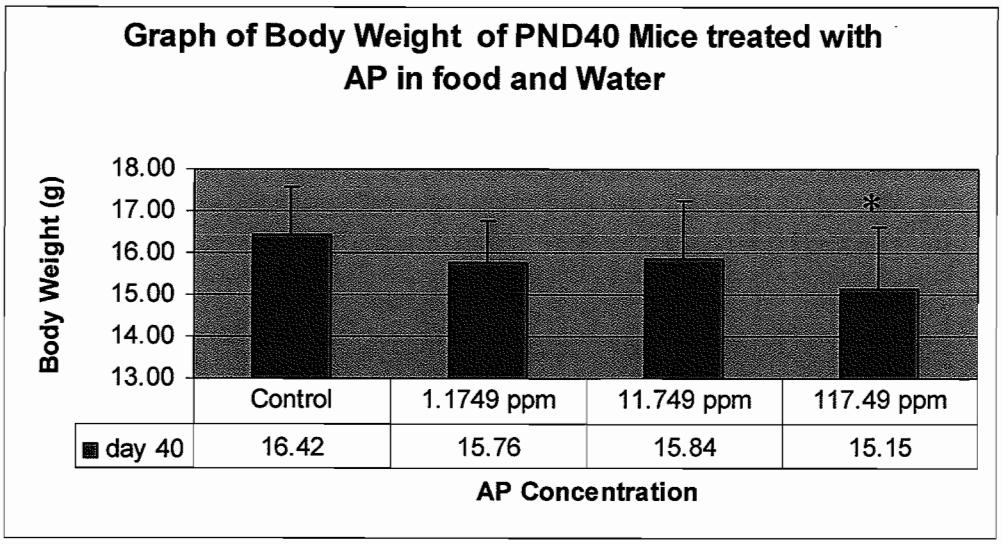

Figure 5 The effect of ammonium perchlorate in food and water on body weight of PND 40 mice.

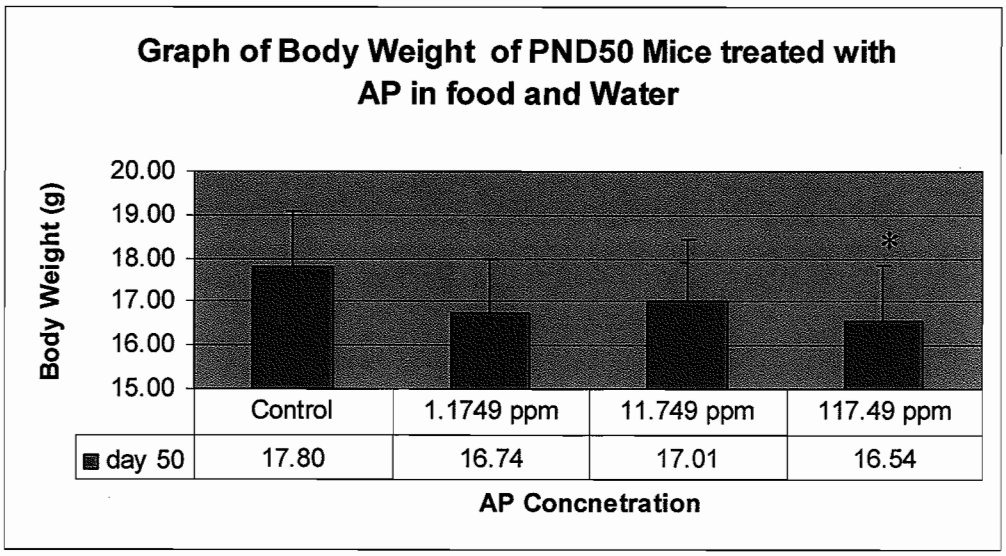

Figure 6 The effect of AP in food and water on body weight of PND 50 mice.

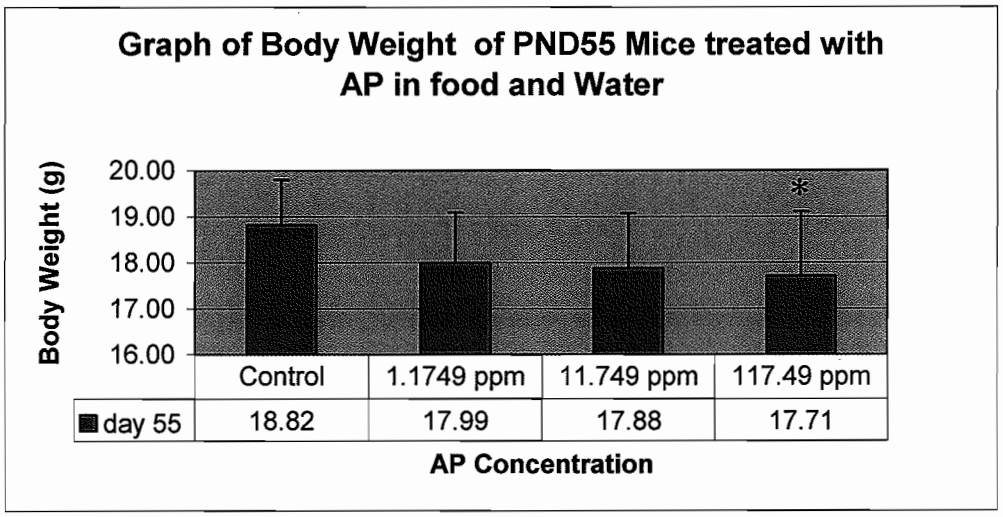

Figure 7 The effect of ammonium perchlorate in food and water on body weight of PND 55 mice.

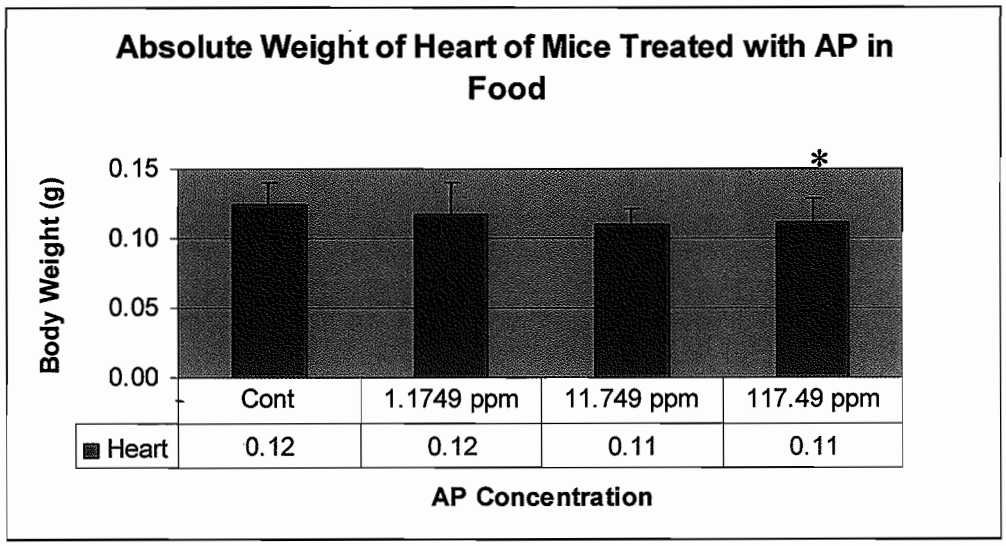

Figure 8 The effect of ammonium perchlorate on heart weight.

The following data (Tables 1-6) represent data for body and organ weights for food, water and food-water exposure scenarios. These endpoints were not statistically significantly different from the control groups. The data are represented as mean ± SD of the mean

Table 2. Average body weights from day 25 to day 55 of mice treated with AP in food.

AP[2]	Body Weights (g)[1]					
	Day 30	Day 35	Day 40	Day 45	Day 50	Day 55
Control	14.55 ± 1.05	15.57 ± 1.07	16.25 ± 1.15	17.15 ± 1.2	17.61 ± 1.25	18.41 ± 1.12
0.01mM	13.94 ± 0.87	15.54 ± 1.28	16.06 ± 1.40	16.69 ± 1.46	17.50 ± 1.53	18.04 ± 1.45
0.1mM	14.34 ± 1.35	15.53 ± 1.07	16.06 ± 1.22	16.56 ± 1.10	16.89 ± 1.35	17.76 ± 1.27
1.0mM	13.95 ± 1.41	15.35 ± 1.62	16.28 ± 1.65	16.85 ± 1.49	17.13 ± 1.67	17.79 ± 1.76
p-value	p = 0.33	p = 0.93	p = 0.92	p = 0.53	p = 0.34	p = 0.48

[1]Mean ± standards deviation
AP[2] = Concentration of ammonium perchlorate

Table 3. Showing average body weights from day 25 to day 55 of mice treated with AP in water

AP[2]	Body Weights (g)[1]						
	Day 25	Day 30	Day 35	Day 40	Day 45	Day 50	Day 55
Control	12.1 ± 1.2	14.7 ± 0.9	15.7 ± 1.4	16.4 ± 1.1	16.8 ± 1.2	17.3 ± 1.4	18.1 ± 1.2
0.01mM	11.1 ± 1.4	14.8 ± 1.3	15.2 ± 1.6	15.9 ± 1.6	16.3 ± 1.7	17.0 ± 1.4	18.8 ± 1.5
0.1mM	10.7 ± 1.9	13.9 ± 1.9	15.5 ± 2.4	16.4 ± 1.9	16.7 ± 2.3	17.2 ± 2.5	18.0 ± 2.4
1.0mM	11.5 ± 1.7	14.8 ± 1.4	15.7 ± 1.5	16.4 ± 1.3	16.9 ± 1.3	17.4 ± 1.4	18.1 ± 1.5
p-value	p = 0.13	p = 0.44	p = 0.85	P = 0.85	p = 0.76	p = 0.91	p = 0.97

[1]Mean ± standards deviation
AP[2] = Concentration of ammonium perchlorate

Table 4. Average organ weights of mice treated with AP in Food.

AP[2]	Organ Weights (g)[1]						
	Adrenal	Brain	Heart	Kidney	Liver	Ovary	Testes
Control	0.007 ± 0.002	0.46 ± 0.03	0.12 ± 0.02	0.24 ± 0.01	0.74 ± 0.13	0.025 ± 0.01	0.22 ± 0.02
0.01mM	0.009 ± 0.002	0.46 ± 0.03	0.12 ± 0.02	0.22 ± 0.02	0.75 ± 0.10	0.032 ± 0.01	0.20 ± 0.03
0.1mM	0.009 ± 0.003	0.46 ± 0.02	0.11 ± 0.01	0.22 ± 0.02	0.722 ± 0.08	0.027 ± 0.01	0.19 ± 0.02
1.0mM	0.010 ± 0.006	0.45 ± 0.02	0.11 ± 0.02	0.23 ± 0.02	0.74 ± 0.10	0.032 ± 0.01	0.20 ± 0.03
p-value	p = 0.32	p = 0.53	p = 0.72	p = 0.61	p = 0.69	P = 0.13	p = 0.08

[1]Mean ± standards deviation
AP[2] = Concentration of ammonium perchlorate

Table 5. Average Organ Weights of Mice Treated with AP in Water

AP[2]	Weights (g)[1]						
	Adrenal	Brain	Heart	Kidney	Liver	Ovary	Testes
Control	0.0092 ± 0.004	0.48 ± 0.08	0.12 ± 0.01	0.24 ± 0.03	0.71 ± 0.07	0.027 ± 0.00	0.22 ± 0.03
0.01mM	0.0087 ± 0.004	0.46 ± 0.05	0.11 ± 0.10	0.22 ± 0.02	0.70 ± 0.08	.036 ± 0.02	0.22 ± 0.02
0.1mM	0.0094 ± 0.003	0.45 ± 0.04	0.11 ± 0.04	0.22 ± 0.02	0.70 ± 0.09	0.08 ± 0.12	0.22 ± 0.04
1.0mM	0.0094 ± 0.005	0.45 ± 0.03	0.11 ± 0.01	0.23 ± 0.02	0.72 ± 0.07	0.023 ± 0.00	0.22 ± 0.03
p-value	p = 0.96	p = 0.32	p = 0.84	p = 0.61	p=0.919	p = 0.37	p = 1.00

[1]Mean ± standard deviation
AP[2] = Concentration of ammonium perchlorate

Table 6. Average Organ Weights of Mice Treated with AP in Food and Water

AP[2]	Organ Weights (g)[1]						
	Brain	Heart	Kidney	Liver	Ovary	Testes	Adrenal
Control	0.47 ± 0.03	0.13 ± 0.02	0.25 ± 0.02	0.78 ± 0.07	0.027 ± 0.01	0.21 ± 0.03	0.008 ± 0.003
0.01mM	0.46 ± 0.02	0.14 ± 0.02	0.23 ± 0.03	0.72 ± 0.08	0.027 ± .005	0.21 ± 0.03	0.009 ± 0.003
0.1mM	0.45 ± 0.03	0.12 ± 0.01	0.23 ±0.04	0.74 ± 0.09	0.030 ± 0.03	0.21 ± 0.03	0.008 ± 0.004
1.0mM	0.45 ± 0.04	0.12 ± 0.01	0.23 ± 0.04	0.75 ± 0.08	0.020 ± .006	0.18 ± 0.03	0.009 ± 0.003
p-value	p = 0.16	p = 0.59	p=0.178	p = 0.11	p = 0.54	p = 0.16	p = 0.81

[1]Mean ± standard deviation
AP[2] = Concentration of ammonium perchlorate

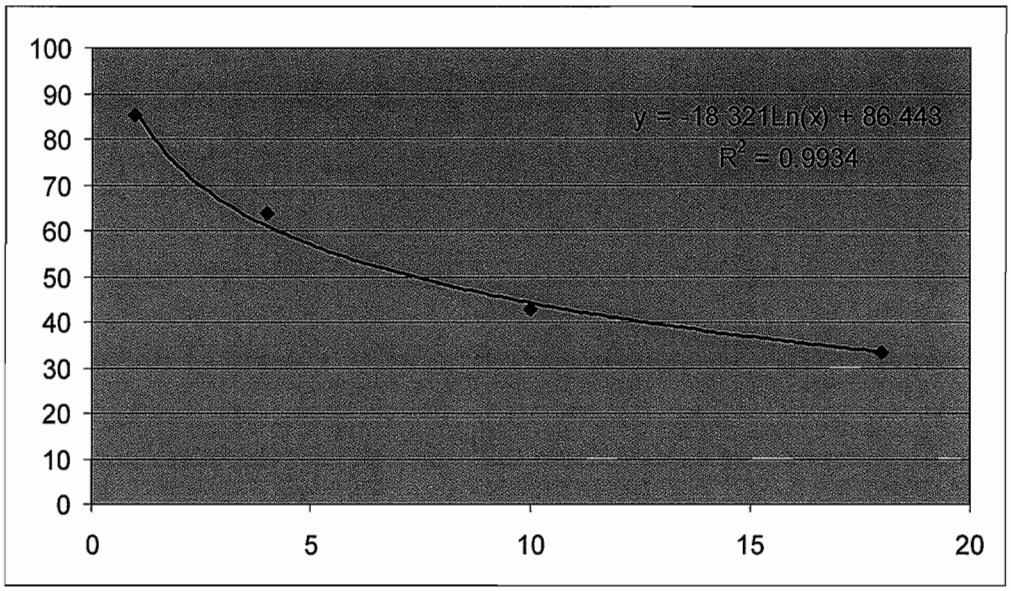

Figure 9 Standard Curve for Total T4 measurement

Table 7. Average Total T4 concentration in Deer Mice.

Exposure Route	T4 Concentration (µg/dl)
Control	3.378 ± 0.721
Food	3.281 ± 0.729
Food and water	2.714 ± 1.092
Water	3.768 ± 0.527

Thyroid Histological Analysis

Histological slides of the thyroid gland of three mice from the highest dose group of mice treated with AP in food, water, and food and water as well as three mice from the control group were assessed qualitatively for the effect of AP. Three sections from the beginning, the middle and the end of the thyroid were assessed in each of the mice. The sections were observed at 40X. The mice were arbitrarily chosen from each group.

In the control animals the thyroid glands had large follicles that were filled with colloid. The follicular cells in these thyroid glands were squamous to cuboidal in shape. No evidence of hyperplasia was observed in any section from the control thyroid glands in these animals. These sections contained few inactive follicles. See Figure 10

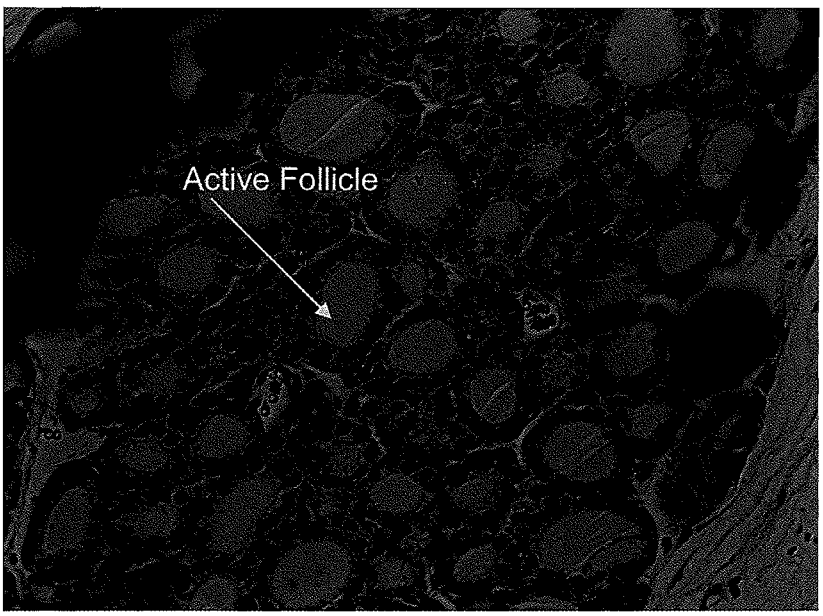

Figure 10 Photomicrograph of the thyroid gland from a control mouse.

Generally, the thyroid glands of mice treated with 1mM AP in food, water, or food and water showed hyperplastic regions (Figure 11 and 12) throughout the sections observed. The lumen of several of the follicles in these sections had a lacy appearance and some were void of colloid. Also, many microfollicles (Figure 12 and 13) were observed in these tissues. The follicular cells were columnar in shape and in many instances the cells appeared to be hypertrophic

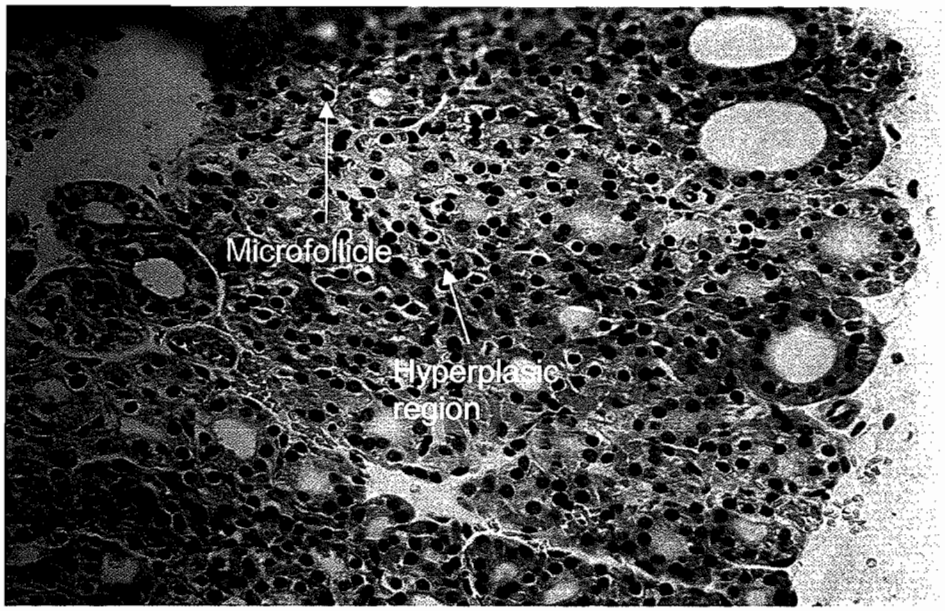

Figure 11 Photomicrograph of the thyroid from a mouse exposed to 1 mM AP in water.

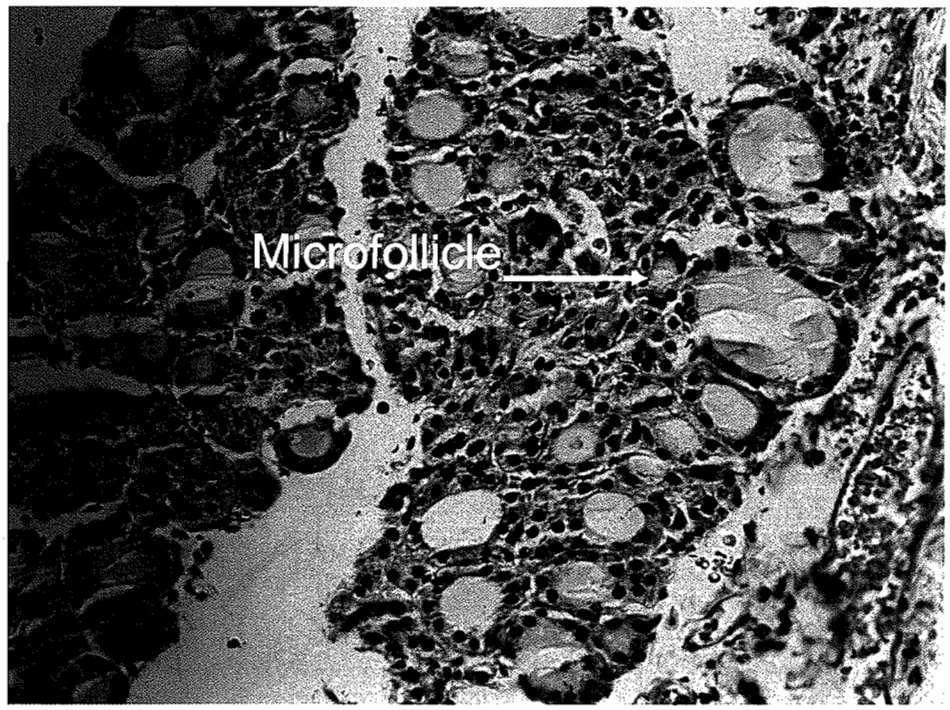

Figure 12 Photomicrograph of the thyroid from a mouse exposed to 1 mM AP in food.

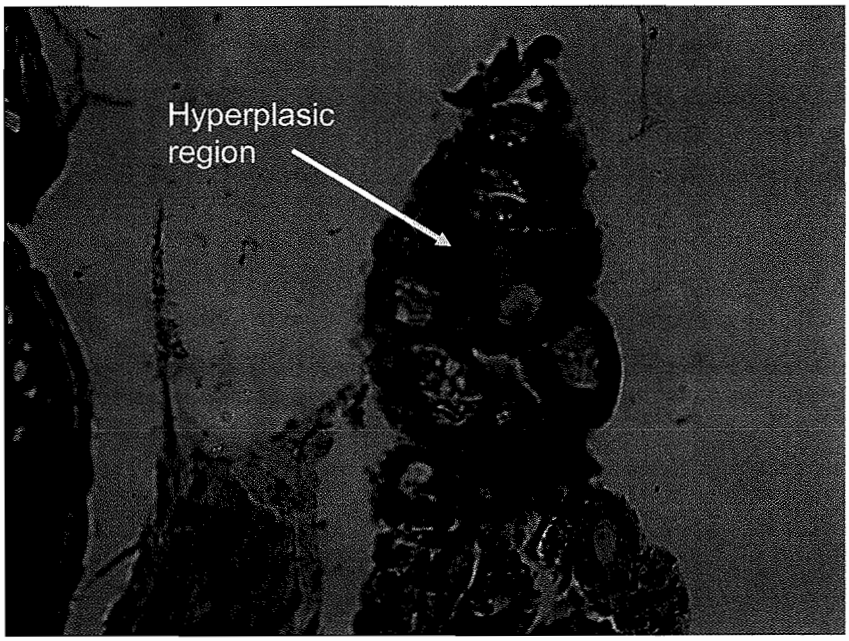

Figure 13 Photomicrograph of the thyroid from a mouse exposed to 1 mM AP in food and water.

16.0 DISCUSSION-DEER MICE

The developmental effect of *in utero* and lactational exposure to AP in drinking water was investigated in a previous set of experiments associated with this research program project. The results of that study indicated that AP at 1nM and 1mM caused an increase in plasma total T4 concentration, a reduction in the number of active follicles in the thyroid gland, and a reduction in body weight of neonatal animals (Thuett et al., 2002; 2003).

In this phase of the study, deer mice (PND25) were treated with AP in food, water, and food and water at concentrations of 1.1749ppm, 11.749ppm, and 117.49ppm. AP administered in food and water simultaneously at the 117.49 ppm level resulted in a significant reduction in body weight at PND 25, 30, 35, and 40 when compared to control animals. AP at concentrations of 117.49ppm and 1.1749ppm caused a slight but significant reduction in body weight of mice. At this time point body weight showed a hormetic response (Calabrese and Baldwin, 2003) which was also observed in a previous study (Thuett et al., 2002). Interestingly, by PND55 (30 days) of exposure AP at each concentration was effective in reducing bodyweight of the mice.

In the control animals the thyroid glands had large follicles that were filled with colloid. The follicular cells in these thyroid glands were squamous to cuboidal in shape. No evidence of hyperplasia was observed in any section from the control thyroid glands in these animals. These sections contained few inactive follicles.

AP in food alone caused a slight reduction in heart weight that approached statistical significance (p=0.048). When administered in food and water or water-alone AP had no effect on absolute or relative organ weight.
The difference seen in response when AP was administered via food and water as opposed to food-alone or water-alone is probably due to the fact that in the food and water group the animals were exposed to twice the amount of AP as in the food-alone group and the water-alone group. The concentration was not adjusted as it was expected

Generally, the thyroid glands of mice treated with 1mM AP in food, water, or food and water showed hyperplastic regions throughout the sections observed. The lumen of several of the follicles in these sections had a lacy appearance and some were void of colloid. Also, many microfollicles were observed in these tissues. The follicular cells were columnar in shape and in many instances the cells appeared to be hypertrophic.

The results of the somatic indices for the food-alone and water-alone exposure support the idea that AP is a developmental toxicant and that toxic response seen in more mature animals are secondary to AP thyrotoxicosis.

17.0 RESULTS-VOLES

The effect of exposure to sodium perchlorate via food or water was also investigated in adult voles. The animals were exposed to either 2.1ppm or 700ppb of sodium perchlorate in food or water, respectively. Separate one-way ANOVA on selected parameters revealed that sodium perchlorate had no statistically significant effect on average daily body weight, organ weight or plasma T4 concentration ($p > 0.05$). The descriptive statistics for these parameters are presented in Tables 7 and 8 below.

Table 8. Averages for Organ Weight and Plasma T4 Concentration in Voles.

Dose	Organ Weights (g) [1]					
	Intestine	Muscle	Heart	Kidney	Liver	T4 (µg/ml)
Control	4.79 ± 1.63	0.57 ± 0.20	0.25 ± 0.06	0.52 ± 0.17	2.06 ± 0.92	1.89 ± 0.75
Food	5.54 ± 1.80	0.67 ± 0.27	0.28 ± 0.09	0.54 ± 0.13	2.13 ± 1.03	1.99 ± 0.65
Water	6.08 ± 1.77	0.66 ± 0.31	0.28 ±0.07	0.57 ±0.12	2.33 ± 1.13	2.19 ± 0.92

Table 9. Averages for Body Weight of Voles.

Dose	Organ Weights (g) [1]		
	Week I	Week II	Week III
Control	49.38 ± 11.14	7.5825.98 ± 8.37	8.44 ± 2.56
Food	51.29 ± 15.16	25.31 ± 6.81	8.29 ± 2.25
Water	55.32 ± 14.20	27.87 ±	9.06 ± 2.90

Vole Thyroid- Histopathological Analysis

Histological slides of the thyroid gland of three voles per treatment group were assessed qualitatively for the effect of sodium perchlorate. Three sections from the beginning, the middle and the end of the thyroid were assessed in each of the voles. The sections were observed at 40X.

Generally, sodium perchlorate did not appear to affect the morphology of the thyroid gland from treated groups. The thyroid gland of animals in the control as well as the treated group had active follicle of varying sizes .The follicular cells are squamous to cuboidal in shape. The lumen of the thyroid follicles in the control group and the treated group were filled with colloid (Figure 14). The colloid however, appeared lacy around the periphery of the lumen in each group (Figure 15, and 16). The follicles seemed to be lined with a single layer of vacuoles around its periphery. This appearance was observed in follicles of various sizes and in all the treatment groups as well as the control group. The thyroid gland of treated and control animals had small regions of hyperplasticity. See Figure 14 through 17 below.

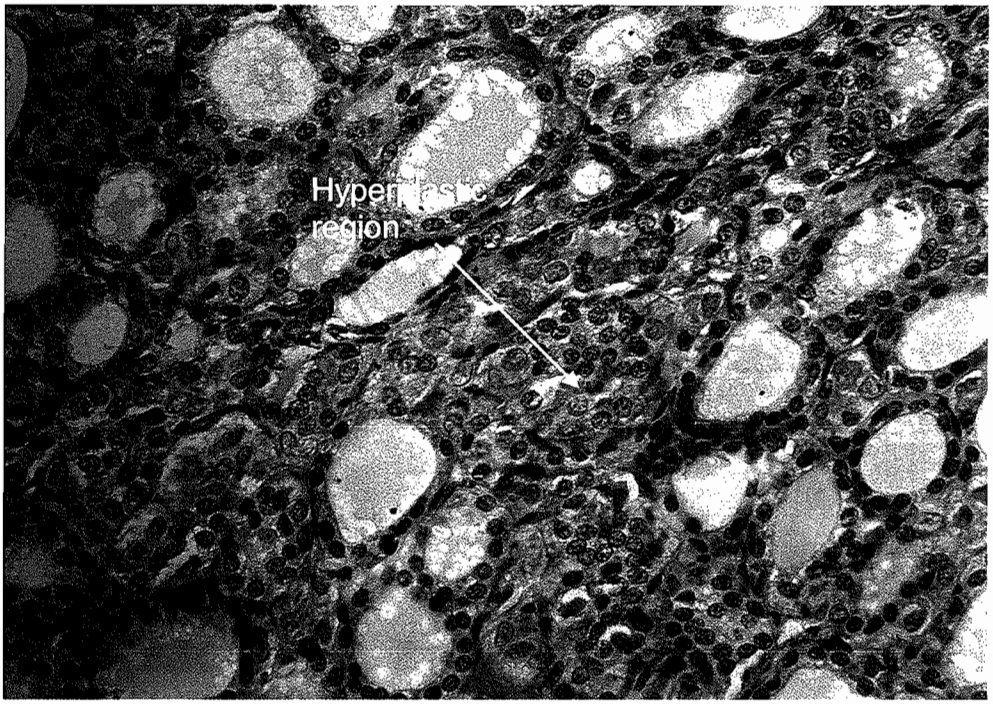

Figure 14 showing a photomicrograph of the thyroid from a control vole.

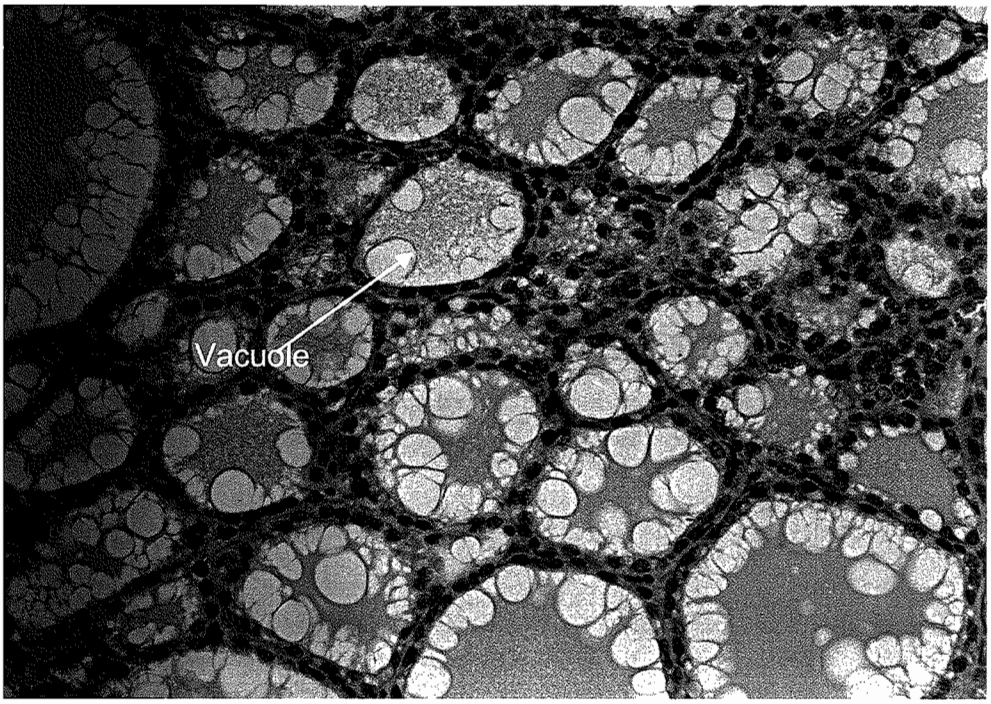

Figure 15 showing a photomicrograph of the thyroid from a control.

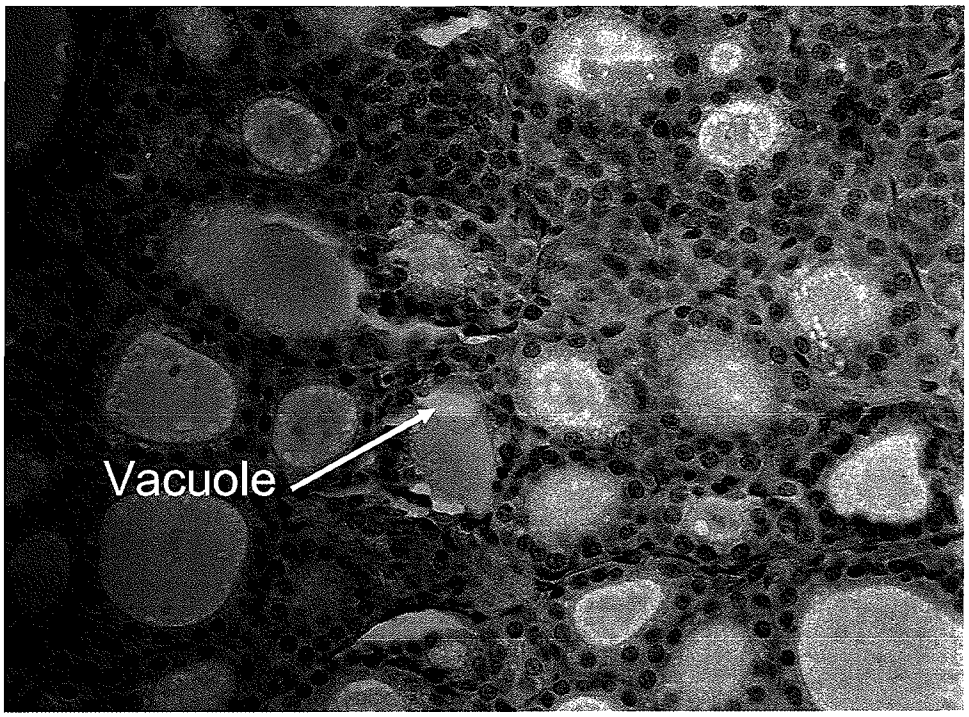

Figure 16 showing a photomicrograph of the thyroid from a vole treated with 2.1ppm sodium perchlorate in food.

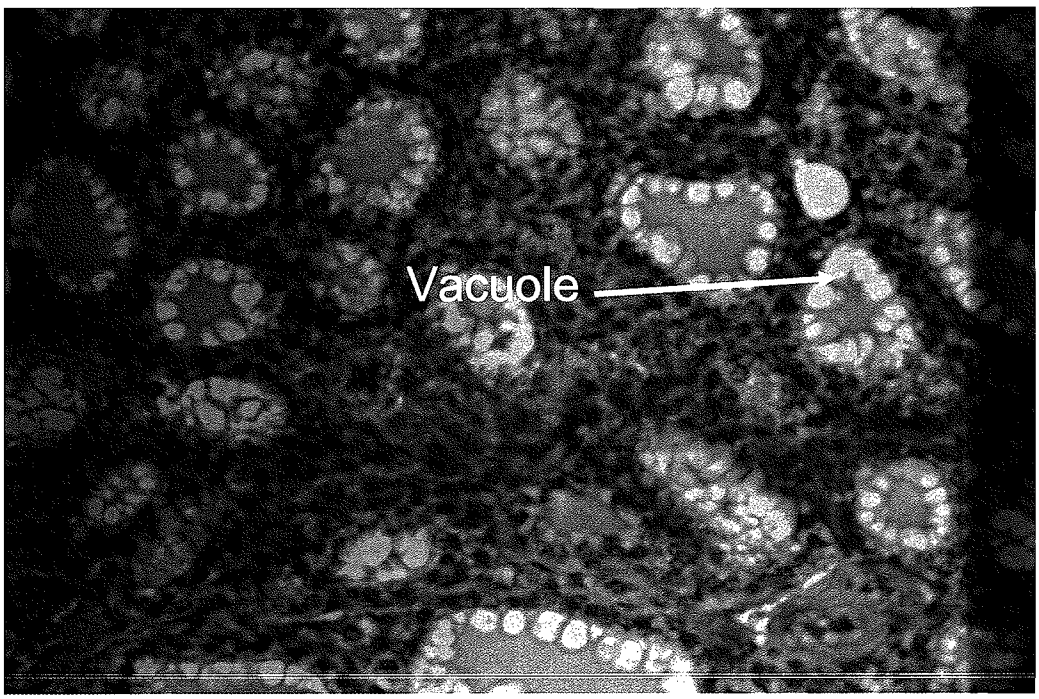

Figure 17 showing a photomicrograph of the thyroid from a vole exposed to 700ppb sodium perchlorate in water.

18.0 DISCUSSION-VOLES

Adult voles were treated with sodium perchlorate in food (2.1 ppm) or water (700 ppb) for 21 days. Statistical analysis of the results the above concentrations, did not reveal that perchlorate in either food or water had observable toxic effect on adult voles. Histopathological analysis of the thyroid gland, the target organ of perchlorate toxicity, revealed no difference in morphology when treated animals were compared to control animals. These results are probably an indication that subchronic exposure to low concentrations of perchlorate for this duration requires more sensitive endpoint for evaluation. It also indicates that perchlorate effect on adult voles should be evaluated over a longer duration of exposure and at other concentrations.

It was interesting to note, however, that there appeared to be a significant difference in morphology of the vole thyroid gland compared to that of the deer mouse. The follicles of the vole thyroid gland (in control and treated animals) appeared vacuolated around the periphery of the lumen as opposed to that of the deer mouse which has lumen that is colloid filled in normal animals. This also suggests that thyroid and related physiological endpoints should be further evaluated to establish baseline data for future risk assessment.

19.0 STUDY RECORDS AND ARCHIVE:

Study records will be maintained at The Institute of Environmental and Human Health Archive for a minimum of one year from study completion date.

20.0 REFERENCES

Calabrese EJ and Baldwin LA. 2003. Toxicology rethinks its central belief: Hormesis demands a reappraisal of the way risks are assessed. Nature. Vol. 421:691-692.

Thuett KA, Roots EH, Mitchell LP, Gentles BA, Anderson T, Kendall RJ, and Smith EE. 2002. Effects of *in utero* and lactational ammonium perchlorate exposure on the thyroid gland histology and thyroid and sex hormones ion developing deer mice (*Peromyscus maniculatus*) through postnatal day 21. J Toxicol. and Environ. Hlth. Part A 65: 2119-2130.

Project No.: T9700

A STUDY PROTOCOL

ENTITLED

Food Item Transfer of Perchlorate into Food: Laboratory Dosing with Perchlorate Contaminated Foods

STUDY NUMBER: FIT-02-01

SPONSOR: United States Air Force
AFIERA/RSRE
2513 Kennedy Cir
Brooks AFB, TX 7235-5123

TESTING FACILITY
Name/Address: The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. Ronald J. Kendall

Study Director: Ms. Lisa Perlmutter

PROPOSED EXPERIMENTAL START DATE: APRIL 28, 2002

1. **DESCRIPTIVE STUDY TITLE:**
Response of Deer Mice and Vole to Perchlorate Contaminated Food and Water Following Oral Exposure

2. **STUDY NUMBER: FIT-02-01**

3. **SPONSOR:**
United States Air Force
AFIERA/RSRE
2513 Kennedy Cir
Brooks AFB, TX 7235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: April 28, 2002
Termination Date: December 31, 2002

6. **KEY PERSONNEL:**
Ernest E. Smith, Co-Principal Investigator
Lisa Perlmutter, Study Director
Lance Williams, Histopathologist
Ryan Bounds, Quality Assurance Manager
James Surles, Statistical support
Ron Kendall, Principal Investigator and Testing Facility Management

7. **DATED SIGNATURES:**

E Smith	5/13/02	Dr. Ernest Smith Co-Principal Investigator
Lisa Perlmutter	5/13/02	Ms. Lisa Perlmutter Study Director
	5/16/02	Mr. Ryan Bounds Quality Assurance Manager
	3 Jun 02	Dr. Lou Chiodo Asst. Dir. for Science

5/15/02 Dr. James Surles
Statistical Support

6/6/02 Dr. Ron Kendall
Testing Facility Management

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

9. STUDY OBJECTIVES / PURPOSE:

To determine the response of deer mice and prairie voles to perchlorate by assessing general toxicity and histological end points of selected organs.

10. STUDY SUMMARY:

This study will consist of exposing rodents to food items containing ammonium perchlorate and comparing those accumulation and perchlorate-related endpoints to those of rodents exposed simply through water. This experiment provides a realistic assessment of exposures that are likely to occur where environmental contamination with perchlorate occurs.

11. TEST MATERIALS:

Test Chemical:	Ammonium Perchlorate 99.999% pure
Source:	Sigma-Aldrich
Characterization:	Oxidizer; explodes when heated
Test Medium:	Deionized Water

12. JUSTIFICATION OF TEST SYSTEM

This project is intended to evaluate risks of perchlorate exposure among organisms consuming perchlorate-contaminated food. Rodents will be used as surrogates to livestock (the most likely exposed organisms) because they are easily handled, permit large sample sizes, and are more economically feasible in a study of this nature. There are no other suitable models for assessing exposure risks in an ecological environmental setting.

This study represents the second phase of an ongoing project and requires live animals for each experiment and cannot be substituted with culture or computer generated models. Culture and computer models cannot simulate changes in general homeostasis and thyroid hormone alteration. In addition it would not provide pertinent scientific data for furture use in risk assessment.

13. TEST ANIMALS:

Species: Deer Mice and Prairie Voles
Strain: Wild type
Age: Juvenile (Post natal day PND 22)
Sex: Males and Females
Number: Approximately 836; Deer mice = 418 and Prairie Voles = 418
Source: In house breeding colony

14. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each cage will be labeled as indicated in TIEHH SOP **IN-3-06,** which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. Each cage will also be labeled to include sex of the individuals (if appropriate), date of birth, date of exposure, the name of the test substance and its concentration. Rodents will be ear marked with unique identification numbers according to SOP ET-3-18.

15. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Perchlorate Treated Water Dosing Only:
10 males and 10 females of each species (deer mice and prairie voles) X 4 treatment groups (perchlorate contaminated water; control (0 ppm), nanomolar (0.117 ppb), micromolar (117 ppb), and millimolar (117 ppm) = 160 rodents.

Perchlorate Treated Food Dosing Only:
10 males and 10 females of each species (deer mice and prairie voles) X 4 treatment groups (perchlorate contaminated food; control (0 ppm), nanomolar (0.117 ppb), micromolar (117 ppb), and millimolar (117 ppm) = 160 rodents.

Perchlorate Treated Food and Water Dosing for 30 Day Study:
10 males and 10 females of each species (deer mice and prairie voles) X 4 treatment groups (perchlorate contaminated food and water; control (0 ppm), nanomolar (0.117 ppb), micromolar (117 ppb), and millimolar (117 ppm) = 160 rodents.

Table 1. Number of rodents for experimental exposure.

Treatment	Deermouse Female	Deermouse Male	Vole Female	Vole Male
Water x4tmt	40	40	40	40
Food x 4tmt	40	40	40	40
Water/Food x 4tmt	40	40	40	40
Total	120	120	120	120

Grand total for experimental exposure = 480

TOTALS:

Table 1 = 480 + 10% mortality = 528;
Deer mice = 264 and Prairie Voles = 264.

16. METHODS:

All personnel will be required to read ammonium perchlorate material safety data sheet and must wear gloves, lab coat, and hair restraints at all times when working with rodents and/or dosing solutions.

A pilot study will be used to acclimate animal technicians and the study director to the study protocol. This initial study will enable the study director to work out the details of the full experiment as well as tissue collection for method validation following exposure to perchlorate contaminated food and water. All pilot studies and research involving rodents will be conducted in accordance with Standard Operating Procedures (SOP's) described in the protocol.

When pilot studies are completed, prairie voles and deer mice will be randomly assigned to control, perchlorate contaminated food (PCF), perchlorate contaminated drinking water (PCW), or a combination of PCF & PCW. Initial body weights will be recorded for all rodents prior to initiation of the study. Rodents will be caged individually because quantity of treated food and/or water consumption is an important variable that can not be obtained if rodents are housed together. They will be housed in standard rodent cages (11 1/2 x 7 x 5 1/2) lined with aspen sani-chips and allowed to acclimate to test facility conditions of 72°F and 16:8 hours of light:dark for 24 to 48 hours prior to initiation of dosing. Since this is a toxicological study that is reasonably short, animals will not be provided environmental enrichment because they will chew and may consume these items and this will add an unknown variable to our assessment.

Water and food will be available *ad libitum* throughout the experiment. Daily observations will be made on all rodents. Animals showing signs of distress or disease will be treated according to the instructions of the University Veternarian.

All rodent handling will be done according to Standard Operating Procedures.

16.1 Test System acquisition, quarantine, and acclimation.

PND 22 deer mice will be obtained from our breeding colony at Texas Tech University and voles will be purchased through a commercial supplier. They will be maintained in standard cages lined with sani-chip bedding and kept on an 16L: 8D light regimen.

16.2 Assignment of Animals to Study Group and Identification

Animals will be assigned randomly to treatment groups upon selection from the pool.

16.3 Test Material Application

Rodents in the PCF group will be fed a standard rodent chow in ground-up form purchased from Harlan Teklad or another reputable feed company. Ammonium perchlorate will be sprayed on the feed (SOP in development) given to the rodents at concentrations of control (0 ppm), nanomolar (0.117 ppb), micromolar (117 ppb), and millimolar (117 ppm). Total dosage per animal will be calculated by determining the mass of food consumed daily multiplied by the concentration of perchlorate in the food divided by mass of the rodent. Ammonium perchlorate will be dissolved in the drinking water of rodents (SOP IN 3-05) in the PCW group at concentrations that will provide an approximately equal daily dosage to rodents in the PCF group based on average daily water intake (as measured through weighing of water bottles). Drinking water concentrations will be adjusted throughout the study to insure similar exposure concentrations in PCW and PCF treatment groups. Rodents in the control group will be given deionized water and food not treated with ammonium perchlorate. Rodent weights will be recorded on the same day of each week during the study to evaluate weight gain or loss. The technique for applying ammonium perchlorate to rodent chow is in development and will be ammended to the protocol.

Rates/concentrations: perchlorate (0.117 ppb, 117 ppb, and 117 ppm), or no treatment.

Frequency: Test substances will be supplied *ad libitum* and renewed at cage change or when water bottles are below 50% of volume.

Route/Method of Application: PND22 animals will be exposed to water and/or food contaminated with ammonium perchlorate for 30 days. Route of exposure will be via oral.

16.4 Daily Observations

Animals will be monitored daily for changes in general health. Based on previous exposure no dead or moribund animals are expected to result from this exposure.

16.5 Animal Euthanasia and Sample Collections

Treatment in the above manner will be maintained for 30 days for all animals. At dosing day 31, all animals will be weighed using an aluminum animal weighing pan with an even distribution of holes and a lid. After the rodents are weighed, they will be rendered unconscious by carbon dioxide, bled, and euthanized by exsanguination and the following tissues will be collected: livers, kidneys, hearts, muscle, testes, ovaries, adrenal glands and thyroid glands. Blood samples will be placed in EDTA tubes, centrifuged, and the resulting plasma will be used in steroid and thyroid hormone assays.

16.6 Evaluations

Selected organs and blood samples (SOP ET-3-19) will be collected according to Thuett, et al, (2002) for chemical and histological analyses.

17. PROPOSED STATISTICAL METHODS

Organ weight and hormone concentration will be subjected to ANOVA and multi-comparison analysis.

18. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include animal receipt, animal care, test material preparation and application, animal observations, sex ratio results and facility records for personnel, equipment, etc.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

Study Methods
Survival of treatment animals
Chemical analysis results
Gonadal morphology summary
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used

19. RECORDS TO BE MAINTAINED LOCATION:

A final report containing the results of the dosing studies will be delivered to the Sponsor on or before **December 31, 2002**. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility for up to 3 years.

20. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to affect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

21. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

22. REFERENCES:

Thuett et al., 2002. Journal of Environmental Toxicology and Health

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Box 41163
Lubbock, TX 79409-1163
(806) 885-4567
qa@tiehh.ttu.edu

Form No. 014 Rev. 3.06/00
Project No.: T9700
*Change No: 01
Page: 1 of 1

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment ____ Deviation ✓ Addendums

Document Reference Information
Check One: ✓ Protocol ____ SOP ____ Other ____
Title: Food Item Transfer of Perchlorate into Food: Laboratory Dosing with Perchlorate Contaminated Fo
Dated: ____
Document # (if appropriate): FIT-02-01
Page #(s): 8
Section #: 16.5
Text to reference: Sample collections.

Change in Document: Originally we proposed to pull muscles from the deer mice. However, we are not pulling muscle, we are pulling the brains instead.

Justification and Impact on Study: The brain is more useful to us than the muscle. No impact on study.

Submitted by: Signature: Lisa Perlmutter Date: 10/01/02
Authorized by: Study Director: Lisa Perlmutter Date: 10/01/02
Received by: Quality Assurance Unit: Brian Budwell Date: 10/1/02

* Sequentially numbered in order of the date that the change is effective

TIEHH
Box 41163
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Form No. 014 Rev. 3.06/00
Project No.: T9700 FIT-02-01
*Change No: 02
Page: 1 of 1

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ✓ Amendment ____ Deviation ____ Addendums

Document Reference Information
Check One: ____ Protocol ✓ SOP ____ Other _____
Title: Food Item Transfer of Perchlorate into Food: Laboratory Dosing with Perchlorate Contaminated Food
Dated: 12/11/02
Document # (if appropriate): FIT-02-01
Page #(s): 8
Section #: 16.3
Text to reference: SOP in development

Change in Document: SOP AF-1-07

Justification and Impact on Study: SOP was written and approved by QA/QC department. Impact on study is ~~complay~~ (SE) 12/11/02 compliance with GLP.

Submitted by: Signature: Lisa Perlmutter Date: 12/11/02
Authorized by: Study Director: Lisa Perlmutter Date: 12/11/02
Received by: Quality Assurance Unit: Brian Bridwell Date: 12-11-02

* Sequentially numbered in order of the date that the change is effective

TITLE

Response of Mallards to Perchlorate Contaminated Food and/or Water Following Oral Exposure

STUDY NUMBER:	AELS-02-02/13803.6100
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	Springborn Smithers Laboratories 2900 Quakenbush Rd. Snow Camp, North Carolina 27349
RESEARCH INITIATION:	12/17/02
RESEARCH COMPLETION:	01/14/03

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GOOD LORATORIES PRACTICES STATEMENT

Project AELS-02-02/13803.6100 entitled, "Response of Mallards to Perchlorate Contaminated Food and/or Water Following Oral Exposure" was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in (40 CFR Part 160, August 19, 1989).

Submitted By:

Ernest E. Smith, Ph.D

3/28/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Audits were performed on Mallard testing facilities, euthanization and tissue collection. The Quality Assurance Officer notified the Study Director of all findings in writing following each audit.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	12-17-02	12-18-02	12-16-02	1-14-03
Euthanasia and Tissue Collection	1-14-03	1-14-03	1-21-03	1-28-03
Facility Inspection	1-14-03	1-14-03	1-21-03	1-28-03
Final Report	3-3-03	3-20-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/03
Date

1.0 DESCRIPTIVE STUDY TITLE:

Response of Mallards to Perchlorate Contaminated Food and/or Water Following Oral Exposure

2.0 STUDY NUMBER: AELS-02-01

3.0 SPONSOR:

United States Air Force
AFIERA/RSRE
2513 Kennedy Cir
Brooks AFB, TX 7235-5123

4.0 TESTING FACILITY NAME & ADDRESS:

Springborn Smithers Laboratories
2900 Quakenbush Rd.
Snow Camp, North Carolina 27349

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start Date: December 17, 2002
Termination Date: January 14, 2003

6.0 KEY PERSONNEL:

Ernest E. Smith, Study Director
Jennifer Stafford, Testing Facility Management
Lance Williams, Research Technician
Brian Birdwell, Quality Assurance
Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:

The study objective was to determine the response of mallards to perchlorate by assessing general toxicity and histological end points of selected organs.

8.0 STUDY SUMMARY:

This experiment provides a realistic assessment of exposures that are likely to occur where environmental contamination with perchlorate occurs. In this study birds were exposed to ammonium perchlorate in water,food, and food and water. Biomarkers of exposure, liver AP accumulation, and other toxic endpoints were. We observed alteration of thyroid gland morphology, body weight and organ weight following exposure to perchlorate.

9.0 TEST MATERIALS:

Test Chemical:	Ammonium Perchlorate 99.999% pure
Source:	Sigma-Aldrich
Characterization:	Oxidizer; explodes when heated
Test Medium:	Deionized Water

10.0 JUSTIFICATION OF TEST SYSTEM

This project was intended to evaluate risks of perchlorate exposure among organisms consuming perchlorate-contaminated food and/or water. Birds were used as surrogates to livestock (the most likely exposed organisms) because they are easily handled, permit large sample sizes, and are more economically feasible in a study of this nature. Mallards (*Anas platyrhyncus)* provide a useful avian model of exposure and toxicity. Mallards are suitable models for assessing exposure risks in an ecological environmental setting.

This study represents the second phase of an ongoing project and requires live animals for each experiment and cannot be substituted with culture or computer generated models. Culture and computer models cannot simulate changes in general homeostasis and thyroid hormone alteration. In addition it would not provide pertinent scientific data for furture use in risk assessment.

11.0 TEST ANIMALS:

Species:	Mallards (*Anas platyrhyncus)*
Strain:	Wild type
Age:	Adults
Sex:	Mixed
Number:	100 mallards
Source:	Purchased from Whistling Wings, Hanover, Illinois

The mallard (*Anas platyrhyncus*) represents a wildlife species that can be potentially exposed, both directly and indirectly, to ammonium perchlorate in the wild. In addition, they permit large sample sizes and are economically feasible in this type of study. They provide useful avian models of exposure and toxicity and are a standard U.S. EPA test species. Therefore, mallards represent the ideal waterfowl model for exposure risks in an ecological setting. Oral exposure to pesticides is considered appropriate because free-ranging waterfowl, especially dabbling ducks, may ingest pesticides while feeding both on land and in water, and their dabbling style of feeding disturbs sediments that may hold pesticide run-off.

The mallards for this study were obtained from Whistling Wings, Hanover, Illinois. Birds were phenotypically indistinguishable from wild stock. The birds obtained from the supplier were shipped on the day of hatch and arrived at CRC at 2 days of age. Birds

were group housed and acclimated to the testing conditions for 7 days prior to initiation of this study. There was no mortality or abnormal behaviors observed in the colony within 72 hours prior to treatment initiation.

The test system was eight days old at treatment initiation. Test birds weighed between 75.7 and 152.4 g at experimental start. Control and treatment birds used in the study were from the same source and hatch. Birds were group housed at five males and five females per cage upon receipt. Due to mortality during the early days of acclimation (as expected with hatchling ducklings), the number of females in each cage were reduced to 4 to maintain equal representation of this gender and equal number of test animals in all cages prior to treatment.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each animal was labeled using a wing band with a unique identification number. Each cage was labeled with a tag which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. As well as, date of exposure, the name of the test substance and its concentration.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Perchlorate treatment was administered via drinking water only, via feed only and via water plus feed. There were 10 ducklings per treatment group plus one control group. Each treatment scenario consisted of three exposure concentrations (4 ppm, 40 ppm, 80 ppm). The control group received feed and water with no perchlorate added. Table 1 shows the number of birds per group.

Table 1. Number of ducks in each experimental exposure group

Treatment	**Feed Exposure**	**Water Exposure**	**Feed & Water Exposure**	**Total Ducklings**
Control	NA	NA	NA	10
4 ppm	10	10	10	30
40 ppm	10	10	10	30
80 ppm	10	10	10	30
Total	**30**	**30**	**30**	**100**

Total ducks in the experiment = 100
NA = Not applicable

14.0 METHODS:

Prior to any studies and pending approval all personnel were required to read ammonium perchlorate material safety data sheet and must wear gloves, lab coat, and hair restraints at all times when working with birds and/or dosing solutions.

Mallards were randomly assigned to treatment groups. Initial body weights were recorded for all birds prior to initiation of the study. There were 2 to 3 mallards per cage, housed in stacked cages (91 x 71 x 27 cm) in test facility conditions of 72°F and 18:6 hours of light:dark.

Water and food (Bluebonnet poultry feed) were available *ad libitum* throughout the experiment. Daily observations were made on all mallards. Animals showing signs of distress or disease were treated according to the instructions of the University Veternarian.

All avian handling was done according to Standard Operating Procedures.

14.1 Test System acquisition, quarantine, and acclimation.

Ducklings were obtained from Whislting Wings game bird farm located in Hanover, Illinois. For the first week of life, temperatures in the immediate vicinity of the heat source in the brooder units were maintained at approximately 35 to 38 °C. Areas of the brooder units most distant from the heat source may approach room temperature. This temperature gradient is desired to allow the ducklings to seek their own comfort zone at various times throughout the day. Maximum and minimum temperatures, within the brooder units and within the study room were monitored and recorded daily. Temperature within the testing room (outside of brooder units) was maintained at 20 to 30 °C. By the end of week 2 of life, brooder temperatures did approximate room temperature. Maximum and minimum humidity was recorded daily. The target relative humidity range is 40% to 80%. However, because the study room floor is washed down with clean water daily, brief periods of higher humidity may occur. Adequate ventilation was maintained. A photoperiod of 14 hours light and 10 hours dark per day was maintained for the duration of the test. Room lighting may be either incandescent or fluorescent. Lights were positioned so that all pens receive similar illumination. An average light intensity of not less than 6 foot candles was maintained at pen level. Ducklings were given a minimum of a one-week quarantine and acclimation period prior to exposure to the test substance.

14.2 Assignment of Animals to Study Group and Identification

Assignment of Groups

Each individual bird was weighed and identified by a uniquely numbered wing tag upon receipt. Assignment of animals to cages, and of cages to test groups were randomized using EXCEL. Cage assignment was carried out by randomizing the cage numbers, and placing the first tagged and weighed male in the first cage of the randomized list. The second male was placed in the second cage on the list. This process continued sequentially until each cage held one male mallard. This process repeated in the randomized order until all cages held five males. The same process was carried out when assigning females to the same cages. Cage assignment was done at animal receipt. Initially, ten animals (five males and five females) were placed in each cage. However, due to mortality experienced during the first 2 days after receipt, numbers were adjusted to accommodate four females per cage prior to treatment. To maintain equal numbers of test animals, and equal gender distribution in all cages, nine birds (five males and four females) were assigned to each of ten test cages, with each cage corresponding to a unique test group. Extra animals not assigned to test cages were euthanized after treatment initiation.

Prior to treatment, body weights among treatment groups were tested for normal distribution, homogeneity of variance and mean differences via Chi-Square, Levene's test, and ANOVA with Dunnett's test, respectively, in TOXSTAT 3.5 (West, Inc., 1996).

14.3 Test Material Application

Ammonium perchlorate exposure was accomplished in three ways: 1) dissolved in the drinking water of one test group, 2) in feed for a second test group and 3) in both water and feed in a third test group. In each of the exposure test groups, the test material was applied to the feed and water at concentrations of 4 ppm, 40 ppm, and 80 ppm. Ten birds in a single control group were given untreated deionized water and feed. Duckling weights were recorded just prior to initiation of exposure and on the same day of each week during the study to evaluate weight gain or loss. A schematic of the study design follows:

	Water	Treated Feed	Treated Treated Feed and Water
Control (10 ducks)	NA	NA	NA
4 ppm	10 ducks	10 ducks	10 ducks
40 ppm	10 ducks	10 ducks	10 ducks
80 ppm	10 ducks	10 ducks	10 ducks

14.4 Feed and Water

Purina Game Bird Startena (Purina Mills, St. Louis, Missouri), Lot 063 OCT 24 02 was provided as the basal feed. A basal feed component list is available in Appendix II. A representative sample from the lot of feed used during this study was analyzed by GeoLabs, Braintree, Massachucetts, for the presence of pesticides, PCB's and toxic metals using U.S. EPA procedures. None of these compounds were detected at concentrations that are considered toxic in any of the samples analyzed. Therefore, the food source was considered to be of acceptable quality.

Water was supplied from a well located at the test facility. Representative samples of the water are periodically analyzed by GeoLabs, Braintree, Michigan, in agreement with ASTM (2000) standard practices, for the presence of pesticides, PCB's and toxic metals. None of these compounds were detected at concentrations that are considered toxic in

any of the samples analyzed. Treated and clean feed and treated and clean water was provided to the test organisms *ad libitum.*

14.5 *Dosing Solution Preparation and Administration*

The nominal oral concentrations chosen for the definitive exposure were 0 (Control), 4, 40, and 80 mg/kg feed and/or water.

To incorporate the test substance into feed, the material was weighed into a glass beaker and sprinkled over the feed, which was pre-weighed and placed in a Hobart mixer. The feed and test substance were mixed for 20 minutes in the Hobart to allow thorough mixing. Mixed feed was stored in 6 gallon, plastic buckets, sealed with screw-on lids, and the were buckets labeled by study number and concentration level. Feed was offered to the test system in rectangular, anti-scratch feeders, and kept to a level that allowed access *ad libitu*m.

In preparation for water mixing, the test substance was again weighed into a glass beaker. Six, 6 gallon buckets (hereafter termed "mixing buckets") were pre-labeled with study number and concentration level. The six mixing buckets allowed for two batches of water to be mixed and available for each of the two cages offering treated water at the indicated levels. Each mixing bucket was calibrated by adding 19 L (19 kg, 5 gal) of water, and using a permanent marker to mark the inside of the bucket at the top edge of the water. Then, when treated water had to be prepared, a hose with a strong spray nozzle was used to deliver tap water into the mixing bucket, and incorporate the test material. Test material was added after the hose began delivering water. The hose was used to rinse the beaker contents into the mixing bucket. The strong spray of water agitated the water and facilitated complete mixing and dissolution of the test substance. If extra water mixes were prepared, they were stored in these mixing buckets, with the lids on, and re-mixed with a paint-stirring attachment on a power drill, prior to delivery to the test cage. Each test cage that was selected to receive treated water was equipped with a platform attached above one corner of the cage. Upon this platform was placed a 6 gallon PVC bucket (hereafter termed "delivery bucket") with an adjustable spigot on the side towards the bottom. Each cage's delivery bucket remained in place for the duration of the study, and the lids were on at all times except when a fresh supply of water was being added. Vinyl tubing carried the water from the delivery buckets to the automatic waterers within the cage. Cages not receiving treated water had the same automatic waterers, but the water was routed directly from the tap rather than through a delivery bucket. The tap used for water supplies was the same used for both treated and untreated water. The treated water was pumped from the appropriate mixing bucket into the delivery bucket via a small electrical pump. Water was delivered to the 4 ppm delivery buckets first, then the 40 ppm buckets, then the 80 ppm buckets. When all delivery buckets were filled, the hoses and pump were rinsed by pumping approximately 5 gallons of clean tap water through the system. This system allowed accurate mixing of the test substance, and refreshing of each cage's water supply as needed. All water supplies were kept to a level that allowed access *ad libitum.*
Rates/concentrations: 0, 4, 40 and 80 ppm perchlorate.

Frequency: Test substances were supplied *ad libitum* for 14 and 28 days.

Route/Method of Application: Ducklings were exposed to: a) treated water b) treated feed, or c) a combination of treated feed and water for either 14 or 28 days. Route of exposure will be oral.

Rates/concentrations: perchlorate (0.117 ppb, 117 ppb, and 117 ppm), or no treatment.

Frequency: Test substances were supplied *ad libitum* and renewed every day.

Route/Method of Application: Mallards were exposed to water contaminated with ammonium perchlorate for 30 days. Route of exposure will be via oral.

14.6 Daily Observations

Animals were monitored daily for changes in general health. If any serious changes are observed, animals were treated according to the recommendations of the consulting Veterinarian.

14.7 Animal Euthanasia and Sample Collections

At the end of exposure exposure to perchlorate, animals were weighed according to standard laboratory procedures. After the ducklings were weighed, each group was bled, and euthanized by CO_2 asphyxiation and the following tissues were collected: livers, kidneys, hearts, breast muscle, testes, ovaries, adrenal glands, thyroid glands. Blood samples were centrifuged, the resulting plasma drawn off and placed in separate vials and frozen. Samples were used in steroid and thyroid hormone assays.

14.8 Statistical Analysis

Statistical analyses for this study utilized analysis of variance followed by Tukey's multiple comparison test for separation of means. This study was designed to evaluate difference between toxicological efficacy of exposure to AP in food, water and food and water combined. Additional sets of ANOVA were utilized to analyze the effects of exposure to perchlorate via food, water and food and water.

The body weights of the experimental and control mallards were taken once weekly during the Phase I and II exposure period. The recorded data were subjected to ANOVA and Tukey's multicomparison for separation of means. The first series of ANOVA was used to test the effect of exposure to food, water, and food and water combined at all concentrations. The results revealed that there were significant effects by concentration and exposure scenarios. Consequently, additional ANOVAs were used evaluate constant concentration with varying exposure media or constant media with varying concentration. For example one set of ANOVA evaluated the 4ppm concentration with food, water and food-water. The other series of ANOVA's were used to determine 4ppm 40ppm and 80ppm with food only etc.

15.0 RESULTS

General Observations and Mortality

No abnormal observations attributable to the test substance were observed during Phase I or II exposure periods. During the ducklings rapid growth, the wing tags on eight birds slipped below their wrist and began to constrict the wing as it grew. This situation was noticed on day 17, and was remedied by removing the slipped wing tags and replacing them with new ones. None of the animals with replaced tags exhibited serious side effects or permanent damage from the temporary constriction by the slipped wing tags or from the replacement procedure. No fatalities occurred during Phase I or II of this study.

Gross Tissue Morphology

Two abnormal observations were made during day 28 dissection activities. The left liver lobes of a male in cage 4 (tag no.17, 4 ppm feed group), and a female in cage 2 (tag 56, 40 ppm feed group), were small, suggesting atrophy, but were normally vascularized and shaped. Both lobes of these livers exhibited spotty hemorrhaging.

Body Weight: Phase I

It was determined by ANOVA that the body weights of birds exposed to AP in food, water or food and water during Phase I of this study were not significantly different from control p= 0.548, 0.625 and 0.619, respectively, for the mean weights recorded in Phase I.

Tissue weights:Phase I

Evaluation of all exposure scenarios and concentration revealed an overall level of statistical significance (p=0.039) for the liver weight. However, Tukey's separation of means test did not indicate a difference between means. As a result the concentration and exposure media were evaluated by separate ANOVA's. See Figure 1.

Kidney weights (Figure 3) were found to be statistically different (p=0.021) among groups when birds were exposed to constant (40 ppm) concentrations of AP in food, food-water and water alone. AP at 40ppm in food alone caused a significant increase in kidney weight relative to control.

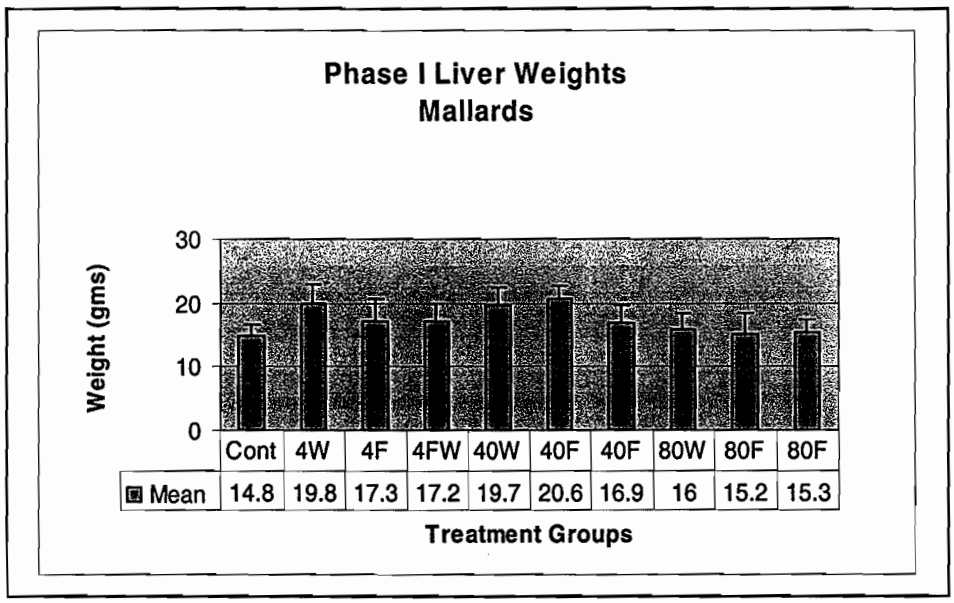

Figure 1. Mallard duckling liver weight following exposure to ammonium perchlorate. Cont=Control; 4W= 4ppm AP in water; 4F = 4ppm AP in food; 4FW = 4ppm AP in food and wate; 40W= 40ppm AP in water; 40F = 40ppm AP in food; 40FW = 40ppm AP in food and water; 80W= 80ppm AP in water; 80F = 80ppm AP in food; 80FW = 80ppm AP in food and water.

Statistical evaluation of the food as the exposure medium and varying concentrations of AP revealed significant differences for liver weight (Figure 2) when compared to the control (p=0.015). AP at 40ppm in food caused a significant increase in liver weight.

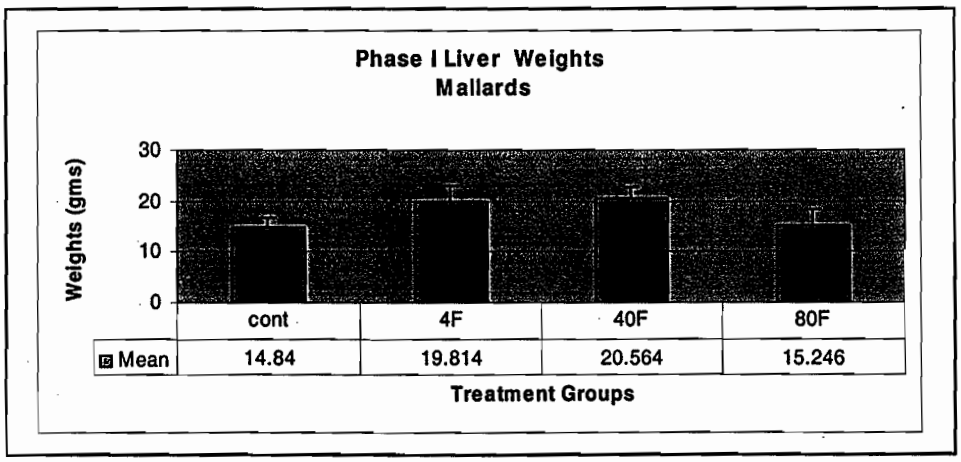

Figure 2. Mallard duckling liver weight following exposure to AP in food media

Cont=Control; 4F = 4ppm AP in food; 40F = 40ppm AP in food; 80F = 80ppm AP in food.

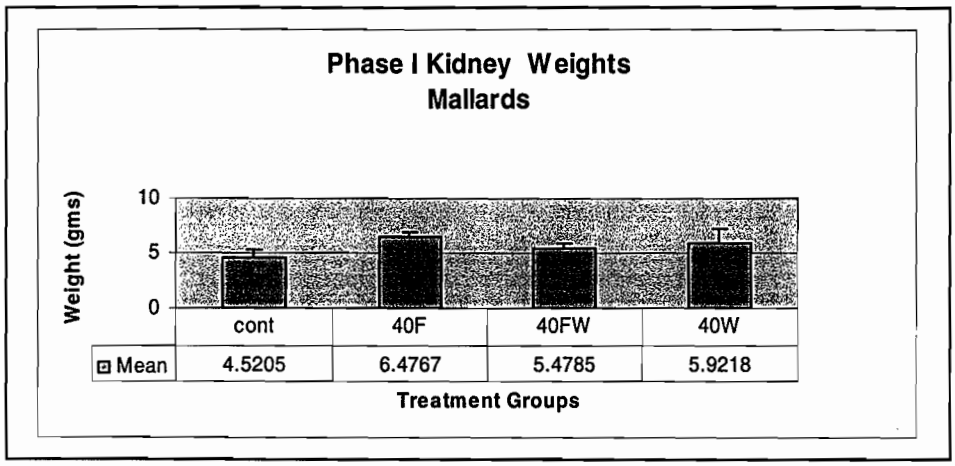

Figure 3. Mallard duckling kidney weight following exposure to 40ppm AP in food food-water or water. Cont=Control; 40F = 40ppm AP in food; 40FW = 40ppm AP in food and water; 40W = 40ppm AP in water.

Kidney weights (Figure 4) were found to be statistically different (p=0.03) among treatment groups in mallards exposed to varying concentrations of AP in food alone. AP at a concentration of 40ppm caused a significant increase in kidney weight compared to control kidney weight.

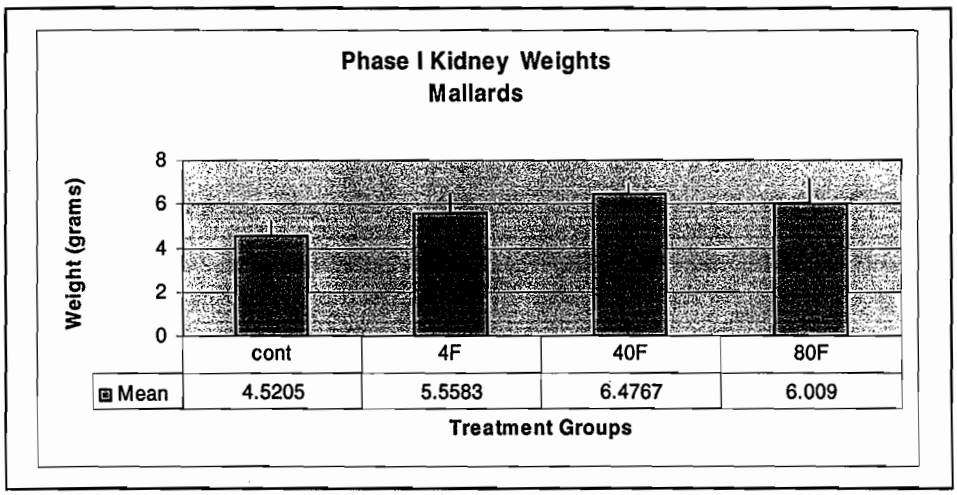

Figure 4. Mallard duckling kidney weights following exposure to varying concentrations of AP in food. AP at a concentration of 40ppm caused a significant increase in kidney weight compared to control kidney weight. Cont=Control; 4F = 4 ppm AP in food; 40F = 40 ppm AP in food; 80F = 80 ppm AP in food.

No other organ from Phase I showed significant difference in relative or absolute weight when compared using exposure scenario with fixed concentration or by varying concentration of AP with fixed exposure scenario. The lowest mean liver (14.840 g) and kidney (4.521 g) weights were presented by the control group (Table 2). The highest liver (20.564 g) and kidney (6.477 g) weights were presented by the 40 ppm feed group. The highest mean heart weight (3.309 g) was presented by the 4 ppm feed group, and the lowest (2.735 g) by the 80 ppm feed-water group. The 4 ppm group presented both the lowest and highest mean gizzard weights, at 20.977 g in the 4 ppm feed group and 16.570 g in the 4 ppm feed-water group.

Body Weight:Phase II Body

No sex related differences were found following statistical analyses.

Analysis of variance for body weight on day 6 of exposure revealed an overall statistically significant (p=0.001) difference among the means. Three treatment groups (80ppm in food; 4ppm in water; and 80ppm in water) were significantly different from the control group. See Figures 5.

On day 20 of exposure, AP at 80 ppm in water resulted in significant (p=0.038) reduction in body weight. At 80ppm in food AP caused a significant reduction in body weight compared to control birds. See Figure 6

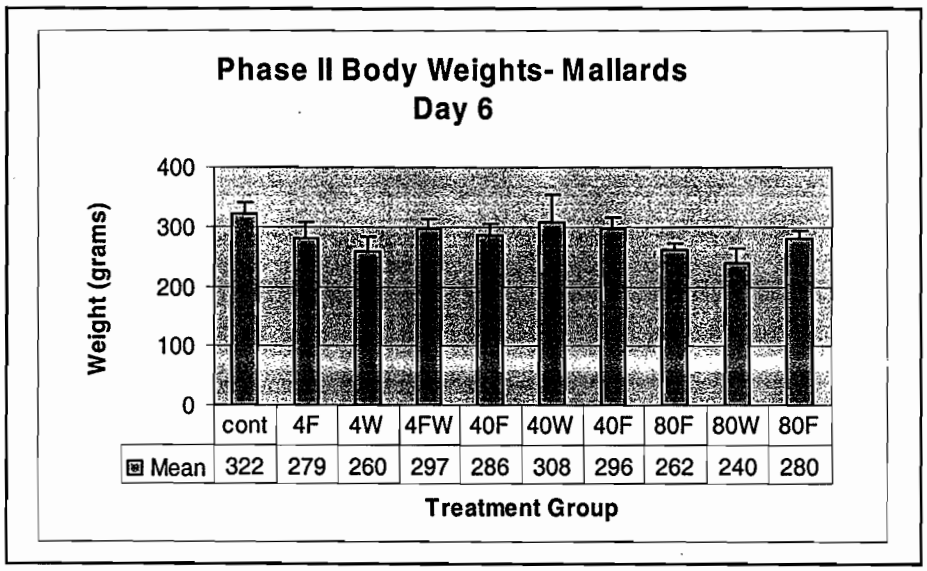

Figure 5. Body weight of ducklings on Day 6 of exposure to AP in food, water, and water-food. 4W= 4ppm AP in water; 4F = 4ppm AP in food; 4FW = 4ppm AP in food and water; 40W= 40ppm AP in water; 40F = 40ppm AP in food; 40FW = 40ppm AP in food and water; 80W= 80ppm AP in water; 80F = 80ppm AP in food; 80FW = 80ppm AP in food and water.

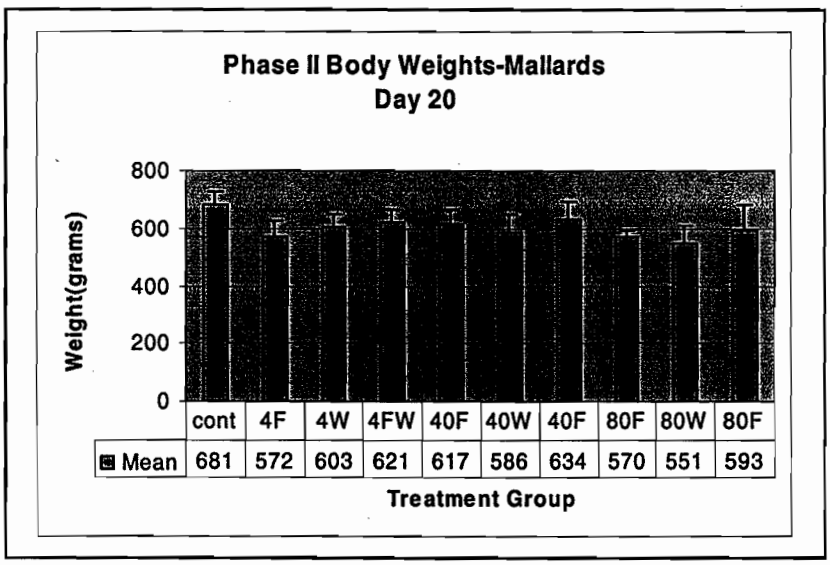

Figure 6. Body weight of ducklings on Day 20 of exposure to AP in food, water, and water-food. Cont=Control; 4W= 4ppm AP in water; 4F = 4ppm AP in food; 4FW = 4ppm AP in food and water; 40W= 40ppm AP in water; 40F = 40ppm AP in food; 40FW = 40ppm AP in food and water; 80W= 80ppm AP in water; 80F = 80ppm AP in food; 80FW = 80ppm AP in food and water.

On day 28 of exposure, AP at 4 ppm and 80ppm in food resulted in significant (p=0.0001) reduction in body weight.

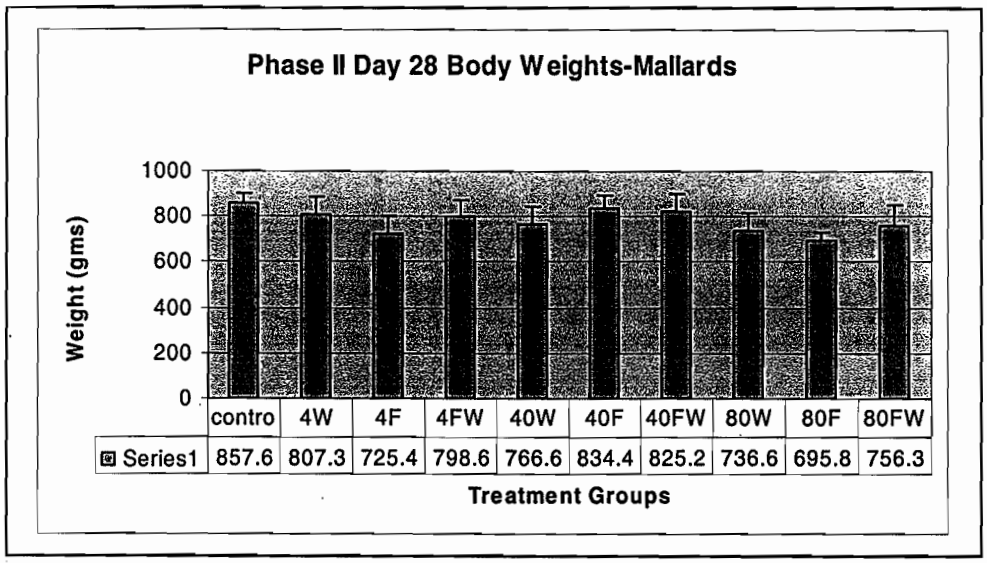

Figure 7. Body weight of ducklings on Day 28 of exposure to AP in food, water, and water-food. Cont=Control; 4W= 4ppm AP in water; 4F = 4ppm AP in food; 4FW = 4ppm AP in food and water; 40W= 40ppm AP in water; 40F = 40ppm AP in food; 40FW = 40ppm AP in food and water; 80W= 80ppm AP in water; 80F = 80ppm AP in food; 80FW = 80ppm AP in food and water.

Effect of AP in Food-alone on Body Weight

The effect of exposure to AP at varying concentrations in food-alone on body weight was analyzed at day 6, day13, day 20, and day 28 of exposure. Compared to controls AP at 4ppm and 40ppm caused a significant (0.002) reduction in body weight on day 6. By day 13, AP at 80ppm caused a significant (p=0.031) reduction in body weight relative to control birds. Exposure to AP at 4ppm and 80ppm caused a significant(p=0.004 and p=0.00, respectively) reduction in body weight by day 20 and 28. See Figures 8-11.

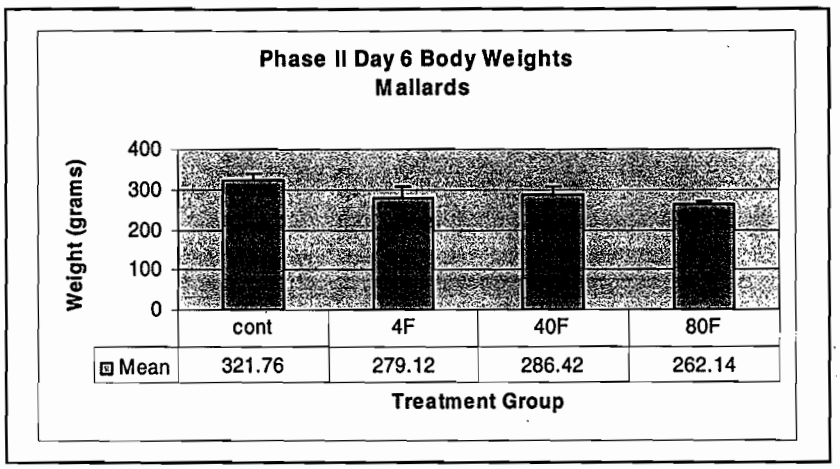

Figure 8. Body weight of ducklings on Day 6 of exposure to AP in food. Cont=Control; 4F= 4ppm AP in food; 40F = 40ppm AP in food; 80F = 80ppm AP in food.

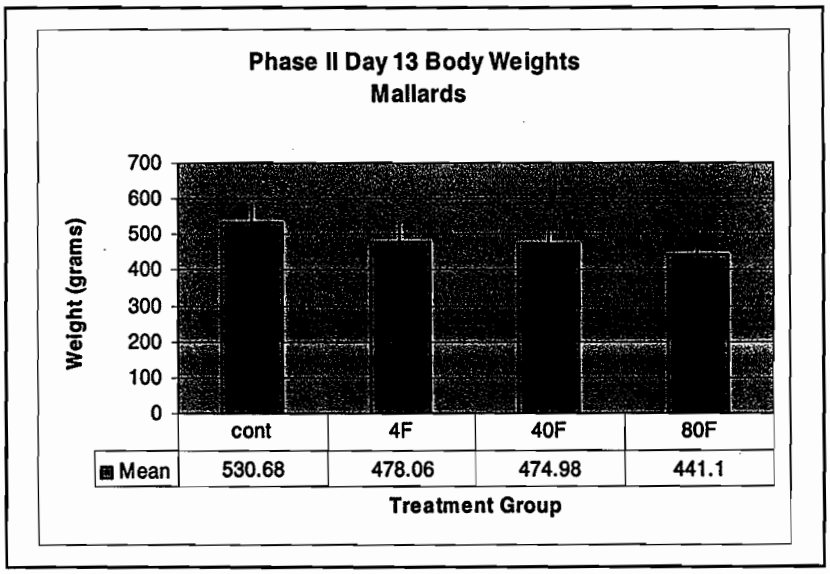

Figure 9. Body weight of ducklings on Day 13 of exposure to AP in food. Cont=Control; 4F= 4ppm AP in food; 40F = 40ppm AP in food; 80F = 80ppm AP in food.

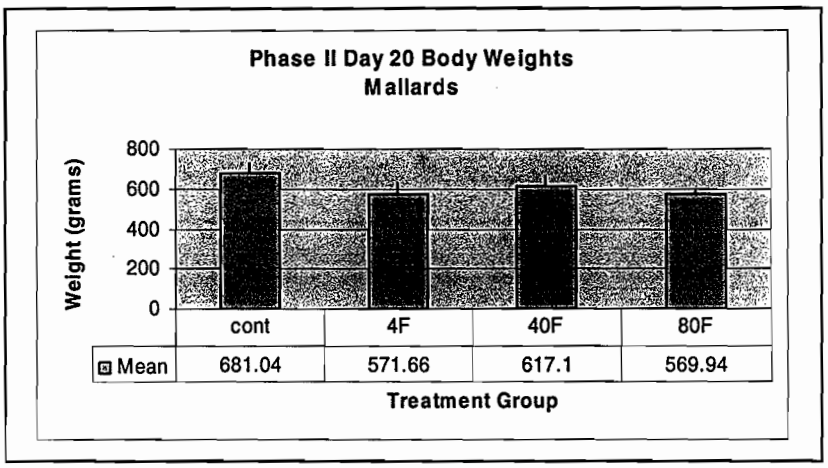

Figure 10. Body weight of ducklings on day 20 of exposure to AP in food. Cont=Control; 4F= 4ppm AP in food; 40F = 40ppm AP in food; 80F = 80ppm AP in food.

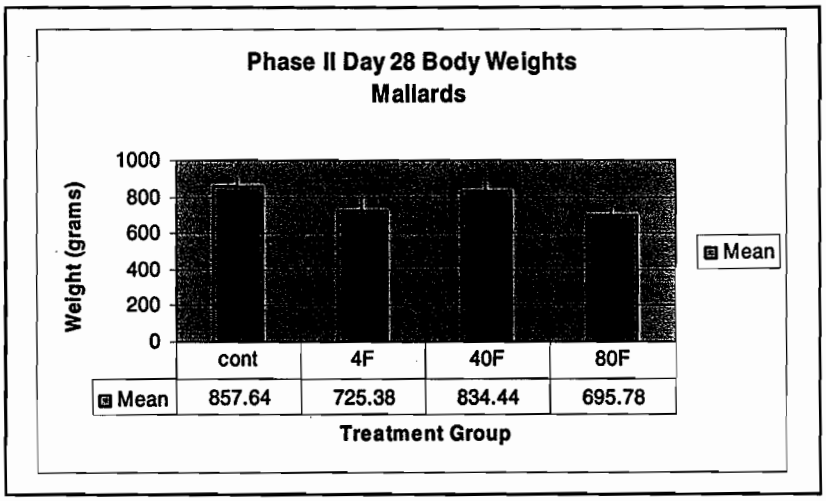

Figure 11. Body weight of ducklings on Day 28 of exposure to AP in food. Cont=Control; 4F = 4ppm AP in food; 40F = 40ppm AP in food 80F = 80ppm AP in food .

Organ Weights:Phase II

The effect of exposure to AP at various concentrations via food, water, and food and water on organ weight was assessed in mallards. At 80ppm, AP in food caused significant reduction in kidney weight (p=0.025). AP at 80ppm in food and water caused a significant reduction in heart weight (P=0.031). AP at 4ppm and 80ppm in food caused a significant reduction in gizzard weight (p=0.007).

The control group presented the highest mean kidney, heart, and gizzard weights (7.581 g, 6.374 g, 30.678 g, respectively). The heart and gizzard weights in particular were noticeably higher than those in the treatment groups. The lowest mean kidney weight was in the 80 ppm feed group (5.898 g). The lowest mean heart weight was in the 80 ppm feed and water group (4.413 g). The lowest mean gizzard weight was in the 4 ppm feed group (21.133 g).

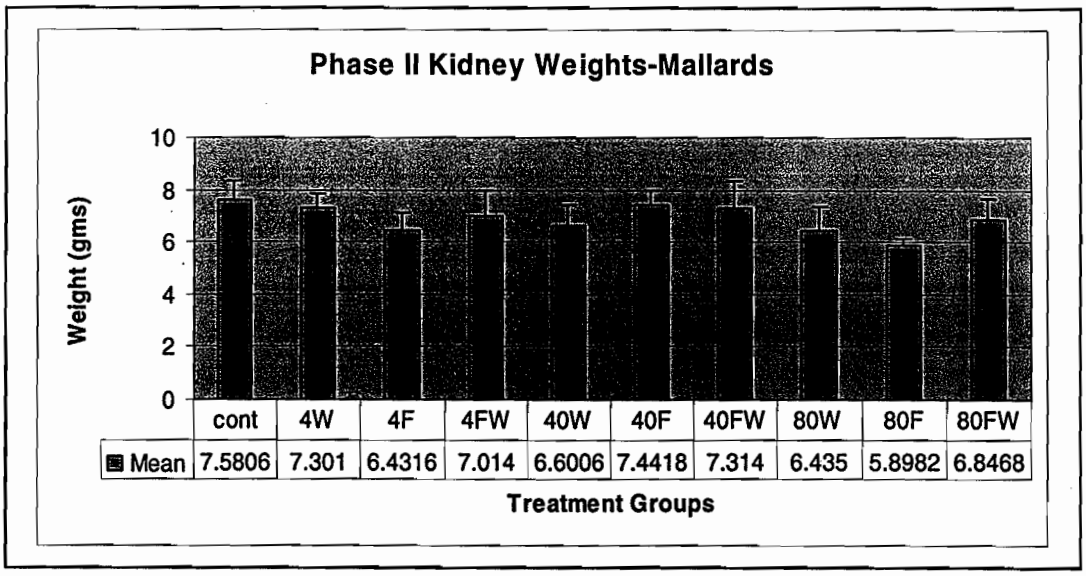

Figure 12. Graph of kidney weight of mallards expose to AP at various concentrations in food-alone, water-alone and food and water. 80ppm AP in food caused significant reduction in kidney weight (p=0.025).

Cont=Control; 4W= 4ppm AP in water; 4F = 4ppm AP in food; 4FW = 4ppm AP in food and water; 40W= 40ppm AP in water; 40F = 40ppm AP in food; 40FW = 40ppm AP in food and water; 80W= 80ppm AP in water; 80F = 80ppm AP in food; 80FW = 80ppm AP in food and water.

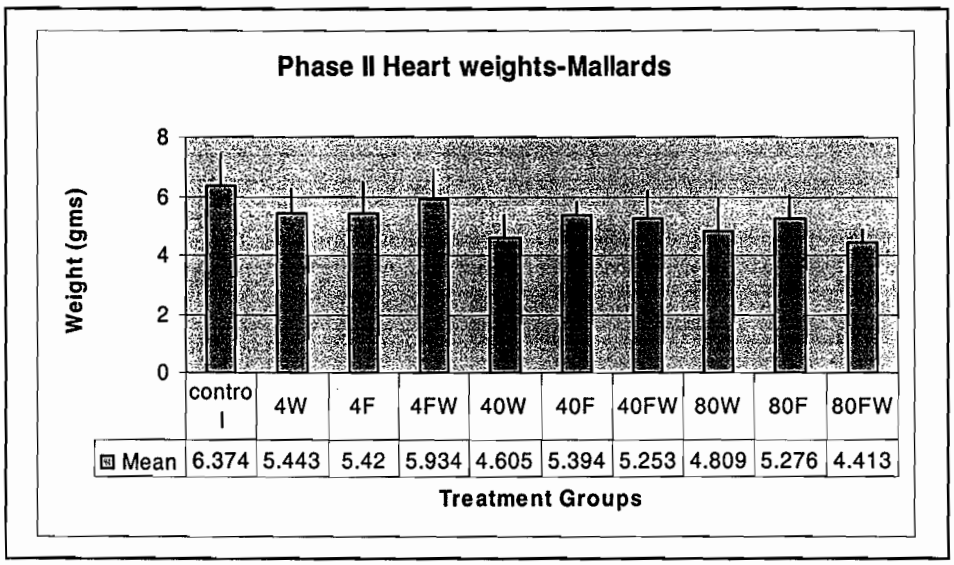

Figure 13. Graph of heart weight of mallards expose to AP at various concentrations in food-alone, water-alone and food and water. 80ppm AP in food and water caused a significant reduction in heart weight (P=0.031). Cont=Control; 4W= 4ppm AP in water; 4F = 4ppm AP in food; 4FW = 4ppm AP in food and water; 40W= 40ppm AP in water

40F = 40ppm AP in food; 40FW = 40ppm AP in food and water; 80W= 80ppm AP in water; 80F = 80ppm AP in food; 80FW = 80ppm AP in food and water.

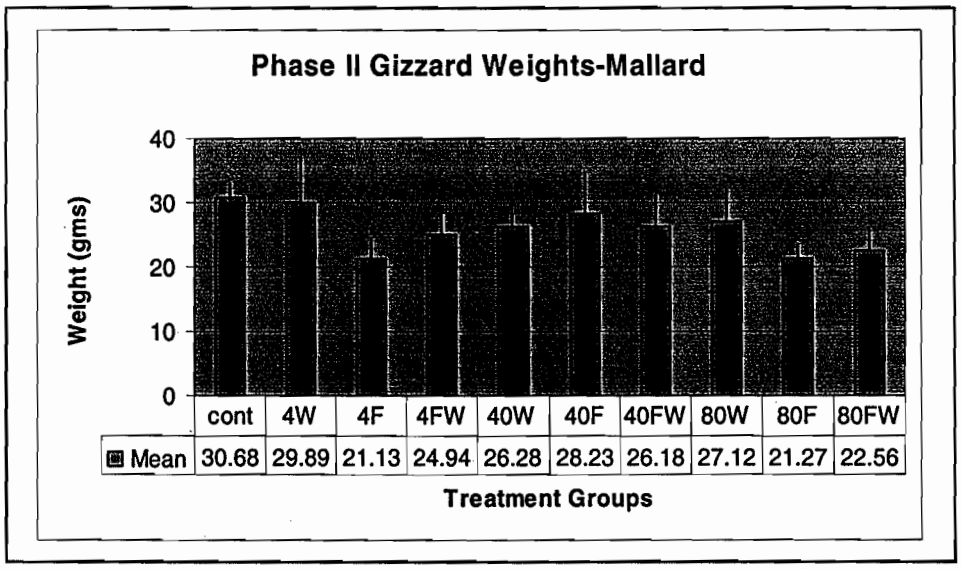

Figure 14. Graph of gizzard weight of mallards expose to AP at various concentrations in food-alone, water-alone and food and water. AP at 4ppm and 80ppm in food caused a significant reduction in gizzard weight (p=0.007).

Cont=Control; 4W= 4ppm AP in water; 4F = 4ppm AP in food; 4FW = 4ppm AP in food and water; 40W= 40ppm AP in water; 40F = 40ppm AP in food; 40FW = 40ppm AP in food and water; 80W= 80ppm AP in water; 80F = 80ppm AP in food; 80FW = 80ppm AP in food and water.

Statistical evaluation of food as the exposure medium and varying concentrations of AP revealed significant difference for liver weight (Figure X) when compared to the control (p=0.034). AP at 40ppm in food caused a significant increase in liver weight

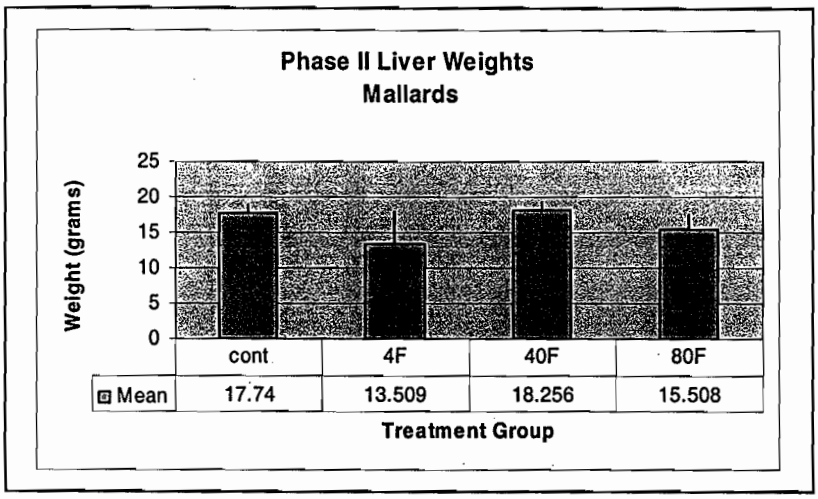

Figure 15. Graph of liver weight of mallards expose to AP at various concentrations in food-alone. AP at 4ppm significantly reduced liver weight relative to control.

Cont=control; 4F = 4ppm AP in food ;40F = 40ppm AP in food; 80F= 80ppm AP in food.

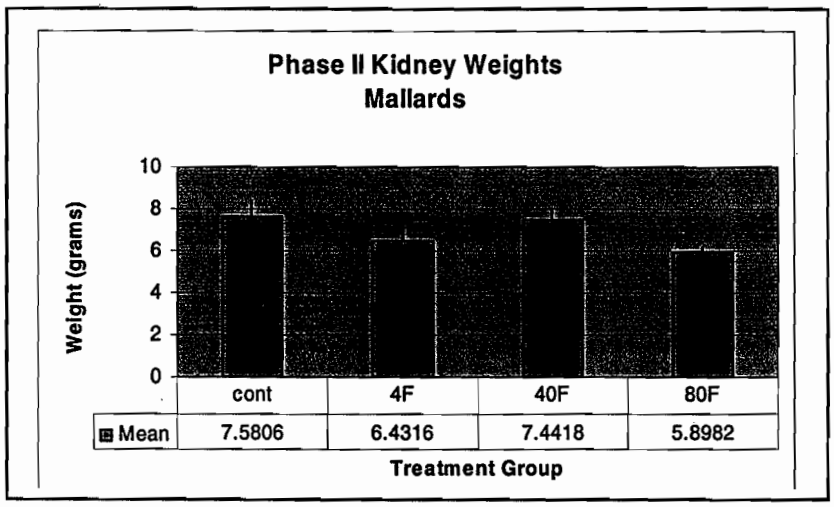

Figure 16. Graph of kidney weight of mallards expose to AP at various concentrations in food-alone. AP at 4ppm and 80ppm significantly reduced kidney weight relative to control. AP at 80ppm also caused significant reduction kidney weight relative to AP at 40ppm. Cont= control; 4F = 4ppm AP in food; 40F = 40ppm AP in food ; 80W= 80ppm AP in food.

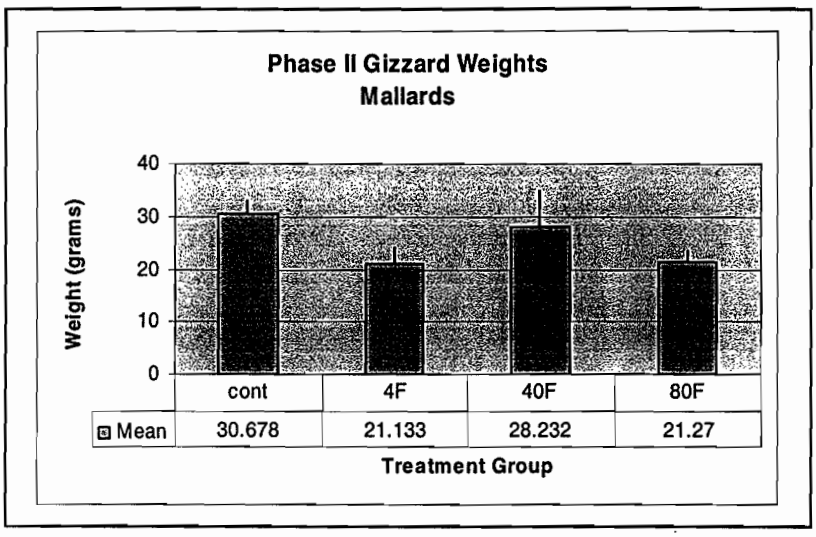

Figure 17. Graph of gizzard weight of mallards expose to AP at various concentrations in food-alone. AP at 80ppm and 4ppm caused significant (p=0.002) reduction in gizzard weight. Cont= control; 4F = 4ppm AP in food; 40F = 40ppm AP in food; 80F= 80ppm AP in food.

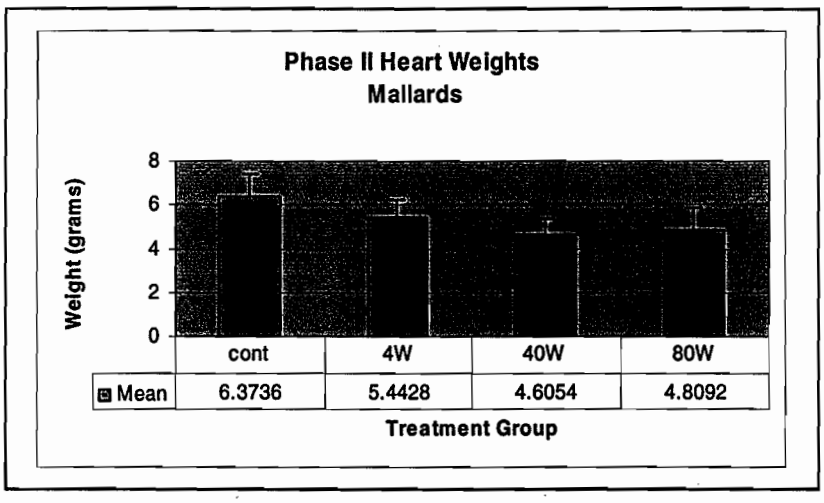

Figure 18. Graph of heart weight of mallards expose to AP at various concentrations in water-alone. AP at 40ppm and 4ppm caused significant (p=0.036) reduction in heart weight. Cont= control; 4F = 4ppm AP in food; 40F = 40ppm AP in food; 80F= 80ppm AP in food.

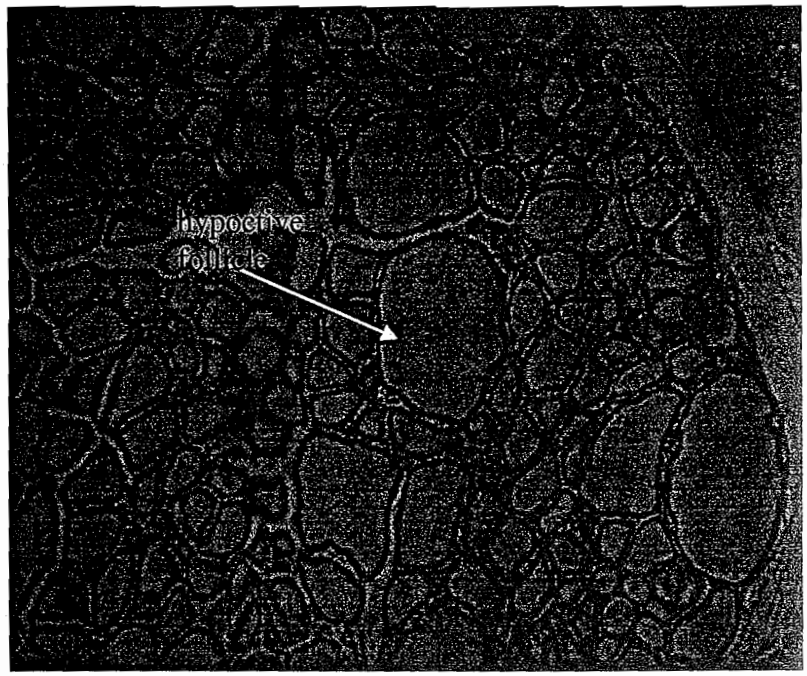

Figure 19. Photomicrograph of Mallard duckling following exposure to AP

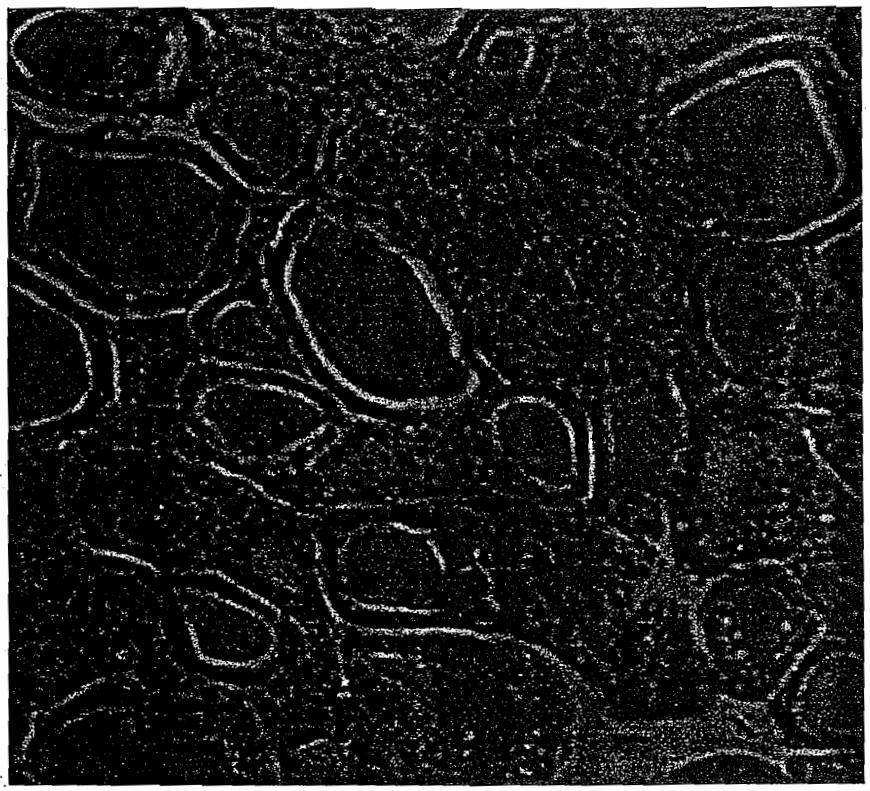

Figure 20. Photomicrograph of Mallard duckling following exposure to AP

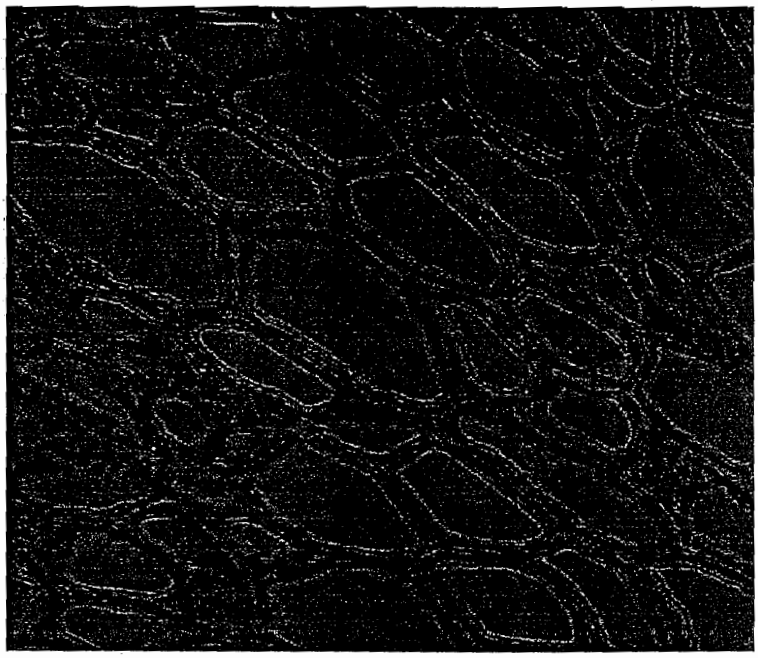

Figure 21. Photomicrograph of Mallard duckling from the control group.

An example of a normal active thyroid with colloid filled lumen.

Thyroid Hormone Analysis

Validation of thyroid hormone assay was accomplished in Dr. James Carr's laboratory with representative plasma samples from mallard duckling. The assay indicated a high level of parallelism with the mallard plasma thyroid hormone. However, due to an inadvertent freeze thaw cycle the variation in the treated samples would provide misleading data and as a result will not be included in this report.

16.0 DISCUSSION

There is little information on the potential effects of perchlorate in birds. We evaluated the effects of perchlorate in mallard ducklings using gross toxicology endpoints. Previously we reported that perchlorate induced alterations of thyroid hormone and reduced the number of active follicle in a rodent species (Thuett, K. A. *et al.* (2002a);Thuett, K. A. *et al.* (2002b). Recently, it was reported that perchlorate affects bobwhites and the order of thyroid variables from most to least sensitive was stated as: Thyroid gland –thyroid hormone content >thyroid gland weight >plasma T4. They reported also, that low doses and short exposure times decrease thyroid gland-thyroid hormone content and high doses and longer exposures decrease plasma thyroid hormone. They stated also that plasma thyroid hormones, the most frequently used

measure of avian thyroid function, is the least sensitive while thyroid gland-hormone content is the most sensitive measure for assessing thyroid disruption. Thyroid glands can maintain extracellular stores of thyroid hormone, so circulating thyroid hormone and organismal thyroid status, can be maintained for some time despite decreases in or cessation of thyroid hormone synthesis (McNabb, et al., 2002a). In another study, McNabb et al., (2002b) investigated the effects of ammonium perchlorate (AP) on thyroid function in embryos, chicks and adult bobwhite quail. They reported a rank order of thyroid variables from most to least sensitive, for all three-life stages were similar to the order stated in the first study above. Thyroid gland-thyroid hormone content >thyroid gland weight >plasma T4. Perchlorate is a competitive inhibitor of iodide uptake into the thyroid gland, and so exposure to perchlorate has the potential to impair the synthesis of thyroid hormone

In the present study mallard ducklings were exposed to varying concentrations of AP in food, water, food-water for approximately 13 day and a 28 day exposure period. We made the following observations in our study. Control animal the thyroid glands had large follicles that are filled with colloid. The follicular cells in these thyroid glands are squamous to cuboidal in shape. No evidence of hyperplasia was observed in any section from the control thyroid glands in these animals. Generally, the thyroid glands of mallard ducklings treated with perchlorate in showed hyperplastic regions. The follicular cells were cuboidal in shape. The results of the somatic indices in this study support the idea that perchlorate is a developmental toxicant and those toxic responses seen in more mature animals are secondary to perchlorate thyrotoxicosis. This concept is supported by limited published data by Thuett et al., (2002a and b) and McNabb et al., (2002a and b).

Data from the present study support the reported effect of perchlorate in developing avian species (McNabb, et al., 2002b). Base on the accumulation of perchlorate in quail eggs (Smith et al., 2003) it is worthy to note that we believe the effect of perchlorate could alter physiological and morpohological endpoints following direct exposure in developing eggs. We have interpreted the present preliminary data to suggest that AP in food appears to induce gross toxicological indicators in mallard duckling more frequently than water alone. It is not clear if these are direct effects of AP or secondary effects following thyrotoxicosis.

17.0 STUDY RECORDS AND ARCHIVE:

Study records are maintained at The Institute of Environmental and Human Health Archive for a minimum of one year from study completion date.

18.0 REFERENCES

McNabb, F.M.Anne, Maher, R.D., Jones, J.E. and Hood, S.A. (2002a). The effects of perchlorate on thyroid function in developing and adult bobwhite quail. SETAC Annual National Meeting, Utah.

McNabb, F.M.Anne[1], Fox, G.A.[2] and Grasman, K.A. (2002b). Comparison of variables for evaluating pollutant effects on thyroid function in birds. . SETAC Annual National Meeting, Utah.

Thuett, K. A., Roots, E. H., Mitchell, L. P., Gentles, B. A., Anderson, T., Kendall, R. J., and Smith, E. E. (2002a). Effects of in utero and lactational ammonium perchlorate exposure on thyroid gland histology and thyroid and sex hormones in developing deer mice (peromyscus maniculatus) through postnatal day 21. J Toxicol Environ Health A **65**, 2119-30.

Smith, Ernest E. Smith, Phillip N. Gentles, Angella. (2003). Response of Quail to Perchlorate Contaminated Water Following Oral Exposure SERDP.

Thuett, K. A., Roots, E. H., Mitchell, L. P., Gentles, B. A., Anderson, T. A., and Smith, E. E. (2002b). *In utero* and lactational exposure to ammonium perchlorate in drinking water: effects on developing deer mice at postnatal day 21. J Toxicol Environ Health A **65**, 1061-76.

1.0 APPENDICES:

Study Protocol
Changes to Study Documentation

A STUDY PROTOCOL

ENTITLED

Avian Laboratory Studies: Mallard Exposure to Perchlorate.

LABORATORY STUDY NUMBER: 13803.6100

SPONSOR:	Texas Tech University TIEHH 1207 South Gilbert Dr. Lubbock, TX
TESTING FACILITY *Name/Address*:	Springborn Smithers Laboratories P.O. Box 620 Snow Camp, NC 27349
Test Facility Management:	Jennifer M. Stafford
Study Director:	Dr. Ernest E. Smith

PROPOSED EXPERIMENTAL START DATE: DECEMBER 16, 2002

1. **DESCRIPTIVE STUDY TITLE:**
Avian Exposure Laboratory Studies: Mallard Exposure to Perchlorate.

2. **LABORATORY STUDY NUMBER:** 1308.6100

3. **SPONSOR:**
Texas Tech University
TIEHH
1207 South Gilbert Dr.
Lubbock, TX

4. **TESTING FACILITY NAME & ADDRESS:**
Springborn Smithers Laboratories
P.O. Box 620
Snow Camp, NC 27349

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: Dec. 16, 2002
Termination Date: Jan. 14, 2003

6. **KEY PERSONNEL:**
Ernest E. Smith, Study Director
Ryan Bounds, Quality Assurance Manager
Jennifer Stafford, Test Facility Manager

7. **DATED SIGNATURES:**

Dr. Ernest Smith — 12/16/02 — Study Director

Ryan Bounds — 12/17/02 — Quality Assurance Manager

Jennifer M. Stafford — 01/14/03 Initially signed on 12/13/02 — Jennifer M. Stafford — Testing Facility Management — 1/14/03

8. **REGULATORY COMPLIANCE STATEMENT**
Quality Control and Quality Assurance
This study will be conducted in accordance with established Quality Assurance program guidelines and in the spirit of the Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989) where possible.

9. STUDY OBJECTIVES / PURPOSE:

The study objective is to determine the response of ducks to perchlorate by assessing general toxicity and histological end points of selected organs.

10. STUDY SUMMARY:

This study will consist of exposing birds to feed and water containing ammonium perchlorate and comparing those accumulation and perchlorate-related endpoints. This experiment provides a realistic assessment of exposures that are likely to occur where environmental contamination with perchlorate occurs.

11. TEST MATERIALS:

Test Chemical:	Ammonium Perchlorate 99.9% pure
Source:	Sigma-Aldrich
Characterization:	Oxidizer; explodes when heated
Test Medium:	Distilled Water and Feed

12. JUSTIFICATION OF TEST SYSTEM

This project is intended to evaluate risks of perchlorate exposure among organisms consuming perchlorate-contaminated water. Ducks will be used as they represent one set of wildlife species that potentially can be directly and indirectly exposed, permit large sample sizes, and are more economically feasible in a study of this nature. Mallards provide a useful avian model of exposure and toxicity and this species has been the one of two U. S. EPA standard test species. There are no other suitable waterfowl model for assessing exposure risks in an ecological setting.

This study represents the second phase of an ongoing project and requires live animals for each experiment and cannot be substituted with culture or computer generated models. Culture and computer models cannot simulate changes in general homeostasis and thyroid hormone alteration. In addition it would not provide pertinent scientific data for furture use in risk assessment.

13. TEST ANIMALS:

Species:	Mallard ducks
Strain:	Wild type
Age:	ducklings 7 – 8 days
Sex:	Mixed (as close to a 50:50 ratio as possible, based on ability to determine sex at one week of age)
Number:	Approximately 100 ducks
Source:	Whistling Wings Hatchery

14. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each cage will be labeled with genus and species name; common name; project name, number, and start date; and the name of the persons responsible for

animal care. Each cage will also be labeled to include date of exposure, the name of the test substance and its concentration. Birds will be leg banded or wing-tagged with unique identification numbers.

15. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Perchlorate treatment will include three exposure scenarios: via drinking water only, via feed only and via water plus feed. There will be 10 ducklings per treatment group plus one control group. Each treatment scenario will be exposed to three concentrations (4 ppm, 40 ppm, 80 ppm). The control group will receive feed and water with no perchlorate added. Table 1 shows the number of birds per group.

Table 1. Number of ducks in each experimental exposure group

Treatment	Feed Exposure	Water Exposure	Feed & Water Exposure	Total Ducklings
Control	NA	NA	NA	10
4 ppm	10	10	10	30
40 ppm	10	10	10	30
80 ppm	10	10	10	30
Total	**30**	**30**	**30**	**100**

Total ducks in the experiment = 100
NA = Not applicable

16. METHODS:

Prior to any studies and pending approval all personnel will be required to read ammonium perchlorate material safety data sheet and must wear gloves, lab coat, and eye protection at all times when working with raw material.

Ducklings will be randomly assigned to treatment groups. Initial body weights will be recorded for all birds prior to initiation of the treated feed and water phase of the study. There will be 10 ducklings per treatment group, housed in PVC-coated wire brooder pens. After 14 days of exposure, approximately 50 randomly selected ducks will be removed and euthanized for sample collection. The remaining 50 ducks will continue exposure until day 28. Water and feed will be available *ad libitum* throughout the experiment. Daily observations will be made on all ducklings. Animals showing extreme signs of distress or disease will be treated according to the instructions of the consulting veterinarian.

All avian husbandry will be done according to Standard Operating Procedures.

16.1 Test System acquisition, quarantine, acclimation and husbandry.

Ducklings will be obtained from Whistling Wings game bird farm located in Hanover, Illinois. For the first week of life, temperatures in the immediate vicinity of the heat source in the brooder units will be maintained at approximately 35 to 38 °C. Areas of the brooder units most distant from

the heat source may approach room temperature. This temperature gradient is desired to allow the ducklings to seek their own comfort zone at various times throughout the day. Maximum and minimum temperatures, within the brooder units and within the study room will be monitored and recorded daily. Temperature within the testing room (outside of brooder units) will be maintained at 20 to 30 °C. By the end of week 2 of life, brooder temperatures will approximate room temperature. Maximum and minimum humidity will be recorded daily. The target relative humidity range is 40% to 80%. However, because the study room floor is washed down with clean water daily, brief periods of higher humidity may occur. Adequate ventilation will be maintained. A photoperiod of 14 hours light and 10 hours dark per day will be maintained for the duration of the test. Room lighting may be either incandescent or fluorescent. Lights will be positioned so that all pens receive similar illumination. An average light intensity of not less than 6 foot candles will be maintained at pen level. Ducklings will be given a minimum of a one-week quarantine and acclimation period prior to exposure to the test substance.

16.2 Assignment of Animals to Study Group and Identification

Animals will be assigned randomly to cages and cages randomly assigned to treatment group. Ducklings will be identified with unique wing-tag numbers. Extra birds will be obtained to allow replacement of any birds that die during acclimation. Typically, three to five percent of hatchlings die during their first week due to natural causes.

16.3 Test Material Application

Ammonium perchlorate exposure will accomplished in three ways: 1) be dissolved in the drinking water of one test group, 2) in feed in a second test group and 3) in both water and feed in a third test group. In each of the exposure test groups, the test material will be applied to the feed and water at concentrations of 4 ppm, 40 ppm, and 80 ppm. Ten birds in a single control group will be given untreated deionized water and feed. Duckling weights will be recorded just prior to initiation of exposure and on the same day of each week during the study to evaluate weight gain or loss. A schematic of the study design follows:

	Treated Water	Treated Feed	Treated Feed and Water
Control (10 ducks)	NA	NA	NA
4 ppm	10 ducks	10 ducks	10 ducks
40 ppm	10 ducks	10 ducks	10 ducks
80 ppm	10 ducks	10 ducks	10 ducks

Rates/concentrations: 0, 4, 40 and 80 ppm perchlorate.

Frequency: Test substances will be supplied *ad libitum* for 14 and 28 days.

Route/Method of Application: Ducklings will be exposed to: a) treated water b) treated feed, or c) a combination of treated feed and water for either 14 or 28 days. Route of exposure will be oral.

16.4 Daily Observations

Animals will be monitored daily for changes in general health. If any serious changes are observed, animals will be treated according to the recommendations of the consulting Veterinarian.

16.5 Animal Euthanasia and Sample Collections

At the end of exposure (for 14 day and 28 day groups), animals will be weighed according to standard laboratory procedures. After the ducklings are weighed, half of each group will be bled, and euthanized by CO_2 asphyxiation and the following tissues will be collected: livers, kidneys, hearts, breast muscle, testes, ovaries, adrenal glands, thyroid glands and heads. Blood samples will be placed in centrifuge tubes, centrifuged, the resulting plasma drawn off and placed in separate vials and frozen. Samples will be used in steroid and thyroid hormone assays.

16.6 Evaluations

Selected organs and blood samples will be collected for chemical and histological analyses.

17. PROPOSED STATISTICAL METHODS

Organ weights and hormone concentrations will be subjected to ANOVA and multi-comparison analysis.

18. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include animal receipt, animal care, test material preparation and application, animal observations, and facility records for personnel, equipment, etc.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

- Study Methods
- Survival of treatment animals
- Chemical analysis results
- Gonadal morphology summary
- Interpretation of all data, including statistical results
- Discussion of the relevance of findings

19. RECORDS TO BE MAINTAINED LOCATION:

A final report containing the results of the dosing studies will be delivered to the Sponsor on or before January 31, 2003. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility for up to 3 years.

20. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at one time point to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to affect study integrity shall be brought to the immediate attention of the Study Director.

21. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

22. REFERENCES:

Thuett et al., 2002. Journal of Environmental Toxicology and Health

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Dec 20 2002 2:28pm

Last Transaction

Date	Time	Type	Identification	Duration	Pages	Result
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Springborn Smithers Laboratories, LLC
Carolina Research Center P.O. Box 620, Snow Camp, North Carolina 27349

PROTOCOL AMMENDMENT

Amendment No.:	1
Effective Date:	19 December 2002
Protocol Title:	Avian Exposure Laboratory Studies: Mallard Exposure to Perchlorate.
Protocol Number:	D9700
Species:	Mallard (*Anas platyrhynchus*)
Study Sponsor:	Texas Tech University
Test Substance:	Ammonium perchlorate 99.9%
Springborn Study No.:	13803.4100

Section 11 of the protocol is amended as follows:

Tap water conventionally supplied to test animals via automatic watering systems will be provided to both treatment and control animals, serving as the test medium for the selected treatment animals.

Justification:

Water supplied to the testing facility is routinely monitored for pesticides, metals, dissolved gasses, and total dissolved solids. Recent analyses indicated acceptable limits. This modification is also more cost effective and does not disrupt the action or delivery of the test material.

Sections 15, 16, 16.3, and 16.5 of the protocol is amended as follows:

Due to delayed shipping, the number of fatalities within the test system reduced the number available to each test group to 9 per group (cage). This also enabled uniform numbers in each group. This adjustment in number of animals per group reduced the number proceeding through the first euthanization and dissection process to 4 per group.

Justification:

It is desirable to maintain equal numbers of animals in each group, and animals from the same hatch, therefore the number of animals per group was adjusted to the number alive after acclimation.

Section 16 of the protocol is amended as follows:

The first half of the test system will be euthanized, bled, and tissues extracted on day 13 of the study.

Justification:

This was necessary to accommodate participant schedules. Noticeable differences in hormone levels are not expected within one day at this time (animals at 19 days old).

Section 16.5 of the protocol is amended as follows:

The following tissues will be collected from each bird during day 13 and 28 processing: liver, kidneys, heart, gonads (as available), and thyroid gland. Gizzards will be weighed but not retained.

Justification:

These tissue samples were expected to provide the most reliable source of hormones and histological indications of hormonal activity in birds at this age (19 days).

This amendment does not result in negative impact on the quality or integrity of the study.

Approval Signatures:

Dr. Ernest E. Smith
Study Director, Texas Tech University

1/14/03
Date

Jennifer M. Stafford
Testing Facility Management

1/14/03
Date

A FINAL REPORT

ENTITLED
Food Item Transfer of Perchlorate into Rodents – Wild Rodent Study

STUDY/PROTOCOL NUMBER: WRS-02-01

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY:

Name/Address:

The Institute of Environmental & Human Health
Texas Tech University / TTU Health Sciences Center
Box 41163
Lubbock, Texas 79409-1163

Test Facility Management:

Dr. Ronald J. Kendall
Director, TIEHH

Study Director:

Dr. Philip N. Smith

RESEARCH INITIATION: October 2001

RESEARCH COMPLETION: December 2002

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Figure 1. Aerial photograph of the Las Vegas Wash near Henderson, Nevada.

GOOD LABOARATORIES PRACTICES STATEMENT

Study Number WRS-02-01 titled "Food Item Transfer of Perchlorate into Rodents – Wild Rodent Study", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

Scott McMurry — 3/28/03
Scott McMurry — Date

Philip N. Smith — 3/28/03
Philip N. Smith — Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Final Report Review	02/25/03	03/14/03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

03/28/03
Date

1.0 DESCRIPTIVE STUDY TITLE:

Food Item Transfer of Perchlorate into Rodents – Wild Rodent Study

2.0 STUDY/PROTOCOL NUMBER:

WRS-02-01

3.0 SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4.0 CONTRACT ADMINISTRATOR:

The Institute of Environmental and Human Health
Texas Tech University / TTU Health Sciences Center
Box 41163
Lubbock, TX 79409

5.0 TESTING FACILITY NAME & ADDRESS:

The Institute of Environmental and Human Health
Texas Tech University / TTU Health Sciences Center
Box 41163
Lubbock, Texas 79409-1163

6.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start Date: October 1, 2001
Termination Date: December 31, 2002

7.0 KEY PERSONNEL:

Dr. Philip N. Smith, Project Manager
Dr. Scott T. McMurry, Project Manager
Mr. Ryan Bounds, Quality Assurance Officer
Dr. Ronald J. Kendall, Principle Investigator / Testing Facility Management

8.0 STUDY OBJECTIVES /TEST SYSTEM JUSTIFICATION:

Recent evaluations of exposure among ecological receptors at a perchlorate-contaminated site revealed that rodents from contaminated areas contained up to 2,328 ppb perchlorate in liver tissue (Smith et al., 2001). These rodents were likely exposed through a number of sources including food items, drinking water, and incidental ingestion of contaminated soils (Beyer et al., 1994). Alternatively, laboratory-dosed deer mice, dosed for up to 70 days with perchlorate dissolved in drinking water at concentrations as high as 117.49 ppm, contained similar concentrations of perchlorate in liver tissues as those collected from the wild (Thuett et al., 2002). The dosing solution used in the laboratory studies contained approximately 6.9 times more perchlorate than the highest concentrations ever found in surface waters where wild rodents were captured, making the similarity in perchlorate concentrations among livers more striking. Although species-specific differences in perchlorate exposure and accumulation in the wild are likely, these data suggest a difference in perchlorate absorption among animals dosed via water and those exposed environmentally.

A number of factors can alter the absorption of xenobiotic substances including the presence of other chemicals, intestinal motility, age, and species-specific differences such as herbivorous versus carnivorous foraging strategies (Rozman and Klaasen, 1996). In fact, numerous interactions between food and toxicants can affect absorption across gastro-intestinal epithelia (Riviere, 1994). Most dosing studies on perchlorate to date have been conducted using drinking water as carrier vehicle for perchlorate (see Siglin et al., 2000). Our preliminary data with laboratory-exposed and wild-caught rodents suggest that exposure routes other than water may contribute to significant perchlorate body burdens and potential effects.

We hypothesized that different species of rodents collected from the same contaminated sites would have different exposure rates dependent on natural history characteristics (e.g. foraging strategies, drinking requirements; see Smith et al., 2002) and perchlorate concentrations in surrounding environmental media. Furthermore, we hypothesized that rodents, plants, and soil collected downstream from a point source will contain lower concentrations of perchlorate than those collected near the source. To test these hypotheses, we selected a heavily contaminated riparian ecosystem located in the western United States where we intensively sampled the rodent and vegetative communities to assess perchlorate movement through the food web.

9.0 TEST ANIMALS (number, weight, source, strain):

Species: House mice (*Mus musculus*), wood rats (*Neotoma lepida*), cactus mice (*Peromyscus eremicus*), canyon mice (*Peromyscus crinitis*)

Strain: Wild

Age: Adult

Number: 58

Source: Collected/captured on or near perchlorate contaminated sites within the Las Vegas Wash, Nevada.

10.0 METHODS:

Sample Collection and Field Procedures

Our research team traveled to the Las Vegas Wash in southern Nevada March 10-15, 2002. The Las Vegas Wash is located just to the southeast of Las Vegas, Nevada. The Wash has been identified as a water body heavily contaminated with perchlorate. Perchlorate moves through the wash into Lake Mead, which then empties into the Colorado River. Perchlorate in the Colorado River can be detected as far south as Yuma, Arizona. Therefore, this site was determined to be an excellent site to study perchlorate exposure among wild small mammal populations.

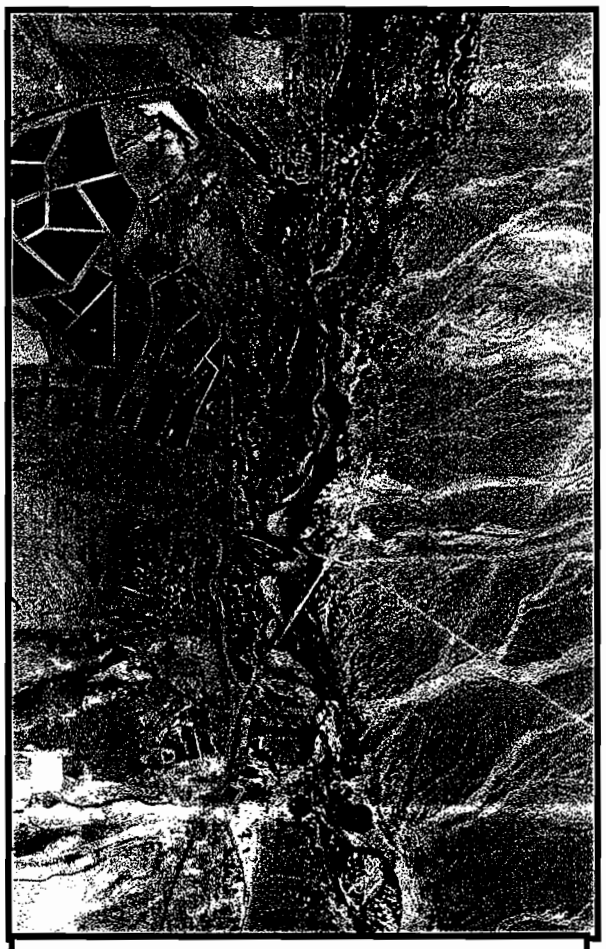

Figure 1. Aerial photograph of the Las Vegas Wash near Henderson, Nevada.

We collected samples from three sites along the Wash, termed LVW1, LVW2, and LVW3. A total of four days was spent, at least in part, at both LVW1 and LVW2, while two days were spent at LVW3. LVW1 is the western-most sampling area located where the Henderson water treatment facility effluent enters into the Wash. The two other sites are respectively to the east, and downstream, of the first.

Museum special and rat snap-traps were baited with peanut butter and set each evening in and around vegetative structure deemed to be suitable rodent habitat. Traps were set near the Wash or on the banks of groundwater seeps where contamination was likely to occur. The following day, captured rodents were collected, labeled and frozen on dry ice. On every possible occasion representative soil, vegetation, and water samples were collected from the immediate area where individual rodents were captured. Collected vegetation was classified into the following categories: terrestrial grass, terrestrial broadleaf, aquatic grass, aquatic

broadleaf, seeding bush, terrestrial tree, tree debris, and detritus. All samples were labeled and stored for transport back to our laboratory.

Kidneys and livers from the collected rodents, and soil, water, and vegetation samples were extracted and analyzed for total perchlorate concentrations to determine body burden, and potential uptake pathways.

Water, Sediment, Soil, and Vegetation Collection:
Water samples were collected into clean vials from just below the water surface wherever possible. All water samples (5 mL) were filtered and either analyzed for perchlorate ion directly, or diluted with distilled, deionized water and then analyzed. Soil samples were taken from the top 5 cm of soil. Soil samples were weighed, placed in glass jars, and extracted (mechanical agitation) with distilled, deionized water (2:1 water:soil). Water extracts were filtered and either analyzed for perchlorate ion directly, or diluted with distilled, deionized water and then analyzed. Vegetation samples were collected from areas adjacent to where animals were collected. Vegetation samples were removed from soil or sediment and placed in plastic bags. Prior to extraction, vegetation samples were air-dried, and weighed.

Tissue and food item analysis: Rodent livers and kidneys were analyzed for perchlorate content using standard tissue extraction and analysis techniques developed in the analytical core of this project (Anderson and Wu, 2002).

Statistical Methods
For calculation of mean detectable concentrations and correlation analyses, "trace" detections were assigned a concentration of 0.5 ppm, or half the detection limit. Concentrations below the detection limit were assigned a value of zero. All data were checked for heteroscedasticity. Non-parametric Wilcoxon tests were used to determine differences between of mean perchlorate concentrations in plants and soils for each sampling area because data did not meet the assumptions necessary for parametric statistical approaches. Spearman's Rho correlation test for ranked data was used to assess potential relationships among rodent tissue, plant, and soil perchlorate concentrations. This test evaluates correlations among ranked data and paired variables similar to Pearson's Product-Moment correlation. Statistical tests were considered significant when $p \leq 0.05$.

11.0 RESULTS

Four rodent species were collected from the Las Vegas Wash including house mice (*Mus musculus*), wood rats (*Neotoma lepida*), cactus mice (*Peromyscus eremicus*), and canyon mice (*Peromyscus crinitis*). Cactus mice appeared to be the dominant species at all three locations accounting for nearly 70% (40/58) of all rodents captured in this study. A total of nine house mice, seven wood rats, and two canyon mice were also collected. A disproportionate amount of trapping effort was applied to the LVW1 sampling location because it was considered to be closest to the main sources of perchlorate entering the Las

Vegas Wash (see Table 1). The LVW2 and LVW3 sampling areas were located downstream from LVW1.

Table 1. Total numbers of samples collected at Las Vegas Wash March 10-15, 2002.

	LVW 1	LVW 2	LVW 3	Total
Small Mammals	36	12	10	58
Plant w/ capture	49	10	12	71
Soil w/ capture	32	9	10	51

Seventy-one total plant specimens were collected in the immediate proximity of traps that successfully captured rodents. In some instances, more than one plant species was collected with a single rodent, and in other instances, no vegetation was present (thus not collected) where rodents were captured. Similarly, some successful traps were located on gravel or stone banks, thus no soil was collected. Most traps were located away from an immediate water source, therefore not all rodent samples had corresponding water perchlorate values.

Perchlorate was detected in a small number of rodents collected at the Las Vegas Wash. The minimum detection limit for rodent tissues in this study was 1 ppm. Perchlorate was detected more often in liver tissue than in kidneys for all rodent species (15 versus 6). Three of nine house mice, 5 of 7 wood rats, 1 of 2 canyon mice, and 8 of 40 cactus mice contained detectable concentrations of perchlorate in liver tissue. Similarly, 2 of 7 wood rats and 4 of 40 cactus mice contained detectable concentrations of perchlorate in kidneys, but none of the house mice or canyon mice were found to contain perchlorate in kidney tissues. Detectable, but not quantifiable (trace) concentrations of perchlorate were found in four cactus mouse livers and two cactus mouse kidney samples.

Vegetation within this affected riparian ecosystem was heavily contaminated with perchlorate, similar to the findings of Urbansky et al. (2000). Perchlorate concentrations ranged from below detection limits to 4,500 ppm with a mean concentration of 290 $\pm$ 94 ppm (S.E.) for all plants analyzed. There was no significant difference in plant concentrations among the three sampling locations (p=0.0758). However, extreme variability in plant concentrations and low sample sizes collected from LVW2 and 3 may have obscured differences. The mean concentration of perchlorate in plants from LVW1 was 390 $\pm$ 134 ppm (S.E.), and was approximately 40 and 4 times greater than mean concentrations in plants from LVW2 and LVW3, respectfully. Multiple plant samples were collected alongside many of the rodents. Therefore, an average plant concentration was calculated for each rodent collection site. There were no significant differences in average plant perchlorate concentrations among the three sampling locations (p=0.0830). Similarly, there was no significant difference between average soil concentrations from the three sampling locations (p=0.6656). Soil concentrations ranged from below detection limits to 1,200 ppm with a mean of 64.5 $\pm$ 25.7 (S.E.) ppm, and median concentration of 0.820 ppm.

There was considerable variation in perchlorate concentrations in plants. The non-parametric Wilcoxon test indicated significant differences (p=0.0227) in plant perchlorate

concentrations among the various plant types collected (e.g seeding bush, terrestrial grass, aquatic grass, etc.). However, we could not discern which plant type or group (e.g. terrestrial vs. aquatic plants, trees versus grasses, etc.) of plants contained the highest concentrations. To eliminate potential site bias, we compared plant type and plant group perchlorate concentrations in LVW1 data only. Although there appeared to be a significant difference in perchlorate concentrations among the plant types at LVW1, multiple comparison and linear contrast tests failed to identify which type or group(s) had the highest or lowest perchlorate concentrations. Overall, terrestrial broadleaf (mean = 645 $\pm$ 514 (S.E.) ppm) and seeding bushes (mean = 525 $\pm$ 492 (S.E.) ppm) contained higher mean perchlorate concentrations than other plant types (although not statistically different).

There appeared to be some significant relationships between rodent tissue, plant, or soil perchlorate concentrations. Average soil perchlorate concentrations were significantly correlated with rodent liver perchlorate concentrations (r_s=0.5101, p=0.0001), but not as strongly with kidney perchlorate concentrations (r_s=0.2792, p=0.0473). Not surprisingly, liver and kidney perchlorate concentrations (r_s=0.4085, p=0.0015) and average soil and average plant concentrations (r_s=0.4411, p=0.0017) were somewhat correlated.

12.0 DISCUSSION

Perchlorate was detected in high concentrations in rodent, plant, and soil samples collected at the Las Vegas Wash. However, there were fewer rodents with quantifiable concentrations of perchlorate in their tissues than we would have anticipated given the highly contaminated environment they inhabited. Undoubtedly, the difficulties associated with quantifying perchlorate anion in small biological tissues contributed to the low number of detects in this dataset. There were likely several rodent samples that contained perchlorate, but at concentrations below the detection limit. Elevated background ions common in biological matrices like livers or kidneys may hide or obscure perchlorate peaks on an ion chromatograph. Additionally, rodent (especially mouse) livers and kidneys are quite small, thus reducing the amount of tissue that could potentially contain perchlorate and the total content of perchlorate. Nonetheless, nearly one quarter of the rodents sampled contained quantifiable concentrations of perchlorate. A greater percentage of wood rats (71%) had quantifiable concentrations of perchlorate in livers and/or kidneys than any other species (house mice, 33%; canyon mice, 50%(1/2); cactus mice, 22.5%), which may have been related to the physical size of wood rat tissues compared to those of the mice rather than actual exposure dynamics (see Smith et al., 2002).

Rodent tissue and vegetation residue levels were found to be correlated with soil perchlorate concentrations, but rodent residues were not significantly correlated with plant perchlorate concentrations. This may indicate that soil is a greater source of perchlorate exposure in rodents than vegetative matter. Soil can make up a significant percentage of the diet of wild rodents, and thus can contribute significantly to contaminant exposure (Beyer et al., 1994). However, these data do not permit us to reject our null hypothesis regarding species-specific differences in perchlorate accumulation

among rodents at the Las Vegas Wash. However, there was limited plant evidence that suggested that perchlorate concentrations changed on a spatial scale. These differences were likely related to distance from the perchlorate source, but no linear pattern of decreasing plant concentrations as a function of distance from source areas was observed in this study.

Previous collections of small mammal tissue (e.g. harvest mice) from the Longhorn Army Ammunition Plant (LHAAP) in east Texas contained perchlorate concentrations ranging from 589 to 2,170 ppb (Smith et al., 2001). Rodents collected in this study contained much higher concentrations of perchlorate than those at the LHAAP. Perchlorate contamination at the Las Vegas Wash is much more widespread and at higher concentration than those at the LHAAP.

These data suggest that measures of perchlorate concentrations in soils may be the best predictor of exposure in both rodents and vegetation at the Las Vegas Wash.

13.0 REFERENCES

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14.0 ACKNOWLEDGMENTS

We acknowledge the substantial contributions of the following individuals to this project: Dr. Todd Anderson, Anna Herboldsheimer, Brandon Law, Stacie Singleton, Jaclyn Canas, and Doug Crockett.

Project No.T9700

A STUDY PROTOCOL
ENTITLED

Food Item Transfer of Perchlorate into Rodents
Wild Rodent Study

STUDY NUMBER: WRS-02-01

SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY:

The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

TESTING FACILITY MANAGEMENT:
Dr. Ronald J. Kendall

STUDY DIRECTOR:
Dr. Philip N. Smith

CO-STUDY DIRECTOR:
Dr. Scott T. McMurry

PROPOSED EXPERIMENTAL START DATE:
November 1, 2001

1. **DESCRIPTIVE STUDY TITLE**: Toxicity of Ammonium Perchlorate Exposure in Small Mammals

2. **STUDY NUMBER:** WRS-02-01

3. **SPONSOR:**

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME AND ADDRESS:**

The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START AND TERMINATION DATES:**

Start Date: November 1, 2001 Termination Date: October 31, 2002

6. **KEY PERSONNEL:**

Study Director: Philip N. Smith
Co-Study Director: Scott T. McMurry
Primary Investigator/Testing Facility Management: Dr. Ronald J. Kendall
Animal Care/Laboratory Veterinarian: Dr. J. Mark Hellman
Quality Assurance Manager: Ryan Bounds

7. **DATED SIGNATURES:**

Philip N. Smith — 3/28/03 Date
Study Director

Scott T. McMurry — 3/28/03 Date
Co-Investigator

Ryan Bounds — 3.28.03 Date
Quality Assurance Manager

Dr. Ronald J. Kendall
Primary Investigator, T.F.M.

3/28/03
Date

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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Phil Smith
The Institute of Environmental and Human Health
P.O. Box 41163
Lubbock TX 79409-1163

9. STUDY OBJECTIVES / PURPOSE:

To assess exposure to ammonium perchlorate and evaluate potential perchlorate-induced thyroid hormone alterations in small mammal populations inhabiting contaminated sites.

10. JUSTIFICATION OF TEST SYSTEM:

Small mammals are important components of ecosystems, and could prove to be valuable models for an ecological risk assessment evaluation of ammonium perchlorate. We will evaluate several rodent species while developing this ecological risk assessment model. These species are potentially present in large numbers, and may be at risk of exposure to perchlorate through food, water, or grooming. Rodents will be collected from contaminated and reference sites.

11. **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system).
The list below indicates potential species targeted, however, species collected will depend upon study site and capture success:

Genus:	*Oryzomys*	**Species:**	*palustris*
Genus:	*Peromyscus*	**Species:**	*leucopus*
Genus:	*Peromyscus*	**Species:**	*gossypinus*
Genus:	*Neotoma*	**Species:**	*floridanus*
Genus:	*Reithrodontomys*	**Species:**	*fulvescens*

Genus: *Sigmodon* **Species:** *hispidus*

Strain: Wild type
Age: Adults and juveniles
Number: A maximum of 210 animals per year with no more than 70 adults and juveniles of one species per year for a maximum of approximately 630 animals over 3 years
Source: Wild caught

12. **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**
Euthanized animals will be given a unique identification number. All pertinent information will be recorded.

13. **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**
Approximately 210 small mammals per year will be trapped with snap traps or live-traps, euthanized (when necessary) with CO_2 (SOP AF-1-3), and have tissues collected according to SOP IN-3-01. Species, trapping location, body mass, estimated age, and gender will be recorded according to SOP IN-3-01 on Form No. 135.

If possible, organs, stomach contents, and potential food items will be collected, weighed, placed in individual containers and frozen (until transfer to a -80°C freezer) for future analysis. Blood will be collected from large individuals following SOP ET-3-19, and centrifuged at approximately 2000 rpm for 12-15 minutes. After centrifugation, plasma will be separated from other blood components and will be frozen in the field (on dry ice), then transported to the lab. Samples will be labeled and batched according to SOP IN-1-02, SOP IN-3-10 and SOP IN-1-05.

Data forms from each field collection will be transported to and permanently stored at The Institute of Environmental and Human Health, Texas Tech University.

14. **METHODS:**

14.1 **Test System Acquisition, Quarantine, Acclimation:**
Target animals will be live-trapped and euthanized or collected lethally in snap traps placed in rodent-producing habitat near contaminated water bodies or on contaminated soils. All non-target species will be released immediately except those captured in snap traps. These will be salvaged for analysis.

14.2 **Test Condition Establishment:**
Animals will be live-trapped using Sherman live traps (SOP ET-3-12) or trapped in snap traps placed in arrays within areas of concern at the contaminated site in question.. Traps will be checked each morning between 0500 and 1000 hrs.

Test Material Application: Preparation of Test Solution:
Not applicable.

Concentrations:
Perchlorate concentrations in nearby bodies of water, soil and native vegetation will be determined near each trapping array according to TIEHH SOPs.

14.3 **Test System Observation:**
N/A

14.4 **Animal Sacrifice and Sample Collections:**
Small mammals will be sacrificed according to SOP AF-1-03. Tissue collection will proceed according to heart stick SOP ET-3-19 and necropsy SOPs AF-1-03 and IN-3-01.

14.5 **Endpoint Analyses:**
Tissues, stomach contents, potential food items, water and soil will be analyzed by the Analytical Department at TIEHH for perchlorate concentrations according to approved SOPs.

15. PROPOSED STATISTICAL METHODS:

We will check for heterogeneity and normal distribution of our data. If the assumptions of normally-distributed data and homogeneity of variances are met, we will use standard parametric statistics, including analysis of variance (ANOVA) and regression to evaluate differences between sites and trends among groups of sites. Otherwise, non-parametric statistics (i.e., Kruskal-Wallace test) will be used.

16. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

- List individual endpoints and analyses.
  - Perchlorate concentrations in tissues, stomach contents, potential food items, soil and water.
- Interpretation of all data, including statistical results
- Discussion of the relevance of findings
- List of all SOPs used
- List of all personnel

17. RECORDS TO BE MAINTAINED / LOCATION:

A final report will be delivered to the Sponsor on or before March 31, 2003. Copies of all data, documentation, records, protocol information as well as the specimens shall be sent to the Sponsor, or designated delivery point, upon request (within six months of

study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility.

18. **QUALITY ASSURANCE:**
The Quality Assurance Unit will inspect the study at intervals to ensure the integrity of the study. Written records will be maintained indicating, but not limited to, the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

19. **PROTOCOL CHANGES / REVISIONS:**
All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

A FINAL REPORT

ENTITLED

ELIMINATION OF PERCHLORATE IN NATIVE FISH

STUDY NUMBER:	FISH 02-01
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	01/01/2002
RESEARCH COMPLETION:	12/31/2002

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GOOD LABORATORIES PRACTICES STATEMENT

Project FISH 02-01, entitled "Elimination of Perchlorate in Native Fish", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

3/27/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase/Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	3-6-02	3-7-02	3-7-02	4-8-02
Test Material Application	3-13-02	3-13-02	3-21-02	3-25-02
Test System Care and Application	3-13-02	3-13-02	3-21-02	3-25-02
Test Material Mixing	3-11-02	3-11-02	3-21-02	3-25-02
Dosing	3-13-02	3-13-02	3-21-02	3-25-02
Final Report and Raw Data Review	1-13-02	1-24-02		

Submitted By:

Ryan Bounds
Quality Assurance Manager

03/27/03
Date

1. DESCRIPTIVE STUDY TITLE:
Elimination of Perchlorate in Native Fish

2. STUDY NUMBER:
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Start: 01/01/2002
Termination: 31/12/2002

6. KEY PERSONNEL:
Ron Kendall, Principal Investigator
Christopher Theodorakis, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Brian Birdwell, Quality Assurance Officer
Jacques Rinchard, Postdoctoral research Associate
June-Woo Park, Graduate Student

7. STUDY SUMMARY:
Mosquitofish and channel catfish were exposed to 100 ppm sodium perchlorate for 2 days. After exposure, they were transferred to clean water and allowed to depurate for up to 20 days. Perchlorate concentration was determined in mosquitofish whole body and catfish tissues (e.g., fillet, liver, GI-tract, gills and head) after 1, 2, 5, 10 and 20 days. The concentration of perchlorate in whole body mosquitofish at the end of the exposure period reached 9.9 ± 1.4 ppm. Perchlorate was then rapidly eliminated from mosquitofish and no perchlorate was detected after 5 days of elimination. The elimination rate constant (K_2) and the half-life of the perchlorate ($T_{1/2}$) were 0.76 day^{-1} and 0.91 day, respectively. In channel catfish, the uptake of perchlorate in each tissue after 2 days exposure appeared in the following order: head > liver > gills > GI-tract > fillet. Perchlorate was also rapidly removed from each tissue. After 1 day of elimination more than 50% of perchlorate was eliminated. The amounts lost from collected tissue are as follows: fillet(80%), head(75%), GI-tract(74%), gills(68%), and liver(43%). However, perchlorate was still present in some

tissues (e.g., liver and GI-tract) after 20 days. The calculation of the elimination rate constant and the half-life of perchlorate in catfish tissues indicated that perchlorate was eliminated at the slowest rate from the liver and at the highest rate from the fillet.

8. STUDY OBJECTIVES / PURPOSE:

The objectives of this study were to determine kinetics of elimination and relative tissue distributions of sodium perchlorate in native fish species.

9. TEST MATERIALS:

Test Chemical: Sodium perchlorate
CAS Number: 7601-89-0
Characterization: Determination of concentration in environmental samples
Source: Aldrich Chemical Company

Reference Chemical: Ultra-pure water with added sea salts ("Instant Ocean®")
CAS Number: Not applicable
Characterization: Determination of pH and conductivity
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultra-pure water. 60 mg/L sea salts ("Instant Ocean®") was added.

10. JUSTIFICATION OF TEST SYSTEM:

Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, fish and wildlife population stability as well as human health. Mosquitofish and catfish were used as the test species because they are native Texas species.

11. TEST ANIMALS:

Species: *Gambusia holbrooki*, mosquitofish and *Ictalurus punctatus*, channel catfish
Strain: Bred in hatcheries
Age: Adults.
Number: Approximately 150 mosquitofish, and 30 catfish
Source: Purchased from hatcheries

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Aquaria were labeled with the aquaria number, species name, animal use protocol number, project number, test system, date of exposure and date of collection, concentration, and person responsible.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Fish were exposed to 100 ppm of sodium perchlorate. Fish were placed into pre-cleaned aquaria. Aquaria were cleaned by washing each aquarium according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays". For exposures, aquaria were

located on shelves capable of supporting such weight. Each shelf held five 40L aquaria. The experimental design consisted of a randomized block design, with each shelf constituting a block. A random number generator determined arrangement of the aquaria within each block. Each block contained at least 1 aquarium of each treatment.

14. METHODS

14.1. Test System acquisition, quarantine, acclimation

Fish were obtained from fish hatcheries. They were treated with commercially available antibiotics for 5 days, as instructed by the manufacturer. After five days, any debris at the bottom of the tank was removed and 1/3 of the tank water replaced with fresh water. Every other day, debris at the bottom of the tank was removed by suction. Water was continuously aerated and filtered using mechanical and biological filtration. Water consisted of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean® sea salts. Animal husbandry was conducted according to SOPs AQ-1-08, "General Fish Husbandry" and AQ-01-09 "Mosquitofish Husbandry". Total acclimatization period was one week. Once acclimated, fish were exposed to sodium perchlorate dissolved in water for 2 days. They were then placed into clean water for elimination of perchlorate anion.

14.2. Test Condition Establishment

Exposures were begun after fish had become acclimatized.

14.3. Test Material Application

Fish were placed into aquaria containing 100 ppm sodium perchlorate for 2 days. They were then moved into aquaria containing clean water. For catfish, there was 1 fish per 15-L aquarium and 4 aquaria per treatment (elimination for 1, 2, 5 or 10 days). For the mosquitofish, 35 fish were placed into 40 L aquaria, and 5 were removed at 0, 1, 2, 5, 10, and 20 days. There were 5 extra to account for mortality, and any extras were disposed of after 20 days. Every other day, 1/3 of the water was replaced in each tank (water was not changed for the 2 first days of the catfish and mosquitofish elimination experiments). Mosquitofish were fed commercial flake goldfish food at the rate of 5 mg per gram of fish, on a daily basis. Catfish were fed pelleted food at the same rate. Debris and uneaten food were removed from the bottom of the tank every other day. Water samples were taken every other day from each aquarium for perchlorate analysis (one sample was taken from each aquarium for the 1 and 2 day catfish and mosquitofish exposures).

Rates/concentrations: Fish were exposed to 100 ppm sodium perchlorate in water for 2 days, thereafter they were moved to clean aquarium water.

Frequency: After moving to clean water, fish were allowed to eliminate perchlorate anion for 1, 2, 5, 10, and 20 days. Elimination period was expended to 20 days for catfish as perchlorate was still found in some tissues (see attached change in study documentation form).

Route/Method of Application: Route was via dermal, oral, and respiratory exposure as the

chemical was in the aquaria water.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification: A sample of each concentration of treated water was tested by chemical analysis.

14.4. Test System Observation

Aquaria were observed on a daily basis. The number of individuals that expired each day was recorded for each perchlorate concentration. In addition, pH, dissolved oxygen, conductivity, temperature, and ammonia were determined at least 3 times per week.

14.5. Animal Sacrifice and Sample Collections

Mosquitofish were removed from each aquarium and then rinsed three times in aquaria with reconstituted fresh water. The fish were sacrificed with an overdose with MS 222 and weighed. An overdose of MS222 consisted of immersing the animal in 1 g/L MS222 for at least 60 seconds after all gill ventilation has ceased, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Small Amphibians and Fish". Individuals were wrapped in aluminum foil and frozen by immersion in liquid nitrogen. Perchlorate concentration was determined in whole bodies (WB) as described in section 14.6. Animals were pooled to obtain sufficient tissue for analysis (a minimum of approximately 2 g for perchlorate analysis).

Channel catfish were anesthetized in 1.5 g/L MS222 until the animal lost righting reflex and did not respond to physical stimuli, but before gill ventilation ceased, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Small Amphibians and Fish". Fish were then sacrificed by cervical scission and dissected to remove the liver (LV), gills (GL), GI-tract (GI), head (HD), and fillet without skin (FL). Livers were pooled into two samples to obtain sufficient tissue for perchlorate analysis.

Labeling: samples were labeled with a unique ID number according to the following scheme: LPE (laboratory perchlorate elimination) - sample number - organ (2 letter abbreviation). E.g., LPE-0001-LV is the liver sample from fish # 0001. LPE-0001-FL is the fillet from fish # 0001.

The individual fish or tissues that were pooled were assigned a prefix LPEC (e.g., LPEC-0001-WB was composite # 1, consisting of whole bodies). The number of individual sampled comprising the composite was indicated on the fish dissection/tissue collection form.

Information included on the label was project number and unique ID (SOP IN-03-02 Sample Labeling/Logging Procedure), date collected, elimination time period, exposure concentration. All of this information was recorded on the fish dissection/tissue collection sheet. Fish weight as well as the standard length for the catfish, were also recorded. Any information not determined was entered as "ND".

14.6. Endpoint Analysis

Tissue or whole body extractions of perchlorate were performed according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Quantification of perchlorate in water and tissue extracts were performed according to SOP AC-2-11 "Analysis of Perchlorate by IC". The endpoint was tissue/whole body concentration of perchlorate.

15. STATISTICAL METHODS

Mean values and standard deviations (SD) were calculated for each group test based on the values obtained for each individual tissue or whole body from 4 replicates. For the exposure period, the uptake rate constant (K_1) was calculated from $K_1 = C_m / (C_w \times T)$, where C_m is the concentration in the selected tissue, C_w the concentration in the water and T is the time of exposure. When the fish were transferred to clean water, the elimination rate constant K_2 was determined using a non-linear regression model: $C_{mT} = C_{mo}e^{-K2T}$ where C_{mo} is the concentration of perchlorate in the selected tissue at the beginning of the experiment, C_{mT} the concentration of perchlorate in the selected tissue at the end of the experiment, and T the time of elimination. The half-life of the perchlorate in selected tissue was estimated according to the equation $T_{1/2} = - \ln 0.5/K_2$

16. PROTOCOL CHANGES/REVISIONS:

Initial results indicated that perchlorate anions were not completely eliminated from catfish tissue after 10 days elimination. Therefore, a new experiment was conducted and elimination was allowed for 20 days. In this experiment, 10 channel catfish were exposed to sodium perchlorate (100 ppm) for 2 days. Five fish were then sacrificed as previously described in section 14.5 and five fish were transferred into aquaria containing clean water and allowed to depurate for 20 days. See attached change in study documentation forms.

17. RESULTS

17.1. Mosquitofish

Analysis of perchlorate in water during the 2 days-exposure period revealed that the actual concentration of perchlorate averaged 74 ± 2 ppm and was close to the nominal concentration (100 ppm). The body burden concentration of perchlorate in mosquitofish at the end of the exposure period reached 9.9 ± 1.4 ppm (Table 1). The uptake rate constant K_1 was 0.07 ± 0.01 day^{-1}. The kinetics of perchlorate elimination in mosquitofish is presented in Figure 1 and Table 1. Perchlorate was rapidly eliminated from mosquitofish and no perchlorate was detected after 5 days of elimination. The elimination rate constant (K_2) and the half-life of the perchlorate ($T_{1/2}$) were 0.76 day^{-1} and 0.91 day, respectively. The concentration of perchlorate in aquarium water during the 2 first days of the elimination period increased (Table 2). No water change was performed during this period. Thereafter, the perchlorate concentration in aquarium water decreased due to the rapid elimination of percholrate from mosquitofish and the water change, which occurred every other day.

17.2. Channel catfish

Analysis of perchlorate in water during the 2 day-exposure period revealed that the actual concentration of perchlorate averaged 84 ± 4 ppm and was close to the nominal

concentration (100 ppm). After the exposure, the amount of perchlorate accumulated in each tissue appeared in the following order: head > liver > gills > GI-tract > fillet (Table 3). Perchlorate was rapidly removed from each tissue (Figure 2). After 1 day of elimination more than 50% of perchlorate was eliminated. The amounts lost from collected tissue are as follows: fillet(80%), head(75%), GI-tract(74%), gills(68%), and liver(43%). However, perchlorate was still present in all tissues after 10 days. The elimination rate constant and the half-life of perchlorate were calculated and are reported in Table 4. The liver eliminated perchlorate at the slowest rate, whereas the fillet at the highest. As in the experiment with mosquitofish, perchlorate concentrations were measured in aquarium water during the entire elimination period (Table 5). Again, the concentration of perchlorate in the water increased during the two first days of the elimination period because no water change occurred.
As perchlorate was still present in channel catfish tissues after 10 days of elimination, we conducted a new experiment. Fish were exposed for 2 days to perchlorate as previously described. Then they were transferred to clean water and allowed to depurate for 20 days. The results of this experiment are reported in Table 6. The measured levels of pechlorate in aquarium water were close to the nominal concentrations (78 ppm vs 100 ppm). The concentrations of perchlorate in the different tissues were similar to the ones reported in the 10 days elimination experiment (Table 3). After 20 days of elimination, perchlorate was still detected in liver and GI-tract of channel catfish indicating that the elimination rate was slower in those two organs. Finally, perchlorate concentrations in water during the elimination were determined and are reported in Table 7.

Table 1: Perchlorate concentration (mean ± SD; n=5) in mosquitofish whole body after exposure and during the elimination period. nd: not detected.

Time (day)	Concentration (ppm)
0	9.9 ± 1.4
1	4.5 ± 0.8
2	2.4 ± 0.3
5	nd
10	nd
20	nd

Table 2: Perchlorate concentration (mean ± SD; n=5) in water prior to and during the elimination period. nd: not detected.

Time (day)	Concentration (ppb)
0	nd
1	12.4 ± 0.9
2	16.2 ± 1.3
5	9.8 ± 0.8
10	nd
14	nd
18	nd
20	nd

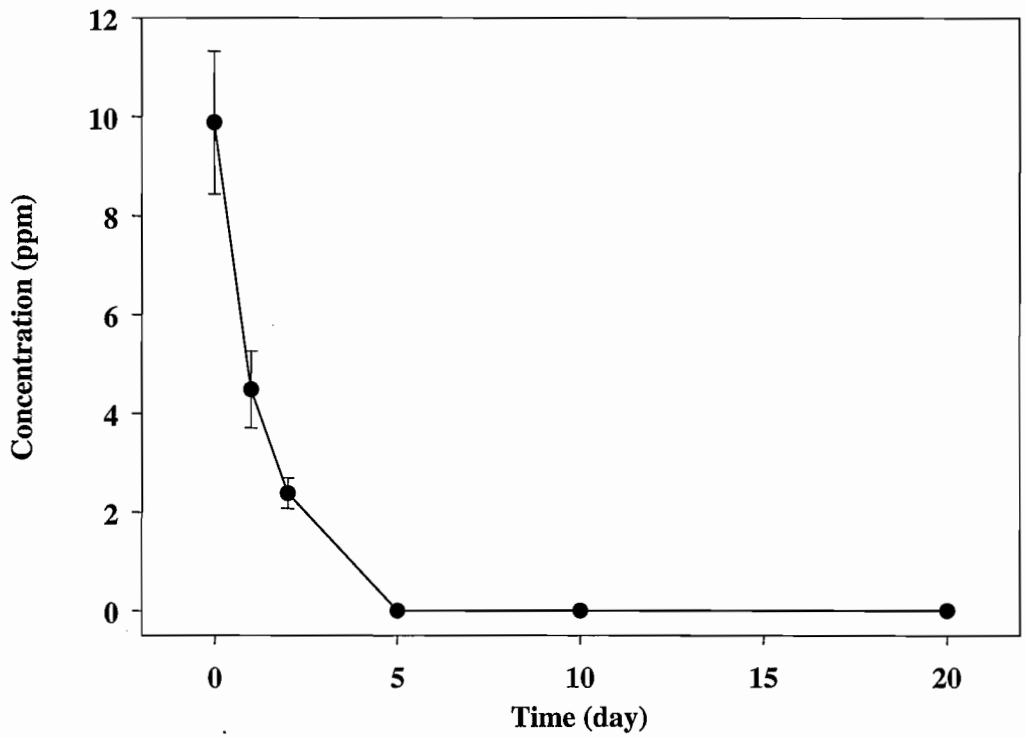

Figure 1: Elimination of perchlorate in mosquitofish whole body over a 20 days period (mean ± SD; n=5).

Table 3: Perchlorate concentration (mean ± SD; n=4 except liver n=2) in channel catfish tissues after exposure and during the elimination period. nd: not detected.

Time (day)	Concentration in channel catfish tissues (ppm)				
	Fillet	Liver	GI-tract	Gills	Head
0	3.4 ± 2.4	17.7 ± 9.4	11.2 ± 6.7	11.9 ± 5.2	17.9 ± 9.6
1	0.7 ± 0.6	10.1 ± 1.7	2.8 ± 1.2	3.8 ± 1.8	4.5 ± 1.3
2	nd	1.6 ± 0.3	1.8 ± 0.6	3.2 ± 1.4	2.8 ± 0.8
5	nd	0.6 ± 0.1	nd	1.0 ± 1.0	nd
10	1.7 ± 2.5	0.6 ± 0.8	1.1 ± 2.2	1.1 ± 1.4	2.0 ± 2.4

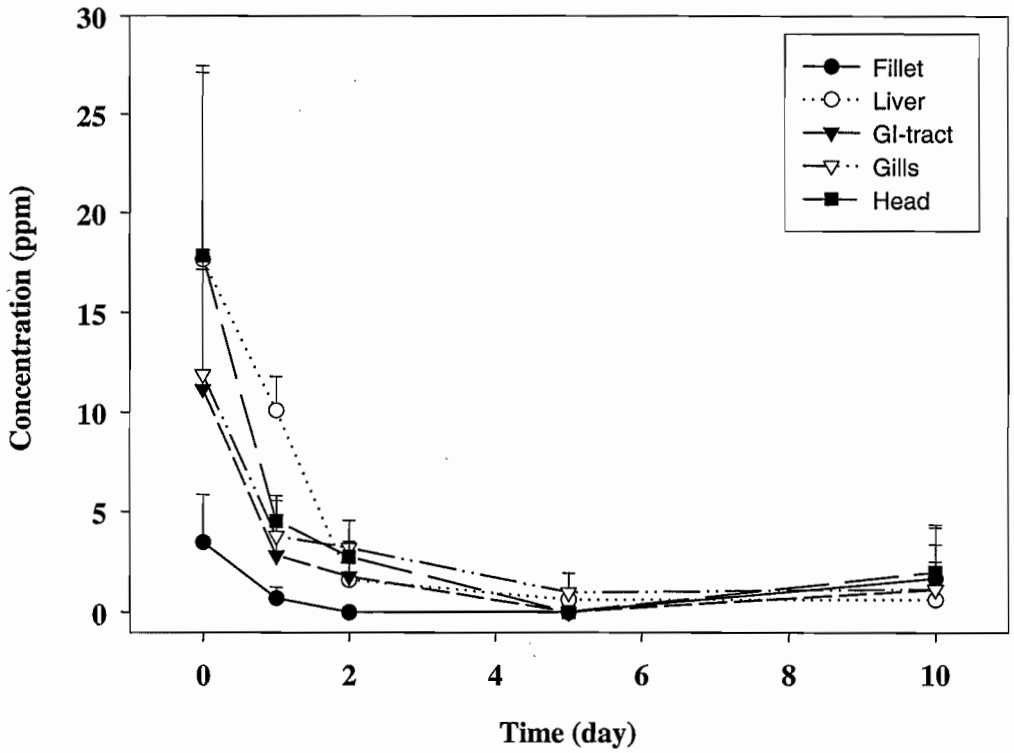

Figure 2: Elimination of perchlorate in channel catfish tissues over a 10 day period (mean ± SD; n=4).

Table 4: Elimination rate constant (K_2) and half-life ($T_{1/2}$) of perchlorate in channel catfish tissues.

Tissue	Elimination rate constant (day^{-1})	Half-life (day)
Fillet	1.67	0.41
Liver	0.79	0.88
GI-tract	1.22	0.57
Gills	0.88	0.79
Head	1.22	0.57

Table 5: Perchlorate concentration (mean ± SD) in water prior to and during the 10 day elimination experiment.

Time (day)	n	Concentration (ppb)
0	20	70 ± 82
1	16	121.2 ± 110.1
2	12	138.2 ± 114.8
3	8	106.6 ± 90.6
5	8	71.2 ± 59.3
7	4	56.0 ± 44.7
10	4	40.5 ± 29.5

Table 6: Perchlorate concentration (mean ± SD, n=5 except liver n=4) in channel catfish tissues after exposure and during the elimination period. nd: not detected.

Time	Concentration in channel catfish tissues (ppm)				
(day)	Fillet	Liver	GI-tract	Gills	Head
0	2.7 ± 0.9	30.6 ± 11.6	25.9 ± 6.4	19.6 ± 7.8	21.8 ± 6.8
20	nd	2.1 ± 1.7	0.7 ± 1.0	nd	nd

Table 7: Perchlorate concentration (mean ± SD; n=4) in water prior to and during the 20 days elimination experiment. nd: not detected.

Time (day)	Concentration (ppb)
0	1.5 ± 3.4
1	70.2 ± 28.4
2	89.3 ± 33.8
6	11.3 ± 5.0
10	6.1 ± 3.6
14	0.8 ± 1.8
18	nd

18. DISCUSSION

To date and to the best of our knowledge, no study has been conducted to determine the elimination of perchlorate in teleost fish. In the present study, the elimination of perchlorate was investigated following a 100 ppm sodium perchlorate exposure for 2 days. As perchlorate anion is highly soluble in water, the respiratory surfaces (gills) of the fish are the main route of perchlorate uptake. As previously reported (Theodorakis et al., unpublished data), under laboratory conditions, perchlorate concentrations in fish were significantly lower than the water exposure concentrations. The body burden concentration of perchlorate in mosquitofish was almost 10 times less than that seen in the water. Similar results were observed in channel catfish. However, in this species, the uptake of perchlorate appeared to be different among the tissues considered with the highest concentrations in the head and the liver. In teleost fish, thyroid tissue is not encapsulated within a discrete gland. Instead, the thyroid tissue is composed of scattered thyroid follicles located mainly in the lower jaw and around the ventral aorta and bulbus arteriosus (Donaldson et al., 1979). Perchlorate competitively inhibits the sodium/iodide symporter of the thyroid follicular cells, which actively transport iodine from the blood into the thyroid. This may explained the high concentrations of perchlorate measured in the channel catfish head.

In both studied species, mosquitofish and channel catfish, the perchlorate was rapidly removed from the fish (whole body or tissues) when the animals were transferred to clean water. The half-lifes of perchlorate in both species were less than 1 day. In rats, the perchlorate half-life ranged from 8 to 20h (Wolff, 1998). Perchlorate has been shown to be primarily eliminated from the body in urine (Clark, 2000). Moreover, perchlorate appears to be excreted virtually unchanged in rat and man despite its high redox potential. In freshwater fish, urine production is reduced and passive elimination of perchlorate probably occurs across the gills. Therefore, further studies are required to determine the route of elimination of perchlorate in fish.

The results obtained in the present study are critical and may be used to develop models of fate, effects and transport of perchlorate in natural systems, as well as to assess ecological risk in affected ecosystems.

19. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20. REFERENCES:

Clark, J.J.J., 2000. Toxicology of perchlorate. In: Perchlorate in the environment (Urbansky, E.T., ed.), pp. 15-29. New-York: Kluwer Academic/Plenum Publishers.

Donaldson, E.M., Fagerlund, U.H.M., Higgs, D.A., and McBride, J.R., 1979. Hormonal enhancement of growth. In: Fish Physiology, vol. 8 (Hoar, W.S. and Randall, D., eds), pp. 455-597. London: Academic Press.

Manzon, R.G., and Youson, JH., 1997. The effects of exogenous thyroxine (T-4) or triiodothyronine (T-3), in the presence and absence of potassium perchlorate, on the incidence of metamorphosis and on serum T-4 and T-3 concentrations in larval sea

lamprey (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Paz, D.A., Dezi, R.E., and Pisano, A., 1996. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

Wolff, J., 1998. Perchlorate and the thyroid gland. Pharm. Rev. 50: 89-105

21. APPENDICES:

Study Protocol
Changes to Study Documentation
List of Key Standard Operating Procedures

Protocol No. FISH-02-01

A STUDY PROTOCOL

ENTITLED

Elimination of Perchlorate in Native Fish

STUDY NUMBER: FISH-02-01

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

ADMINISTRATOR: The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

TESTING FACILITY

Name/Address:

The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

PROPOSED EXPERIMENTAL
START DATE: January 1, 2002

1 **DESCRIPTIVE STUDY TITLE:** Elimination of Perchlorate in Native Fish

2 **STUDY NUMBER:** T9700

3 **SPONSOR:** United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4 **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

5 **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: (date of chemical application) January 1, 2002
Termination Date: (date of last data collected) September 31, 2002

6 **KEY PERSONNEL:**
Christopher Theodorakis, Study Director
Ronald Kendall, Testing Facility Management
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager

7 **DATED SIGNATURES:**

Christopher Theodorakis	3/7/02	Dr. Christopher Theodorakis Study Director
R J Kendall	4-9-02	Dr. Ronald Kendall Testing Facility Management
		Dr. Lou Chiodo Assistant Director for Science
R M	3/8/02	Mr. Ryan Bounds Quality Assurance Manager
Todd Anderson	4-8-02	Dr. Todd Anderson Analytical Chemist

8 REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

9 STUDY OBJECTIVES / PURPOSE:

To determine kinetics of uptake and relative tissue distributions of sodium perchlorate in native fish species.

10 TEST MATERIALS:

Test Chemical name: Sodium perchlorate
CAS number: 7601-89-0
Characterization: Determination of concentration in environmental samples.
Source: Aldrich Chemical Company

Reference Chemical name: (list any standards, positive or negative control materials) ultrapure water with added sea salts ("Instant Ocean®" or any other brand of sea salts with identical or nearly identical composition).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water. 60 mg/L salts will be added.

11 JUSTIFICATION OF TEST SYSTEM

Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently,

fish and wildlife population stability as well as human health. Mosquitofish and catfish will be used as the test species because they are native Texas species.

12 **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: *Gambusia affinis,* Western mosquitofish (any native Texas species weighing less than 5 g may be substituted for mosquitofish); *Amerius spp.* bullhead catfish (any other species native to Texas weighing at least 30 g may be substituted for bullhead catfish).

Strain: Feral organisms or bred in hatcheries

Age: Adults.

Number: approximately 2400

Source: Captured in the wild, or purchased from hatcheries, Carolina Biological Supply or other commercial suppliers

13 **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**

The test system will consist of laboratory exposures constructed according to the experimental design described below. Wild fish will be identified in the field by the project manager or personel trained in the identification of such fish. Identity of all fish will be confirmed in the laboratory by visual inspection before tests are begun. Aquaria will be labeled with the aquaria number, species name, animal use protocol number, project number, test system, date of exposure and date of collection, concentration, and person responsible.

14 **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**

Fish will be exposed to 100 ppm of sodium perchlorate. Fish will be placed into precleaned aquaria or beakers. Aquaria/beakers will be cleaned by washing each aquarium according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays." For exposures, aquaria will be located on shelves capable of supporting such weight. Each shelf will hold five 40L aquaria (for the catfish), ten 20L aquaria or fifteen to twenty 4L beakers (for the mosquitofish). The experimental design will consist of a randomized block design, with each shelf constituting a block. Determine arrangement of the aquaria or beakers within each block by a random number generator, random number table or by rolling dice. Each block will contain at least 1 beaker or aquarium of each treatment.

15 METHODS:

15.1 Test System acquisition, quarantine, acclimation

Fish will be obtained from the wild populations according to SOP AQ-3-05, commercial vendors, or fish hatcheries. They will be treated commercially available antibiotics for 5 days, as instructed by the manufacturer. After five days, any debris at the bottom of the tank and 1/3 of the tank water will be cleaned and replaced with our fish water. Every other day, debris at the bottom of the tank will be cleaned by suction. Water will be continuously aerated and filtered using mechanical and biological filtration. Water will consist of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts or other brands of identical composition. Animal husbandry will be according to SOPs AQ-1-08, "General Fish Husbandry" and AQ-01-09 "Mosquitofish Husbandry". Total acclimatization period will be a minimum of one week. Once acclimated, fish will be exposed to sodium perchlorate dissolved in water for 2 days. They will then be placed into clean water for elimination of perchlorate anion.

15.2 Test Condition Establishment

Exposures will begin after fish have become acclimatized.

15.3 Test Material Application

Fish will be placed into aquaria containing 100 ppm sodium perchlorate for 2 days. They will then be moved into aquaria containing clean water. For catfish, they there will be 1 fish per aquarium and 4 aquaria per treatment (elimination for 1, 2, 5 or 10 days). All catfish will be removed from all aquaria at the end of each treatment, and the next treatment will then be started. For the mosquitofish, 25 fish will be placed into 38 L aquaria, and 5 will be removed at 1, 2, 5, and 10 days. There will be 5 extra to account for mortality, and any extras will be disposed of after 10 days. Every other day, 1/3 of the water will be replaced in each tank (water will not be changed for the 1 or 2 day catfish elimination experiments). Water will be continuously circulated through a filter containing anionic exchange resin in order to remove the perchlorate anion. Mosquitofish will be fed commercial flake goldfish food at the rate of 5 mg per gram of fish, on a daily basis. Catfish will be fed pelleted food at the same rate. Debris and uneaten food will be removed from the bottom of the tank every other day. Water samples will be taken every other day from each aquarium for perchlorate analysis (one sample will be taken from each aquarium for the 1 and 2 day catfish exposures).

Rates/concentrations: Fish will be exposed to 100 ppm sodium perchlorate in water for 2 days, thereafter they will be moved to clean aquarium water.

Frequency: After moving to clean water, fish will be allowed to eliminate perchlorate anion for 1, 2, 5, and 10 days. Elimination periods may be extended if initial results warrant.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker/aquaria water.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification:
A sample of each concentration of treated water will be tested by chemical analysis. If fish are collected from wild populations, a sample of water will be tested by chemical analysis.

15.4 Test System Observation

Tanks or beakers will be observed on a daily basis. The number of individuals that expire each day will be recorded for each perchlorate concentration. In addition, pH, dissolved oxygen, conductivity, temperature, and any other water chemistry parameters deemed appropriate by the project manager will be determined at least 3 times per week.

15.5 Animal Sacrifice and Sample Collections

Mosquitofish will be weighed, sacrificed with an overdose with MS 222, and frozen in liquid nitrogen. An overdose of MS222 will consist of immersing the animal in 1 g/L MS222 for at least 60 seconds after all gill ventilation has ceased, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Small Amphibians and Fish". Individuals will be wrapped in aluminum foil or placed in cryogenic tubes suitable for liquid-phase liquid nitrogen and will be frozen by immersion in liquid nitrogen. Perchlorate concentration will be determined as described in section 15.6. Animals may be pooled to obtain sufficient tissue for analysis (at least 2 g for perchlorate analysis).

Bullhead catfish (or other surrogate) will be anesthetized in 1.5 g/L MS222 until the animal loses righting reflex and does not respond to physical stimuli, but before gill ventilation ceases, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Small Amphibians and Fish". Fish will then be sacrificed by cervical scission and dissected to remove any combination (or all) of the following organs (for labeling purposes, the letter in parentheses will be used, see below):

Whole body (WB); fillet (FL); liver (LV); gill (GL); GI tract (GI); gonad (GD); whole blood (BD); plasma (PS); head (HD); kidney (KY); skin (SK); operculum (OP); base of the bucchal cavity (BB).

For tissue distribution of perchlorate in catfish or surrogate species, fillet, liver, gill, GI tract, and head will be collected. If the gonads are not atrophied, they will also be collected. If at least 1 ml of whole blood can be collected, it will also be analyzed for perchlorate analysis. For mosquitofish, whole bodies will be used.

Labeling: samples will be labeled with a unique ID number according to the following scheme:

LPE (laboratory perchlorate elimination)– sample number- organ (2 letter abbreviation).

e.g., LPE-0001-LV is the liver sample from fish # 0001.
LPE-0001-FL is the fillet from fish # 0001

If samples are to be divided into subsamples, then a suffix is attached. E.g., if the liver sample above is divided into 3 subsamples, these subsamples will be labeled:

LPE-0001-LV.1
LPE-0001-LV.2
LPE-0001-LV.3

If samples are to be composited, then the prefix will be LPEC. E.g., LPEC-0001-WB is composite # 1, consisting of whole bodies. The number of individual samples comprising the composite should be indicated on the fish dissection/tissue collection form and/or in a bound laboratory notebook.

Minimum information to be included on the label is project number and unique ID (SOP IN-03-02 Sample Labeling/Logging Procedure). Additional information can include species, date collected and sex (if known), in decreasing order of importance. All of this information should be recorded on the fish dissection/tissue collection sheet and/or bound laboratory notebook. Fish weight should also be recorded, and standard length (from the tip of the nose to the end of the caudal peduncle) may also be included on this form or in the notebook. Any information not determined should be entered as "ND".

15.6 Endpoint Analysis

Tissue or whole body extraction of perchlorate will be performed according to SOP AC-2-15 "Extraction and Cleanup of Tissue samples to be Analyzed for Perchlorate Using Ion Chromatography". Analysis of water and of tissue extracts will be performed according to SOP AC-2-11 "Analysis of Perchlorate by IC". The endpoint will be tissue/whole body concentration of perchlorate.

16 PROPOSED STATISTICAL METHODS

To statistically determine differences between treatments in terms of perchlorate body concentrations, 2-way ANOVA will be used to determine effects of concentration and time of exposure. Correlation and regression analysis may also be used to determine the relationship between response (body burden) vs. dose or vs. time. If warranted by lack of

normality and/or homogeneity of variances of the data, nonparametric 2-way (e.g. Friedman test) and correlation/regression will be performed.

17 REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include: Room temperature and water temperature, dissolved oxygen, salinity, and pH will be collected. Date, time, and amount of feedings per tank will be recorded. Relative tissue distribution in bullhead catfish, and relationship between perchlorate body burden and exposure concentration will be included in the report. Report content will include presentation of data, interpretation, and discussion of the following endpoints:

List individual endpoints and analyses.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18 RECORDS TO BE MAINTAINED / LOCATION:

A draft of the final report will be delivered to the Sponsor on or before September 31, 2002. The final report will be delivered to the Sponsor on or before November 14, 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point, for final archive within six months of study completion (upon request). All data, the protocol, and a copy of the final report shall be maintained by the testing facility.

19 QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20 PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and Test Facility Manager and maintained with the protocol and the Quality Assurance Unit.

TIEHH
Box 41163
Lubbock, TX 79409-1163
(806) 885-4567
qa@tiehh.ttu.edu

Form No. 014 Rev. 3.06/00
Project No.: _T9700 _____
*Change No: ___1_____
Page: _1____ of ___1__

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment ____Deviation __X__ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Elimination of perchlorate in native fish
Dated: _____5/3/02_____
Document # (if appropriate): ____FISH-02-01_____
Page #(s): _____5_____
Section #: _____15.3_____
Text to reference: _____
For catfish, they will be 1 fish per aquarium and 4 aquaria per treatment (elimination for 1, 2, 5, 10 days). After moving to clean water, fish will be allowed to eliminate perchlorate anion for 1, 2, 5 and 10 days.

Change in Document: _____
An additional elimination period of 20 days will be performed following previously described exposure and elimination procedure.

Justification and Impact on Study: _____
Initial results indicated that perchlorate anions were not completely eliminated after 10 days. Extension of elimination period to 20 days will investigate anion perchlorate presence for a longer period of elimination.

Submitted by: Signature: _____ Date: 5/3/02

Authorized by: Study Director: _____ Date: 5/2/02

Received by: Quality Assurance Unit: Brian Bridwell Date: 5/3/02

* Sequentially numbered in order of the date that the change is effective

TIEHH
Box 41163
Lubbock, TX 79409-1163
(806) 885-4567
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Form No. 014 Rev. 3.06/00
Project No.: _____T9700_____
*Change No: ___2_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: ____ Elimination of Perchlorate in Native Fish_____
Dated: _12/08/02_____
Document # (if appropriate): ___Fish 02-01
Page #(s): _____8_____
Section #: _____18_____
Text to reference: The final report will be delivered to the Sponsor on or before November 14, 2002.

Change in Document: The final report will be delivered to the Sponsor on 31 March 2003

Justification and Impact on Study: _____

Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/20/03

Authorized by: Chris Theodorakis Study Director: Chris Theodorakis Date: 2/20/03

Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 2-21-03

* Sequentially numbered in order of the date that the change is effective

Project No.: T9700
Study No.: FISH-02-02

SOPs Referenced in the Protocol

1. AQ-1-02 Cleaning Glassware and Aquaria for Perchlorate Assays
2. AQ-1-08 General Fish Husbandry
3. AQ-1-09 Mosquitofish (Gambusia spp.) Husbandry
4. IN-4-06 Use of pH Meter for pH Determination in Aqueous Solutions

A FINAL REPORT

ENTITLED

A COMPARATIVE STUDY OF PERCHLORATE CONCENTRATIONS IN MULTIPLE FISH SPECIES AT THE NAVAL WEAPONS INDUSTRIAL RESERVE PLANT, MCLENNAN COUNTY, TEXAS

STUDY NUMBER:	AQUA 02-01
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	02/28/2002
RESEARCH COMPLETION:	12/31/2002

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GOOD LABORATORIES PRACTICES STATEMENT

Project AQUA 02-01, entitled "A Comparative Study of Perchlorate Concentrations in Multiple Fish Species at The Naval Weapons Industrial Reserve Plant, McLennan County, Texas ", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

3/27/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase/Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	4-3-02	4-8-02	4-8-02	4-8-02
Final Report and Raw Data Review	1-28-03	2-4-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

03/27/03
Date

1. DESCRIPTIVE STUDY TITLE:

A Comparative Study of Perchlorate Concentrations in Multiple Fish Species at The Naval Weapons Industrial Reserve Plant, McLennan County, Texas and the Las Vegas Wash Area, Clark County, Nevada.

2. STUDY NUMBER:

AQUA 02-01

3. SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. TESTING FACILITY NAME AND ADDRESS:

The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start: 03/28/2002
Termination: 12/31/2002

6. KEY PERSONNEL:

Ron Kendall, Principal Investigator
Christopher Theodorakis, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Brian Birdwell, Quality Assurance Officer
Jacques Rinchard, Postdoctoral research Associate
June-Woo Park, Graduate Student
Les McDaniel, Graduate Student
Fujun Liu, Graduate Student

7. STUDY SUMMARY:

On March 29, 2002, water samples were collected from 6 creeks in and around the Texas Naval Weapons Industrial Reserve Plant (NWIRP). Harris Creek, North Fork South Bosque, South Fork South Bosque and Station Creek all originated on the NWIRP. Wasp creek does not originate on the NWIRP but a portion of the creek runs through it. Coryell Creek neither originates on nor runs through NWIRP. Perchlorate was detected in water samples from 3 creeks originated on the NWIRP: Harris Creek, North Fork South Bosque, and Station Creek. Several species of fish were also collected from four of those creeks. However, perchlorate was only detected in two fish, a largemouth bass from South Fork South Bosque and a yellow bullhead from Station Creek. There were no fish present in North Fork North

Bosque and the weather conditions (e.g., rain and thunderstorms) did not allow us to collect fish using electrofishing at Wasp Creek, one of our reference sites.

8. STUDY OBJECTIVES / PURPOSE:

To determine comparative body burdens of perchlorate in various species of fish collected from surface waters at the Naval Weapons Industrial Reserve Plant (NWIRP).

9. TEST MATERIALS:

Test Chemical: Perchlorate anion
CAS Number: 7601-89-9
Characterization: Determination of concentration in environmental samples
Source: Wastewater effluent discharge, groundwater seepage, runoff from or percolation through contaminated soil.

10. JUSTIFICATION OF TEST SYSTEM:

Preliminary surveys of NWIRP have revealed that measurable levels of ammonium perchlorate have been found in surface waters and fish. However, little is known about the relative uptake and concentrations of perchlorate in different species of fish collected from these waters. Such information is vital for rigorous and valid ecological risk assessments of perchlorate exposure, as well as for input into models used in predicting fate and food chain transport of perchlorate in natural environments.

11. TEST ANIMALS:

Species: Green sunfish *Lepomis cyanellus*
Longear sunfish *Lepomis megalotis*
Common stoneroller *Campostoma anomalum.*
Yellow bullhead *Ameiurus natalis*
Redfin shiner *Lythrurus umbratilis*
Black bullhead *Ameiurus melas*
Darters *Etheostoma sp.*
Western mosquitofish *Gambusia affinis*
Largemouth bass *Micropterus salmoides*
Bluegill sunfish *Lepomis macrochirus*
Strain: Wild
Age: Various
Number: 112
Source: Captured from natural waters at NWIRP.

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of natural waters within NWIRP. TTU and private contractors have identified contaminated sites in previous surveys. Reference sites were selected based on proximity to NWIRP and similarity to NWIRP water bodies, and were found not to contain detectible levels of perchlorate. Each sampling location was labeled with its whole name or a 4-letter abbreviation. Six sites have been identified on NWIRP. Their names (and 4 letter abbreviations) are Harris Creek (HARC), North Fork South Bosque River (NFSB),

South Fork South Bosque River (SFSB), Wasp Creek (WASP), Coryell Creek (CORC), and Station Creek (STAC). Any other sites added were referenced either by their full name or the 4 letter abbreviation, determined as follow: the names of ponds, creeks, etc, was abbreviated with the 1st three letters of the name followed by P (pond), C (creek), R (river), L (lake), or B (bayou) (e.g., Jim's Bayou = JIMB, Caddo Lake = CADL). If the name of the creek, lake, etc had only 4 letters, this was used in place of a 4-letter abbreviation (Star Pond = STAR). If the water body consisted of 2 or more words, the last letter of the abbreviation indicated the type of water body (P=pond, etc.), and the other letters represented the first letter of each word of a compound name, and additional letters in the name were added to total 4 letters, if needed (e.g., Little Cypress Bayou was abbreviated LCYB or LICB, provided the same abbreviation was used for all samples; East Fork Poplar Creek was abbreviated EFPC). If the name of the water body contained more than 4 words, the abbreviation of the 1st words was used (e.g., for North Fork South Bosque River was abbreviated NFSB). All samples taken from the same water body within 100 meters were counted as a single sample. If 2 or more samples were taken at intervals greater than 100 meters, or if a series of samples were taken from a stretch of lake, creek, etc, that was more than 100 meters long; the samples were suffixed with numbers (e.g., WASP-1, WASP-2, etc.). If a pond, lake, creek did not have a name associated with it, it was labeled with a letter, e.g., Pond A, Pond B, Lake A, Creek A, Creek B, Creek C, etc. The 1st 3 letters of the abbreviation were PND (pond), CRK (creek), LKE (Lake), BYU (bayou) or RIV (river), followed by A, B, C, etc. (e.g., Pond A = PNDA). All names and abbreviations were recorded on data sheets and sample tracking forms and/or in the field notebook for future reference.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Fish were collected from sites of known perchlorate contamination and at least 1-2 reference sites. As many individuals as could be collected were taken from each site, up to 30 individuals per species per site. Individuals were temporarily held in a bucket until processed. The buckets were washed between sampling sites. Before taking previously used buckets into the field or between sampling sites, buckets were rinsed with tap water or other water known to be uncontaminated with perchlorate 3 times. Mud, algae, or other residues remaining on the inside of the bucket were scrubbed off before rinsing (a different brush was used for each sampling site). The length of time animals was held in buckets was minimized, and was as similar as possible for all sites. Fish were weighed prior to processing and weights were recorded on sampling form.

14. METHODS

14.1. Test System acquisition, quarantine, acclimation

Water Sampling

At each location where fish were captured, 60 ml of water was also taken for perchlorate analysis, according to SOP AQ-3-03. Water was collected before collection of fish. Water samples were collected at either end of the section of the stream from which fish were collected, plus at least one sample in between these two points. Samples were taken at least 10 m apart, unless the section of stream to be sampled was less than 10 m long. Water

samples were collected in precleaned glass vials (Wheaton), and were collected from just under the water surface. Water samples were stored away from direct sunlight and excessive heat (> 50° C). Before samples were taken, the pH, dissolved oxygen, conductivity, and temperature was measured according to SOP IN-2-01 and recorded on TIEHH Form 181.

Fish Collection
Fish were collected with backpack electroshocker set at a current of 2-4 amps and a frequency of 30-60 cps and with a seine or dip nets as described in SOP AQ-3-05 "Fish and Amphibian Field Collection Methods". Because mosquitofish were not susceptible to electroshocking they were collected by seining. The seine was 10' long and 4' deep, with a mesh of ¼" or smaller. Holding buckets were cleaned as described in section 13.

14.2. Test Material Application

Rates/concentrations: Concentration determined by laboratory analysis.

Frequency: Perchlorate is discharged into surface waters in NWIRP continuously from ground water or wastewater effluent, or is discharged into surface waters after rainfall events via runoff or percolation of rainwater through soil.

Route/Method of Application: Ingestion of absorption of perchlorate from water and natural food items.

Justification for Exposure Route: The animals were exposed to perchlorate in water and food items in their natural environment.

Exposure Verification: Water samples were collected for determination of perchlorate concentrations wherever biota samples were collected.

14.3. Test System Observation

At every location from which water samples were taken, the following environmental parameters were evaluated: water temperature, pH, salinity, dissolved oxygen, and conductivity.

14.4. Animal Sacrifice and Sample Collections

Data Recording and Sample Labeling
Prior to processing any samples, they were given a unique ID number and species and weight were recorded on sample collection/dissection forms, as well as tissues collected and method of preservation. Fish weight was measured on a portable balance to the nearest gram (if the fish weighed 10 grams or more) or to the nearest 1/10 gram (if it weighed less than 10 grams). Prior to use, the scale was calibrated according to IN-4-01, "Field Scale Operations and Maintenance" and calibration was recorded on TIEHH Form 60.
According to the SOP IN-3-02, the information to be recorded on labels was the project number and unique ID. We also included on labels the tissue collected, the species, as well

as the date of collection. The unique ID, species, date of collection, sex and tissue were also recorded on a sample processing form (e.g. forms 027 "Multiple Fish/Amphibian Dissection/Collection Form" or 182 "Fish Dissection Form"). Labels were written in waterproof ink as the samples were frozen in liquid nitrogen.

The unique ID followed the following format: For samples collected from NWIRP, the ID was LW-(2-letter abbreviation of the sampling site)-(sample number). For example, LW-SB-1 was the unique ID for sample #1 collected from the South Fork South Bosque River (SFSB). The letter LW preceded all samples ID, signifying "Lake Waco", the closest lake to NWRP. The 2-letter abbreviation of the sampling site was derived from the 1st and last letters of the 4-letter abbreviation. The abbreviations were recorded on the data record forms. Sample numbers were assigned in the order in which they were processed. If a sample was a composite, then the letter C was added immediately before the sample number. For example: LW-SB-C-1 was a composite sample collected from South Fork South Bosque River. If a sample was divided into sub samples, a suffix consisting of a decimal point and a number were assigned. For example, if LW-SB-C-1 was divided into 2 sub samples, the IDs for these sub samples were LW-SB-C-1.1 and LW- SB-C-1.2. If a fish was dissected into constituent organs, the label of each sample was suffixed with a 2-letter abbreviation designating the organ, as follows:

Tissue	Abbreviation
Fillet	FL
Head	HD
Gills	GL
Whole blood	BD
Plasma	PL
Liver	LV
Gonad	GD
GI tract	GI

For example, LW-SB-1-BD was the whole blood sample from fish 1, LW-SB-C-1-PL was a composite sample #1 of plasma, and LW-SB-C-1.2-PL was a sub sample #2 from the blood of fish #1. Labels for whole bodies did not contain a suffix (e.g., LW-SB-1 implies this sample was a whole body, LW-SB-C-1 was a composite of whole bodies).

Perchlorate Analysis

Various species of different trophic levels were collected for perchlorate body burden analysis. Fish collected were anesthetized with an overdose of MS222 (0.5 g/L) and frozen in liquid nitrogen for perchlorate analysis. Individual fish were wrapped in aluminum foil and labeled prior to freezing. Smaller fish were consolidated into composite samples (at least 5 g of tissue as needed for perchlorate analysis). Composite samples were placed into Ziploc freezer bags and stored on ice until transport back to the laboratory. After transport to the laboratory, samples were stored in the freezer (temperature -20°C) until analysis. Water samples also were transported back to the laboratory for perchlorate analysis. Once in the laboratory, they were stored in a refrigerator (4°C) until analysis.

14.5. Endpoint Analysis

Perchlorate concentration in fish tissues were extracted according to SOP AC-2-15

"Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Analysis and quantification of perchlorate in water or extracted from tissues were performed according to SOP AC-2-11 "Analysis of Perchlorate by IC".

15. STATISTICAL METHODS

All data were checked for normality using the Shapiro-Wilk *W* test. Homogeneity of variances was checked using Bartlett's test. Comparisons between sites were accomplished by Analysis of Variance (ANOVA) for multiple mean comparisons. If the data did not meet assumptions for testing using parametric statistics, they were analyzed using nonparametric analogs such as Kruskall-Wallis test.

16. PROTOCOL CHANGES/REVISIONS:

The Las Vegas Wash Area was not sampled during this study because perchlorate was not detected in almost all our fish samples collected from the NWIRP although perchlorate was detected in the water. Moreover, it appears form our previous study that perchlorate did not bioconcentrate in fish. See attached change in study documentation forms.

17. RESULTS

On March 29, 2002, water samples were collected from 6 creeks in and around the Texas NWIRP. Harris Creek, North Fork South Bosque, South Fork South Bosque and Station Creek all originated on the NWIRP. Wasp creek does not originate on the NWIRP but a portion of the creek runs through it. Coryell Creek neither originates on nor runs through NWIRP. Perchlorate was detected in water samples from 3 creeks originated on the NWIRP: Harris Creek, North Fork South Bosque, and Station Creek (Table 1). The concentrations measured at North Fork South Bosque were significantly higher than the one measured in the other contaminated sites. No perchlorate was detected in the water from the reference sites. Several species of fish were also collected from four of those creeks (Table 2). Perchlorate was only detected in two fish, a largemouth bass from South Fork South Bosque and a yellow bullhead from Station Creek. The concentrations of perchlorate measured in those two ecological receptors were higher than the ones measured in the water where they were collected. In South Fork South Bosque, no perchlorate was detected in the water. There were no fish present in North Fork North Bosque and the weather conditions (e.g., rain and thunderstorms) did not allow us to collect fish using electrofishing at Wasp Creek, one of the reference sites. However, a preliminary study (Theodorakis et al., unpublished data) indicated that perchlorate was not detected in the different species (e.g., green sunfish, longear sunfish, stoneroller fish, mosquitofish, and blacktail shiner) collected at this reference site.

Table 1: Perchlorate concentrations (mean ± SD; n=3) in water collected near the NWIRP in March 2002. nd = not detected.

Collection sites	Perchlorate concentration (ppb)
Reference sites	
Wasp Creek	nd[a]

Coryell Creek	nd[a]
Contaminated sites	
Harris Creek	23.9 ± 0.3[b]
North Fork South Bosque	324.0 ± 4.1[c]
South Fork South Bosque	nd[a]
Station Creek	22.4 ± 0.9[b]

Table 2: Body burden concentrations of perchlorate (ppb) in fish collected near the NWIRP in March 2002. nd = not detected and - = fish not collected.

Species	Collection sites			
	Coryel Creek	Harris Creek	South Fork South Bosque	Station Creek
Green sunfish	nd (n=6)	nd (n=9)	nd (n=8)	nd (n=9)
Longear sunfish	nd (n=5)	-	nd (n=11)	nd (n=5)
Stoneroller fish	nd (n=12)	-	nd (n=6)	nd (n=2)
Yellow bullhead	nd (n=6)	-	nd (n=2)	nd (n=4), 250 (n=1)
Redfin shiner	nd (n=2)	-	-	nd (n=8)
Blacktail shiner	nd (n=2)	-	nd (n=5)	-
Black bullhead	-	nd (n=4)	-	-
Darter	-	-	nd (n=1)	-
Mosquitofish	-	-	nd (n=1)	-
Largemouth bass	-	-	nd (n=1), 373 (n=1)	-
Bluegill sunfish	-	-	-	nd (n=1)

18. DISCUSSION

The present study indicates that perchlorate exposure occurs among fish species at the NWIRP. Perchlorate concentrations in water and fish were similar to those reported at the Longhorn Army Ammunition Plant, Karnack, TX (Smith et al., 2001). In that study, body burden concentrations of perchlorate in fish ranged from below detection limits (nd) to 207 ppb.

Comparative analysis of perchlorate accumulation in fish and water from field collected specimens indicates that perchlorate levels may be higher in fish than in water, but laboratory analysis indicates that perchlorate does not bioconcentrate in fish (Theodorakis et al., unpublished data). This suggests that fish collected in the field are being exposed by some routes other than direct absorption from the water. One possible route of uptake in fish may be through the food chain. In the stream ecosystems, the primary productivity is due to periphyton (algae film growing on rocks and other solid objects) or introduction of detritus (dead and decaying leaf litter) from terrestrial sources the associated epiflora (epiphytes, bacteria, and fungi) that grow on detritus. Therefore, one possible route of exposure of fish is via accumulation of perchlorate by the periphyton or saprobes. An alternative explanation for the higher concentrations of perchlorate in fish than in water would be that there is a temporal variation in the amount of perchlorate in the water, and there is a lag time between

reductions in water concentrations and elimination of perchlorate (i.e., perchlorate persists in the body after exposure ceases). Thus, simple measurement of perchlorate in the water may be not adequate to assess fish exposure.

Finally, human exposure to perchlorate through consumption of fish caught in those creeks is highly unlikely.

19. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20. REFERENCES:

Smith, N.S., Theodorakis, C.W., Anderson, T.A., and Kendall, R.J., 2001. Preliminary assessment of pechlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. Ecotoxicology 10: 305-313.

21. APPENDICES:

Study Protocol
Changes to Study Documentation
List of Key Standard Operating Procedures

Project No. T9700

A STUDY PROTOCOL

ENTITLED

A COMPARATIVE STUDY OF PERCHLORATE CONCENTRATIONS IN MULTIPLE FISH SPECIES AT THE NAVAL WEAPONS INDUSTRIAL RESERVE PLANT, MCLENNAN COUNTY, TEXAS AND THE LAS VEGAS WASH AREA, CLARK COUNTY, NEVADA

STUDY/PROTOCOL NUMBER: AQUA-02-01

SPONSOR: US Air Force
AFIERA/RSRE
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ADMINISTRATOR: The Institute of Environmental and Human Health
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TESTING FACILITY:

Name/Address: The Institute of Environmental and Human Health
Texas Tech University/Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-41163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

PROPOSED EXPERIMENTAL
START DATE: 3/28/02

1. **DESCRIPTIVE STUDY TITLE:**
A Comparative Study of Perchlorate Concentrations in Multiple Fish Species at The Naval Weapons Industrial Reserve Plant, McLennan County, Texas and the Las Vegas Wash Area, Clark County, Nevada.

2. **STUDY NUMBER:** AQUA-02-01

3. **SPONSOR:**

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**

The Institute of Environmental and Human Health
Texas Tech University/Texas Tech University Health Sciences Center
Box 41163
Lubbock TX 79406-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: 3/28/02
Termination Date: 9/31/02

6. **KEY PERSONNEL:**
Dr. Christopher Theodorakis, Study Director
Dr. Todd Anderson, Analytical Chemist
Dr. Ronald Kendall, Testing Facilities Management/Principal Investigator
Ryan Bounds, Quality Assurance Manager
Carrie Hendrickson, Technician
Dr. Jacques Rinchard, Technician
Les McDaniel, Technician
June-Woo Park, Technician
Liu Fujun, Technician

7. DATED SIGNATURES:

_____	4/8/02	Dr. Christopher Theodorakis Study Director
_____	4/9/02	Dr. Ronald Kendall Testing Facility Management/PI
_____	4/8/02	Ryan Bounds Quality Assurance Manager
Todd Anderson	4-8-02	Dr. Todd Anderson Analytical Chemist
_____	_____	Dr. Lou Chiodo Assistant Director for Science

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:
Dr. Ronald J. Kendall
Texas Tech University
The Institute of Environmental and Human Health
Lubbock, TX 79409-1163 USA

9. STUDY OBJECTIVES / PURPOSE:

To determine comparative body burdens of perchlorate in various species of fish collected from surface waters at the Naval Weapons Industrial Reserve Plant (NWIRP) and Las Vegas Wash Area (LVWA)

10. TEST MATERIALS:

Test Chemical name: Perchlorate anion
CAS number: 7790-98-9
Characterization: Determination of concentration in environmental samples.
Source: Wastewater effluent discharge, groundwater seepage, runoff from or percolation through contaminated soil.

11. JUSTIFICATION OF TEST SYSTEM

Preliminary surveys of NWIRP and LVWA have revealed that measurable levels of ammonium perchlorate have been found in surface waters and fish. However, little is known about the relative uptake and concentrations of perchlorate in different species of fish collected from these waters. Such information is vital for rigorous and valid ecological risk assessments of perchlorate exposure, as well as for input into models used in predicting fate and food chain transport of perchlorate in natural environments.

12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, sub-strain, and age of test system):

Species: Representatives of all species that can be captured by electroshocking, dipnetting, trapping, and/or seining.

Strain: Wild animals.

Age: Various

Number: Maximum of 300 per species.

Source: Captured from natural waters at NWIRP and LVWA.

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system will consist of natural waters within NWIRP and LVWA. TTU and private contractors have identified contaminated sites in previous surveys. Reference sites will be selected based on proximity to NWIRP and LVWA and similarity to NWIRP and LVWA water bodies, and are found not to contain detectible levels of perchlorate. Each sampling location will be labeled with its whole name or a 4-letter abbreviation. To date, six sites have been identified on NWIRP. Their names (and 4 letter abbreviations) are Harris Creek (HARC), North Fork South Bosque River (NFSB), South Fork South Bosque River (SFSB), Wasp Creek (WASP), Coryell Creek (CORC), and Station Creek (STAC). Any other sites that may be added will be referenced either by

their full name of the 4 letter abbreviation, determined as follow: The names of ponds, creeks, etc, will be abbreviated with the 1st three letters of the name followed by P (pond), C (creek), R (river), L (lake), or B (bayou) (e.g., Jim's Bayou = JIMB, Caddo Lake = CADL). If the name of the creek, lake, etc has only 4 letters, this may be used in place of a 4-letter abbreviation (Star Pond = STAR). If the water body consists of 2 or more words, the last letter of the abbreviation will indicate type of water body (P=pond, etc.), and the other letters will at least represent the first letter of each word of a compound name, and additional letters in the name may be added to total 4 letters, if needed (e.g., Little Cypress Bayou may be abbreviated LCYB or LICB, provided the same abbreviation is used for all samples; East Fork Poplar Creek would be abbreviated EFPC). If the name of the water body contains more than 4 words, the abbreviation of the 1st words will be used (e.g., for North Fork South Bosque River is abbreviated NFSB), All samples taken from the same water body within 100 meters may be counted as a single sample. If 2 or more samples are taken at intervals greater than 100 meters, or if a series of samples are taken from a stretch of lake, creek, etc, that is more than 100 meters long; the samples will be suffixed with numbers (e.g., WASP -1, WASP -2, etc.). If a pond, lake, creek does not have a name associated with it, it will be labeled with a letter, e.g., Pond A, Pond B, Lake A, Creek A, Creek B, Creek C, etc. The 1st 3 letters of the abbreviation will be PND (pond), CRK (creek), LKE (Lake), BYU (bayou) or RIV (river), followed by A, B, C, etc. (e.g., Pond A = PNDA). All names and abbreviations must be recorded on data sheets and sample tracking forms and/or in the field notebook for future reference.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Fish will be collected from sites of known perchlorate contamination and at least 1-2 reference sites. As many individuals as can be collected will be taken from each site, up to 30 individuals per species per site. Individuals will be temporarily held in a bucket until processed. Different buckets will be used for each site, if possible, or else buckets will be washed between sampling sites. Before taking previously used buckets into the field or if washing between sampling sites is required, buckets will be rinsed with tap water or other water known to be uncontaminated with perchlorate 3 times. If mud, algae, or other residues remain on the inside of the bucket, it will be scrubbed off before rinsing (a different brush will be used for each sampling site). The length of time animals are held in buckets should be minimized, and should be as similar as possible for all sites. Fish will be weighed prior to processing and weight will be recorded on sampling form.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation

Water Sampling

At each location where fish are captured, 60 ml of water will also be taken for perchlorate analysis, according to SOP AQ-3-03. Water should be collected before collection of fish, if at all possible. Water samples should be collected at either end of the section of the stream from which fish were collected, plus at least one sample in between these two points. Samples should be taken at least 10 m apart, unless the section of stream to be sampled is less than 10 m long. Water samples will be collected in precleaned glass vials (Wheaton), and will be collected from just under the water surface. Water samples should be stored away from direct sunlight and excessive heat (> 50° C). Before samples are taken, the pH, dissolved oxygen, conductivity, and temperature should be measured according to SOP IN-2-01 and recorded on TIEHH Form 181.

Fish Collection

Fish may be collected with backpack electroshocker set at a current of 2-4 amps and a frequency of 30-60 cps; or they may be collected with a seine, dip net or baited traps as described in SOP AQ-3-05 "Fish and Amphibian Field Collection Methods". Traps should be placed at least 1 m. apart and checked at most every 24 hours. Traps should be anchored to a non-movable object on the shore with highly visible nylon twine or firmly attached to a highly visible floating buoy and anchored to the bottom. Placement of traps may be regularly spaced, or concentrated in habitats where target species are known to occur. Because mosquitofish and topminnows are not susceptible to electroshocking, they may be collected by seining. The seine should be at least 10' long and 4' deep, with a mesh of ¼" or smaller. Smaller seines may be used if seining smaller water bodies. If the water is too deep for backpack shocking, shocking may be done by boat. In smaller water bodies, the backpack shocker generator and power supply may be disconnected from the backpack frame and secured on the boat. In larger bodies of water, a boat-electroshocking device may be used. Any captured fish will be placed in plastic buckets with aeration until processing. A different bucket will be used for each site, or the buckets will be cleaned as described in section 14. If any fish is captured that is not readily identifiable in the field, one or more specimens will be anesthetized in 0.5 g/L MS222 (SOP AQ-1-03) and preserved in 10% neutral buffered formalin (approx. 10 ml for each g body weight) and transported back. If it can be identified as belonging to a particular family, it will be labeled as an unidentified member of that family (see SOP IN-1-06). If it cannot be identified to family, it will be labeled as an unidentified fish (see SOP IN-1-06).

15.2 Test Material Application

Rates/concentrations: Concentration determined by laboratory analysis.

Frequency: Perchlorate is discharged into surface waters in NWIRP and LVWA continuously from ground water or wastewater effluent, or is discharged into surface

waters after rainfall events via runoff or percolation of rainwater through soil.

Route/Method of Application: Ingestion of absorption of perchlorate from water and natural food items.

Justification for Exposure Route: The animals are exposed to perchlorate in water and food items in their natural environment.

Exposure Verification: Water samples will be collected for determination of perchlorate concentrations wherever biota samples are collected..

15.3 Test System Observation

At every location from which water samples are taken, the following environmental parameters will be evaluated: water temperature, pH, salinity, dissolved oxygen, and conductivity.

15.4 Animal Sacrifice and Sample Collections

Data Recording and Sample Labeling

Prior to processing any samples, they will be given a unique ID number and species and weight will be recorded on sample collection/dissection forms, as well as tissues collected and method of preservation. Fish length is also an optional parameter than can be recorded. Fish weight will be measured on a portable balance to the nearest gram (if the fish weighs 10 grams or more) of the nearest 1/10 gram if it weighs less than 10 grams. Prior to use, the scale needs to be calibrated according to IN-4-01, "Field Scale Operations and Maintenance" and calibration should be recorded on TIEHH Form 60, or in bound field notebook.

According to the SOP IN-3-02, the minimum information to be recorded on labels is the project number and unique ID. The unique ID will contain enough information to identify the tissue. If there is enough room on the label, the species will be the next most important piece of information. Date of collection, sex and tissue may also be included on the label, if space permits. The unique ID, species, date of collection, sex and tissue must be recorded in a bound notebook or on a sample processing form (e.g. forms 027 "Multiple Fish/Amphibian Dissection/Collection Form" or 182 "Fish Dissection Form", or other appropriate form approved by the study director). Pre-printed labels may be used. If labels are used on samples to be frozen or chilled on ice, the labels must be printed or written in waterproof ink.

The unique ID will follow the following format: For samples collected from NWIRP, the ID will be LW- (2-letter abbreviation of the sampling site) – (sample number). For example, LW-SB-1 is the unique ID for sample #1 collected from the South Fork South Bosque River (SFSB). The letter LW will precede all samples ID, signifying "Lake Waco", the closest lake to NWRP. The samples collected from LVWA will be identified similarly, but will have LV- (for "Las Vegas") instead of LW as the first part of the ID (e.g., LV-SB-1). The 2-letter abbreviation of the sampling site will be derived from the 1st and last letters of the 4-letter abbreviation. If this designation is already used, the 2nd and last letters of the 4-letter abbreviation shall be used. The abbreviations will be recorded in the field notebook and/or the data record forms. Sample numbers will be assigned in the order in which they are processed. If a sample is a composite, then the letter C will be added immediately before the sample number. For example: LW-SB-C-1 is a composite sample collected from South Fork South Bosque River. If a sample is divided into subsamples, a suffix consisting of a decimal point and a number will be assigned. For example, if LW-SB-C-1 and is divided into 2 subsamples, the IDs for these subsamples would be LW-SB-C-1.1 and LW- SB-C-1.2. If a fish is dissected into constituent organs, the label of each sample will be suffixed with a 2-letter abbreviation designating the organ, as follows:

Tissue	Abbreviation
Fillet	FL
Head	HD
Gills	GL
Whole blood	BD
Plasma	PL
Liver	LV
Gonad	GD
GI tract	GI

For example LW-SB-1- BD would be the whole blood sample from fish 1, LW-SB-C-1-PL would be composite sample #1 of plasma, and LW-SB-C-1.2-PL would be subsample #2 from the blood of fish #1. Labels for whole bodies do not need to contain a suffix (e.g., LW-SB-1 implies this sample is a whole body, LW-SB-C-1 is a composite of whole bodies).

Perchlorate Analysis:

Various species of different trophic levels will be collected for perchlorate body burden analysis. Fish collected will be anesthetized with an overdose of MS222 (0.5 g/L) and frozen in liquid nitrogen for perchlorate analysis. Alternatively, fish may be chilled on wet ice until transport back to the laboratory. Fish should not chilled on wet ice for more

than 5 days before processing or being frozen. Individual fish will be wrapped in aluminum foil and labeled prior to freezing or chilling. Smaller fish may also be consolidated into composite samples. Composite samples will be placed into Ziploc freezer bags and stored on ice until transport back to the laboratory. At least 5 g of tissue is needed for perchlorate analysis; fish smaller than this must be composited.

After transport to the laboratory, samples will be stored in the freezer (temperature -20° C or colder) until analysis. Water samples also are transported back to the laboratory for perchlorate analysis. Once in the laboratory, they will be stored in a refrigerator (4° C) until analysis.

15.5 Endpoint Analysis

Perchlorate concentration in fish tissues will be extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Analysis and quantification of perchlorate in water or extracted from tissues will be according to SOP AC-2-11 "Analysis of Perchlorate by IC".

16. PROPOSED STATISTICAL METHODS

All data will be checked for normality using the Shapiro-Wilk *W* test. Homogeneity of variances will be checked using (Bartlett's or Lavine's test. Comparisons between sites will be accomplished by Analysis of Variance (ANOVA) for multiple mean comparisons. If the data does not meet assumptions for testing using parametric statistics, they will be analyzed using nonparametric analogs such as Kruskall-Wallis test.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include information entered on Form No. 181 "Aquatic Sampling Form"; Form No. 182 "Fish Dissection Form" or Form No. 027 "Multiple Fish/Amphibian Dissection/Collection Form". Data on these forms will include identity, number, mass, sex and location of animals captured; and identity, amount and location or water or sediment samples collected. Alternatively, this information may be recorded in QA-approved, bound field notebooks. Additional records to be maintained include Form No. 026 "Aquatic Sample Tracking Log"; Form No. Scale calibration log; Form no. 64 b and 64c "Batched Sample tracking log"; any entries in laboratory and field notebooks; and raw data from perchlorate analysis, thyroid hormone analysis and thyroid histology.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

Perchlorate concentrations in biota collected and water concentrations of perchlorate at sites from which these biota were collected.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. RECORDS TO BE MAINTAINED / LOCATION:

A final report will be delivered to the Sponsor on or before 15 November 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point, upon request. All data, the protocol and the final report shall be archived at the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

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qa@tiehh.ttu.edu

Form No. 014 Rev. 3.06/00
Project No.: _T9700 ____
*Change No: ___1_____
Page: _1____ of ___1__

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: A Comparative Study of Perchlorate Concentrations in Multiple Fish Species at The Naval Weapons Industrial Reserve Plant, McLennan County, Texas
Dated: _____10/3/02_____
Document # (if appropriate): ____AQUA-02-01_____
Page #(s): _____3_____
Section #: _____9_____
Text to reference: To determine comparative body burdens of perchlorate in various species of fish collected from surface waters at the Naval Weapons Industrial Reserve Plant (NWIRP) and Las Vegas Wash Area (LVWA)

Change in Document: To determine comparative body burdens of perchlorate in various species of fish collected from surface waters at the Naval Weapons Industrial Reserve Plant (NWIRP).

Justification and Impact on Study:
The Las Vegas Wash Area was not sampled during this study because perchlorate was not detected in almost all our fish samples collected from the NWIRP although perchlorate was detected in the water. Moreover, it appears form our previous study that perchlorate did not bioconcentrate in fish.

Submitted by: Jacques Rinchard Signature: _____ Date: 12/8/02

Authorized by: Chris Theodorakis Study Director: _____ Date: 2/4/03

Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 2-18-03

* Sequentially numbered in order of the date that the change is effective

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Form No. 014 Rev. 3.06/00
Project No.: _____T9700_____
*Change No: ___2_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: A COMPARATIVE STUDY OF PERCHLORATE CONCENTRATIONS IN MULTIPLE FISH SPECIES AT THE NAVAL WEAPONS INDUSTRIAL RESERVE PLANT, MCLENNAN COUNTY, TEXAS AND THE LAS VEGAS WASH AREA, CLARK COUNTY, NEVADA

Dated: 12/08/02
Document # (if appropriate): AQUA 02-01
Page #(s): _____10_____
Section #: _____18_____
Text to reference: A final report will be delivered to the Sponsor on or before 15 November 2002.

Change in Document: The final report will be delivered to the Sponsor on 31 March 20003

Justification and Impact on Study: _____

Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/20/03

Authorized by: Chris Theodorakis Study Director: Chris Theodorakis Date: 2/20/03

Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 2-21-03

* Sequentially numbered in order of the date that the change is effective

Project No.: T9700
Study No.: AQUA-02-01

SOPs Referenced in the Protocol

1. AQ-3-03 Collecting Water Samples in Natural Waters
2. IN-2-01 Measuring Water Chemistry in Natural Waters
3. AQ-3-05 Fish and Amphibian Field Collection Methods
4. AC-2-15 Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate using Ion Chromatography
5. AC-2-11 Analysis of Perchlorate by Ion Chromatography (IC)
6. AQ-1-03 MS-222 Anesthesia and Euthanasia of Amphibians and Fish
7. IN-1-06 Standard Abbreviations for Common Names of Fish
8. IN-4-01 Field Scale Operation and Maintenance
9. IN-3-02 Sample Labeling/Logging Procedure

A FINAL REPORT ENTITLED:
ANALYTICAL EVALUATIONS IN SUPPORT OF TOXICOLOGICAL INVESTIGATIONS

STUDY NUMBER:	NA
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	09/28/01
RESEARCH COMPLETION:	12/31/02

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GOOD LABORATORIES PRACTICES STATEMENT

This project, entitled "Analytical Evaluations in Support of Toxicological Investigations", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

Submitted By:

Todd Anderson

Todd A. Anderson, Ph.D

3-27-03

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Audits were performed on the analytical portions of this project. The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection.

Submitted By:

Quality Assurance Manager

03/27/03
Date

1.0 DESCRIPTIVE STUDY TITLE:

Analytical Evaluations in Support of Toxicological Investigations

2.0 STUDY NUMBER:

N/A

3.0 SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4.0 TESTING FACILITY NAME AND ADDRESS:

The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start: 1/1/02
Termination: 12/31/02

6.0 KEY PERSONNEL:

Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:

The focus of the subproject outlined here was to provide analytical support for ongoing toxicological evaluations of perchlorate in fish, amphibians, and mammals. In addition, because of the paucity of relevant food chain data on perchlorate, basic environmental analyses of potential food items (vegetation and seeds) were also carried out. Our goal was to support laboratory and field studies by measuring and quantifying potential perchlorate exposure among organisms.

8.0 STUDY SUMMARY:

Perchlorate concentrations in various samples were determined using ion chromatography. Samples submitted for analysis included soil, sediment, water, plant and vegetable matter, seeds, and various biological tissues and fluids. These analyses utilized equipment and analytical methods developed and validated during previous studies on perchlorate carried out by this laboratory.

A variety of tests were used throughout the course of sample analysis to ensure optimum performance of the analytical instrument as well as the data generated.

These tests included calibration on the days of sample analysis, blank samples (DI Water), check standards, sample carryover analysis, and sample replicates among others.

9.0 TEST MATERIALS:

Test Material: Laboratory and Environmental Samples

Test Chemical: Sodium Perchlorate
CAS Number : 7601-89-0
Characterization: NIST Certified.
Source: AccuStandard, Inc.

Test Chemical: Sodium Perchlorate
CAS Number : 7601-89-0
Characterization: ACS Certified.
Source: Fisher Scientific, Inc.

Test Chemical: Ammonium Perchlorate
CAS Number : 7790-98-9
Characterization: ACS Certified.
Source: Aldrich, Inc.

Reference Chemical: deionized water (18MΩ)
CAS Number : NA
Characterization: The quality of the water was confirmed by analytical tests.
Source: Milli-Q

10.0 JUSTIFICATION OF TEST SYSTEM:

Evaluating tissue levels and identifying possible sources of perchlorate contamination are critical in determining toxicological and ecological exposure. The unique characteristics of perchlorate make accurate quantitation in biological matrices difficult. The presence of additional ions, proteins, lipids, and other biomolecules that can foul ion exchange columns further confounds accurate determination of perchlorate concentrations in biological tissues and fluids.

11.0 TEST ANIMALS:

NA.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Samples were logged in at the time of receipt and logged out at the time of analysis. As the samples were not provided in any particular order (chronologically), they were not analyzed in any particular order. Sample tracking numbers and dates were included in log sheets.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

A variety of tests were used throughout the course of sample analysis to ensure

optimum performance of the analytical instrument as well as the data generated (Skoog and Leary, 1992). These tests included calibration on the days of sample analysis, blank samples (DI Water), check standards, sample carryover analysis, and sample replicates among others.

14.0 METHODS:

Dionex Corp. (1998) originally developed an ion chromatography method which allowed detection of perchlorate in water down to 1 µg/L (ppb). We have used this method for analysis with modifications as necessary for extraction, cleanup, and detecting perchlorate in tissue and vegetation samples (Anderson and Wu, 2002; Tian et al., 2003).

General operation of the ion chromatograph (DX-500, Dionex Corp.) is described in **SOP AC-4-03-01**. The operation of the ion chromatograph for perchlorate analysis is described in **SOP AC-2-11-01** and **SOP AC-2-15-01**. These SOPs provided the basis for determining AP in dosing solutions used in the toxicological tests. As described in **SOP AC-2-11-01**, the analysis of perchlorate using the Dionex instrument is controlled by PeakNet software using a method within the software package.

15.0 RESULTS:

A variety of tests were used throughout the course of sample analysis to ensure optimum performance of the analytical instrument as well as the data generated (Skoog and Leary, 1992). These tests included calibration on the days of sample analysis, blank samples (DI Water), check standards, sample carryover analysis, and sample replicates among others. A summary of the individual data quality tests are described below.

As a calibration curve is run each time a set of samples is analyzed, we routinely include an analysis of the calibration curves as part of our evaluation. We use a certified perchlorate standard (100 µg/mL) to determine possible dilution errors as well as to prepare calibration curve standards. These calibration curves represent the analysis of identical calibration standards on different days as well as different calibration standards (calibration standards expire after 60 days). Overall, variation in detector response for the calibration standards is low. As expected, the lowest calibration standard (2.5 ppb) has the highest %CV. This concentration is also our typical method limit of quantitation. The calibration curves are very linear over the range of calibration standards. The regression coefficient (r2) for all of the calibration runs (> 100) has never been below 0.995. This represents the r2 from the untransformed data.

Throughout the analyses, we include check standards (calibration standards of known concentrations treated as samples) to ensure the performance of the calibration curve in calculating sample concentrations. In addition, we also perform individual check standard tests in which 2 perchlorate standards are analyzed repeatedly. This test also included DI water blanks in between each

check standard. The results of these tests indicate low standard deviations and coefficient of variation. The values of SD and %CV for the check standards are typical for analytical measurements and represent the precision of the analytical method. The % differences between the actual and analytically determined concentrations are indicative of method accuracy expressed in terms of relative error.

As part of our analysis of sample reproducibility, we conduct tests using environmental samples. These tests also allow us to determine potential matrix effects for the aqueous samples. The %CV (precision) from these tests are always similar to those obtained in the check standard test described above.

As part of our analysis of sample reproducibility, we also conduct tests to determine possible dilution errors with calibration standards. For example, a 100 ppb perchlorate standard is diluted with DI water to make the following perchlorate sample array: 100 ppb, 50 ppb, 20 ppb, 10 ppb, 5 ppb, and 2.5 ppb. A perchlorate calibration curve is then used to determine the concentrations of the samples and these analytically determined concentrations are compared to the expected concentrations. The results of these tests indicate that analytically determined concentrations agree with expected concentrations indicating no dilution errors. The % differences (accuracy expressed in terms of relative error) from one test ranged from –6.56% to 2.43%. These values are typical and consistent with our check standard and replicate sample analyses.

16.0 DISCUSSION

More than 1000 samples were analyzed for perchlorate in 2002. These samples included laboratory dosing solutions and exposure tank waters as well as environmental samples (water, vegetation, biological tissues). Samples were received from several research investigators (E. Smith, J. Carr, P. Smith, C. Theodorakis).

In summary, the perchlorate analyses we have conducted in support of toxicological investigations have been critically evaluated for precision and accuracy. These analyses were conducted utilizing equipment and analytical methods developed and validated during previous studies on perchlorate carried out by this laboratory.

17.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

18.0 REFERENCES:

Anderson, T. A., and T. H. Wu. 2002. Extraction, cleanup, and analysis of the perchlorate anion in tissue samples. Bulletin of Environmental Contamination and Toxicology. 68:684-691.

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Tian, K., P. K. Dasgupta, and T. A. Anderson. 2003. Simple determination of trace perchlorate in high salinity water samples by ion chromatography with online preconcentration and preelution. Analytical Chemistry. (In press).

Project No.: T9700
Study No.: N/A (Analytical Evaluations-Todd Anderson)

SOPs Referenced in the Report

1. AC-4-03 General Operation and Maintenance of the Dionex DX-500 Ion Chromatograph
2. AC-2-11 Analysis of Perchlorate by Ion Chromatography (IC)
3. AC-2-15 Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate using Ion Chromatography

A FINAL REPORT ENTITLED: ENVIRONMENTAL MODELING

STUDY NUMBER:	MOD-02-03
SPONSOR:	United States Air Force AFIERA/RSE 2413 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
RESEARCH INITIATION:	September 28, 2001
RESEARCH COMPLETION:	December 31, 2002

GOOD LABORATORIES PRACTICES STATEMENT

Project MOD-02-03, entitled "Environmental Modeling ", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

Ken Dixon

Dr. Ken Dixon

3/28/03

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

The final report was reviewed by the Quality Assurance department.

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/03
Date

1) **Sub- Project Title:**
Environmental Modeling

2) **Project Background:**
This research project will address perchlorate exposure to several animal species.

3) **Objective:**
The objectives of this sub-project are: 1) to develop mathematical models of the uptake, distribution and effects of perchlorate, 2) use computer simulation to predict long-term effects of the contaminants on wildlife populations, and 3) generate simulation results that can be used in a probabilistic risk assessment.

4) **Technical Approach:**
The modeling approach was to develop and apply mathematical models, either through modification of off-the-shelf models or *de novo* development, as appropriate, and parameterize the models using data from Phase IV laboratory studies above.

Software tests are intended to challenge the application software and other parts of the overall system functionally and structurally. Functional testing demonstrates only that the system outputs appear to be correct. It does not allow an assessment of whether the software is actually performing according to specifications and requirements. A complete functional test of every combination of inputs may not be feasible except for very small programs. Functional testing is essentially a subset of structural testing.

Structural testing is designed to exercise all modules and branches of the software and their interrelationships with the hardware and peripheral devices. Structural testing is performed to ensure that all relevant functions in the software perform as intended.

Each of the testing types described below was conducted. Performing only one type of test will not prove that the system is working properly.

A. Normal Testing includes cases that test the functional and structural integrity of the computerized system. The input data for these test cases all fall within the range the user considers to be normal. Performing enough test cases can give a reasonable level of confidence that the system behaves as intended under normal conditions.

B. Boundary Testing is performed using values that force the system to discern whether the input is valid or invalid, or to make a decision as to which branch of the program to execute. Boundary test values are set at the edges (i.e., slightly below and above) of valid input ranges. Boundary testing does not mean making the computerized system "crash" or involuntarily stop.

C. Special Case Testing, also known as "exceptional case testing", documents the system's reactions to specific types of data or lack of data and is intended to ensure that the computerized system does not accept unsuitable data. These tests should be

designed to document what happens when values that are not included in the ranges defined in the specifications are entered. Use of test cases with no data entry in a field will assist in establishing software system defaults.

D. Parallel Testing is one of the most common types of tests performed by software developers. Parallel testing is performed by running two systems in parallel and comparing the outputs (e.g., two software application versions or software compared with a manual procedure). The comparison of the outputs from the same software release on different systems or different releases on the same system is part of parallel testing plans. Parallel testing can be a valuable tool when it is used in conjunction with other testing types for validation, or to train personnel to use a new computer system.

5) Project Accomplishments:

A. Model Development.

A mathematical model was developed and computer simulations were conducted of the effects of perchlorate on populations of avian species at the Longhorn Army Ammunition Plant (LHAAP) in Karnack, Texas. The modeling objectives were to (1) predict the uptake and distribution of perchlorate in avian body tissues, (2) predict maternal transfer of perchlorate to eggs, and (3) predict the effects of ammonium perchlorate on the thyroid hormone system. The model consists of two sub-models: (1) a physiologically based toxicokinetics (PBTK) model of the uptake and distribution of perchlorate in bird body tissues for each individual in the population, including maternal transfer of perchlorate from mother to egg, and (2) a model of the thyroid hormone secretion as affected by the perchlorate concentration at the thyroid. The model is stochastic in that it contains random variables for the concentrations of perchlorate in drinking water and in other dietary components, as well as the number of eggs produced by female quail. These random variables provide the capability to conduct Monte Carlo simulations.

The model was tested with each of the structural tests described above and passed each test.

B. Model Description

The PBTK model includes compartments for blood plasma, kidney, liver, skin and fat, thyroid, heart, gut contents, gut wall, and egg (Figure 1).

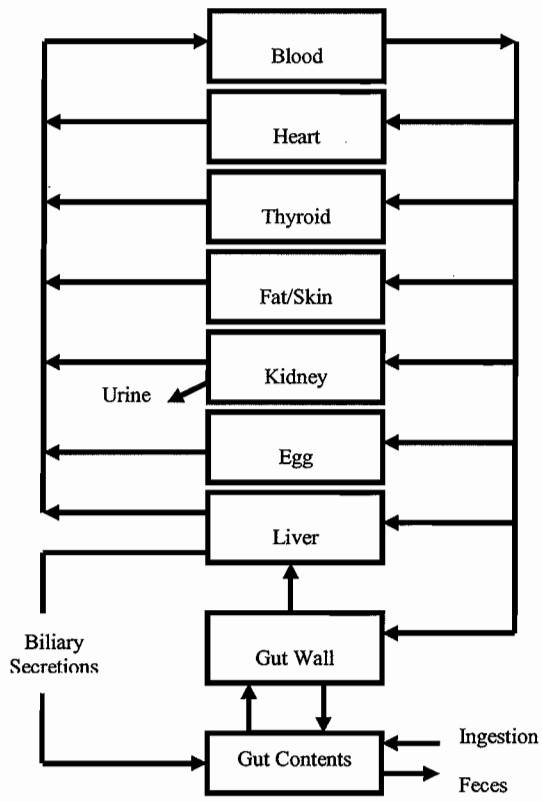

Figure 1. Flow diagram of ammonium perchlorate PBTK model.

The organ compartment concentrations of perchlorate in an individual between time t and time $t+1$ results from the rates of ingestion and elimination of perchlorate in the interval. The dose at time $t+1$, Q_{t+1}, then can be described by the general difference equation (Equation 1):

Equation 1.

$$Q_{t+1} = Q_t - \left(\frac{F \bullet (Q_t / v)}{P} \right) + \left(\frac{F \bullet (Qplasma_t / v)}{P} \right)$$

where,

Q_t = perchlorate compartment burden at time t, mg • L^{-1}

Q_{t+1} = perchlorate compartment burden at time $t+1$, mg • L^{-1}

F = Blood flow rate into the compartment, L/hour

v = Volume of compartment, L

P= Compartmental partitioning coefficient for perchlorate

$Qplasma_t$ = perchlorate plasma burned at time t, mg • L^{-1}

Equation 1 holds for the heart, thyroid, fat, and egg compartments. The plasma compartment burden is described by the difference equation (Equation 2):

Equation 2.

$$Qplasma_{t+1} = Qplasma_t - \sum\left(\frac{F \bullet (Qplasma_t / v)}{P}\right) + \sum\left(\frac{F \bullet (Q_t / v)}{P}\right)$$

where,

$Qplasma_t$ = perchlorate plasma burden at time t, mg • L^{-1}

$Qplasma_{t+1}$ = perchlorate plasma burden at time $t+1$, mg • L^{-1}

Q_t = individual perchlorate compartment burden at time t, mg • L^{-1}

F = Blood flow rate into the individual compartments, L/hour

v = Volume of individual compartments, L

P= Individual compartmental partitioning coefficients for perchlorate

The kidney burden may be described as (Equation 4):

Equation 4.

$$Qkidney_{t+1} = Qkidney_t - \left(\frac{F \bullet (Qkidney_t / v)}{P}\right) - U \bullet Qkidney_t \ldots + \left(\frac{F \bullet (Qplasma_t / v)}{P}\right)$$

where,

$Qkidney_t$ = perchlorate kidney burden at time t, mg • L^{-1}

$Qkidney_{t+1}$ = perchlorate kidney burden at time $t+1$, mg • L^{-1}

F = Blood flow rate into the kidney, L/hour

v = Volume of kidney, L

P = Compartmental partitioning coefficient for perchlorate,

U = Coefficient for the loss of perchlorate through urinary secretion

$Qplasma_t$ = perchlorate plasma burned at time t, mg • L^{-1}

The perchlorate liver burden may be determined by (Equation 5):

Equation 5.

$$Qliver_{t+1} = Qliver_t - \left(\frac{F \bullet (Qliver_t / v)}{P}\right) - B \bullet Qliver_t \ldots + \left(\frac{FG \bullet (Qgutwall_t / v)}{P}\right) + \left(\frac{F \bullet (Qplasma_t / v)}{P}\right)$$

where,

$Qliver_t$ = perchlorate liver burden at time t, mg • L^{-1}

$Qliver_{t+1}$ = perchlorate liver burden at time $t+1$, mg • L^{-1}

F = Blood flow rate into the liver, L/hour

v = Volume of liver, L

P = Compartmental partitioning coefficient for perchlorate,

B = Coefficient for the loss of perchlorate through biliary secretion

FG = Blood flow rate for the gut wall, L/hour

$Qplasma_t$ = perchlorate plasma burned at time t, mg • L^{-1}

$Qgutwall_t$ = perchlorate gut wall burden at time t, mg • L^{-1}

The perchlorate gut wall burden at time t can be described as (Equation 6):

Equation 6.

$$Qgutwall_{t+1} = Qgutwall_t - \left(\frac{FG \bullet (Qgutwall_t / v)}{P}\right) - D \bullet Qgutwall_t \ldots$$
$$+ \alpha \bullet Qgutcontent_t + \left(\frac{FG \bullet (Qplasma_t / v)}{P}\right)$$

where,

$Qgutwall_t$ = perchlorate gut wall burden at time t, mg • L^{-1}

$Qgutwall_{t+1}$ = perchlorate gut wall burden at time $t+1$, mg • L^{-1}

F = Blood flow rate of the gut wall, L/hour

v = Volume of gut wall, L

P = Compartmental partitioning coefficient for perchlorate

D = Coefficient for secretion of perchlorate out of the gut wall

FG = Blood flow rate for the gut wall, L/hour

$Qplasma_t$ = perchlorate plasma burned at time t, mg • L^{-1}

$Qgucontent_t$ = perchlorate gut content burden at time t, mg • L^{-1}

α = absorption coefficient for perchlorate contaminated gut contents

The burden of perchlorate in the gut contents may be determined as follows (Equation 7):

Equation 7.

$$Qgutcontent_{t+1} = Qgutcontent_t - \beta \bullet Qgutcontent_t - \alpha \bullet Qgutcontent_t \ldots$$
$$+ B \bullet Qliver_t + D \bullet Qgutwall_t + If_t + Iw_t$$

where,

$Qgutcontent_t$ = perchlorate gut content burden at time t, mg • L^{-1}

$Qgutcontent_{t+1}$ = perchlorate gut content burden at time $t+1$, mg • L^{-1}

D = Coefficient for secretion of perchlorate out of the gut wall

B = Coefficient for the loss of perchlorate through biliary secretion

α = Absorption coefficient for perchlorate contaminated gut contents

β = Coefficient for the loss of perchlorate through defecation

If_t = Ingestion rate of perchlorate in food items (mg•h^{-1})

Iw_t = Ingestion of perchlorate in water (mg•h $^{-1}$)

The ingestion rate of perchlorate in food, If_t, (mg•h $^{-1}$) may be written as (Equation 8.):

Equation 8.

$$If_t = \sum_{i=1}^{m} p_i \times Cf_i \times \upsilon_i$$

where

p_i = proportion of total diet contributed by item i at time t

Cf_i = consumption rate of food item i , mg•h $^{-1}$

v_i = perchlorate concentration in food item i , mg•kg $^{-1}$

Similarly, for ingestion of perchlorate in water, Iw_t (mg•h $^{-1}$) is (Equation 9):

Equation 9.

$$Iw_t = Cw \times \upsilon$$

where

Cw_i = consumption rate of water, L•h $^{-1}$

v_i = perchlorate concentration in food item i , mg•L $^{-1}$

Parameters for the PBTK model were obtained from various sources. Organ blood flow rates, weights, and volumes were scaled to represent steady-state quail values (Strukie, 1986). Blood flow rates, weights, and volumes for the egg compartment were also scaled to represent reproductive processes in quail (Freeman, 1984). Data for calibrating the PBTK model currently are lacking for all compartments except for the maternal transfer to

egg compartment (personal communication, E. Smith, Texas Tech University). We are awaiting perchlorate -dosing study data from ongoing laboratory studies that will enable us to determine more accurate parameters for the organ compartments. As such, the model output for the blood plasma, kidney, liver, skin and fat, thyroid, heart, gut contents, and gut wall compartments are merely qualitative representations for perchlorate distribution in quail.

The thyroid hormone sub-model was adapted from a model developed for the human thyroid system by DiStefano, *et al.* (1975), DiStefano and Fisher (1976), Saratchandran, et al. (1976), and DiStefano and Mori (1977). The number of parameters in the model developed by these authors were reduced or simplified in order to better represent the thyroid hormone system of wildlife species. The model is currently running with parameter values calibrated to generate output that falls within two percent of the steady-state values as reported by Saratchandran, et al. (1976). Therefore, the thyroid hormone system simulations provide only a qualitative representation of the effects of perchlorate on the avian system, and not a quantitative representation. As data becomes available from laboratory and field studies on avian thyroid hormone systems and the effects of perchlorate, the model will be calibrated accordingly. Figure 2 depicts the flow diagram for the thyroid hormone system sub-model.

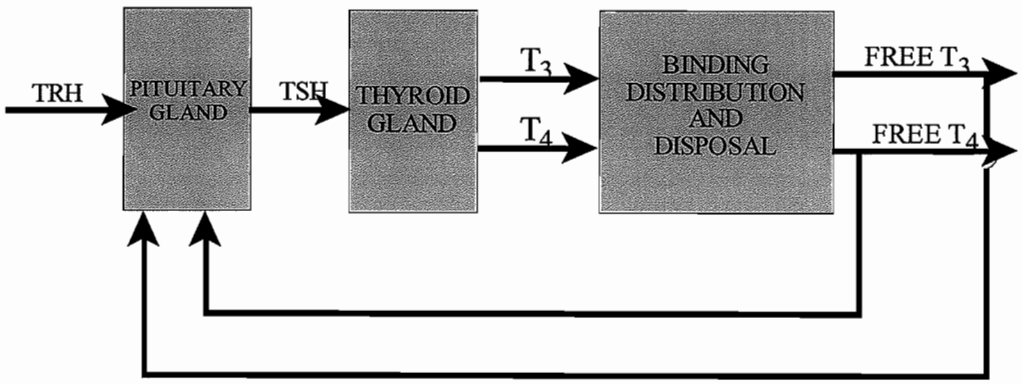

Figure 2. Flow diagram of the thyroid hormone system sub-model.

Internal dose and maternal transfer to egg were related to concentrations of perchlorate in the components of the diets of the avian species feeding in different drainages of the LHAAP watershed. The model was used to predict the dynamics of perchlorate uptake and distribution, based on stochastic feeding rates, elimination rates, and the effect of perchlorate on thyroid function (Figure 3-14). Several individuals were simulated to develop a mean distribution of perchlorate in the various tissues at the population level.

C. Analysis of Simulation Experiments

Simulations were run for quail populations feeding in two drainages of LHAAP (Harrison Bayou, and Central Creek). Each population consisted of 100 individuals with a mean body weight of 181 g. For comparison purposes, we assumed that the quail obtained their food and drinking water solely from a single area. The model is preliminary with many parameters having unknown values. Therefore, simulations do not reflect the actual exposure of quail to perchlorate at LHAAP. Perchlorate concentrations for each area are presented in Table 1.

Table 1. Perchlorate concentrations (ppb) in food items and drinking water used in model simulations.

	LHAAP Area			
	Harrison Bayou		Central Creek	
	Mean	S.D.[a]	Mean	S.D.[a]
Food				
Reptiles/ Amphibians	261.5	84.15	669.67	803.9
Insects	0.00	0.0	44.0	62.2
Misc.[b]	115.75	67.35	1582	1216.22
Water	1.0	2.36	3.0	4.73

[a] Standard deviation estimate based on range

[b] Vegetation, seeds, and fruits

RESULTS

Harrison Bayou

Simulation output for Harrison Bayou included perchlorate concentrations in quail organs and tissues (Figures 3 and 4), quail eggs (Figure 5), the concentrations of TSH in the plasma (Figure 6), the secretion rates of T3 and T4 (Figure7), and the concentration of T3 and T4 in plasma and slow and fast pools (Figure 8).

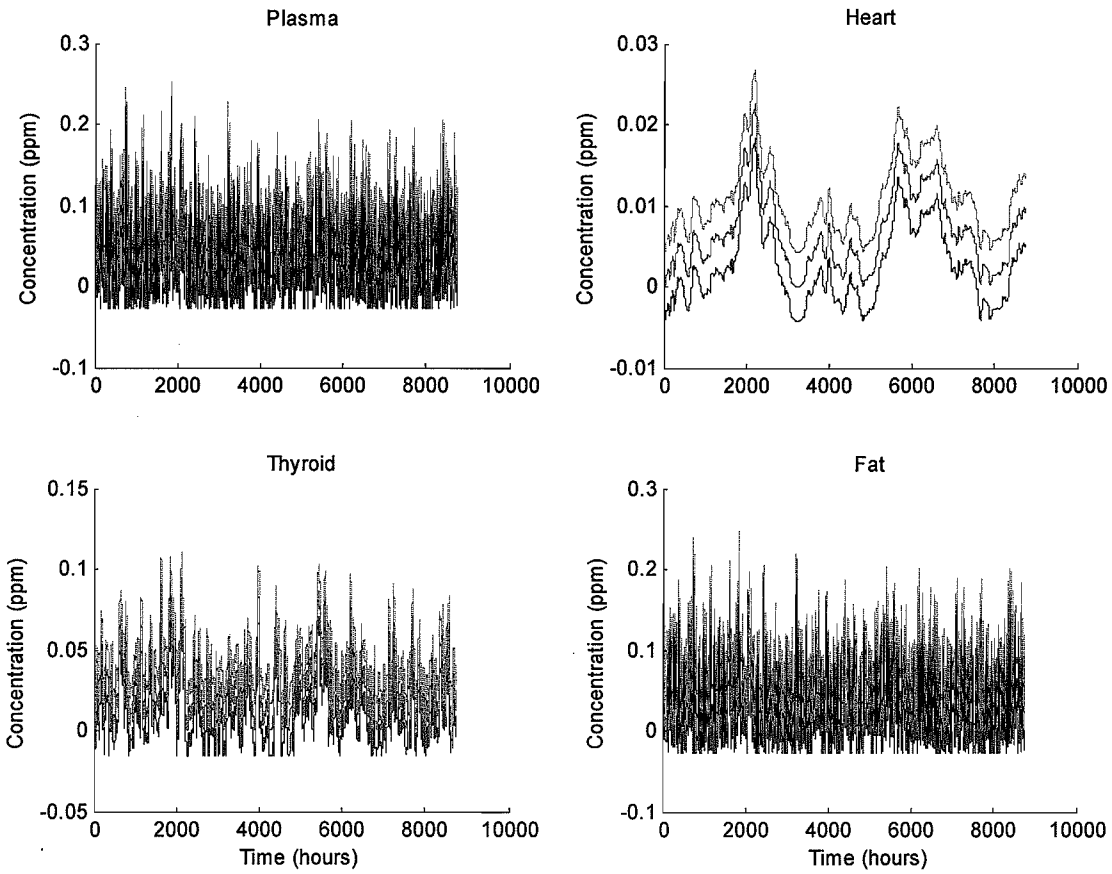

Figure 3. Simulated perchlorate concentrations in quail plasma, heart, thyroid, and fat from Harrison Bayou drainage.

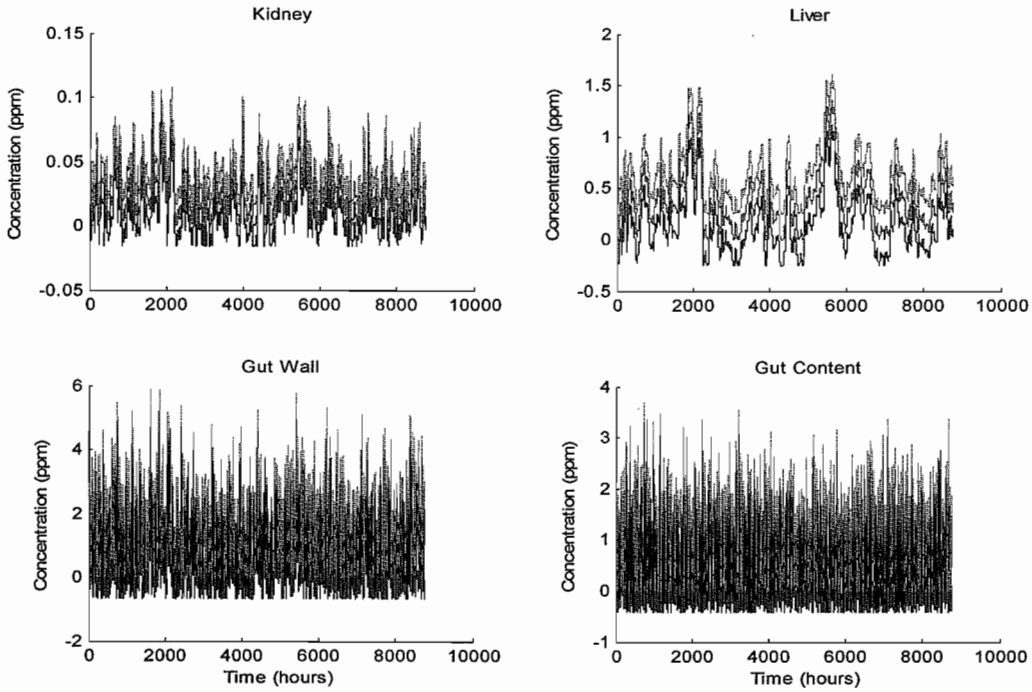

Figure 4. Simulated perchlorate concentrations in quail kidney, liver, gut wall, and gut contents from Harrison Bayou drainage.

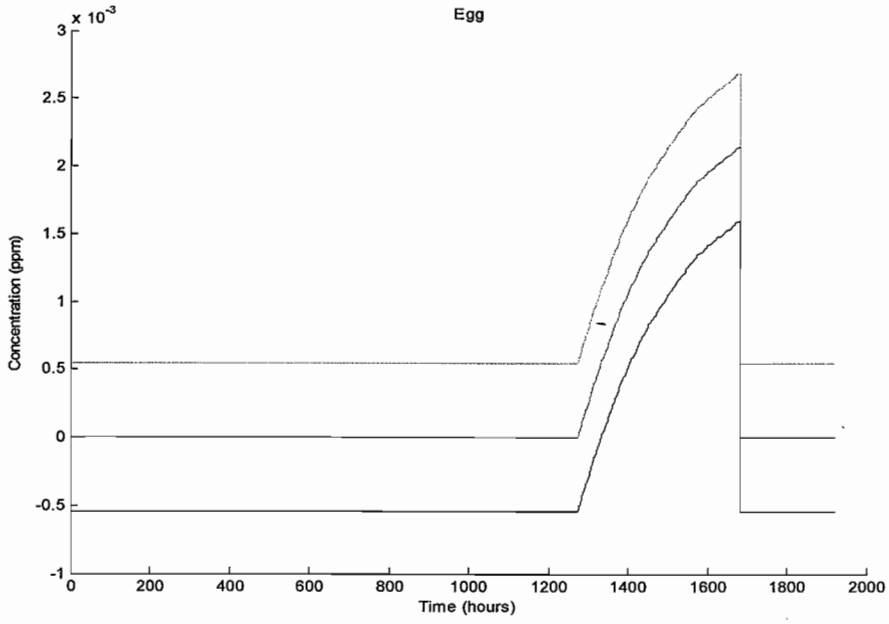

Figure 5. Simulated perchlorate concentrations in quail eggs from Harrison Bayou drainage.

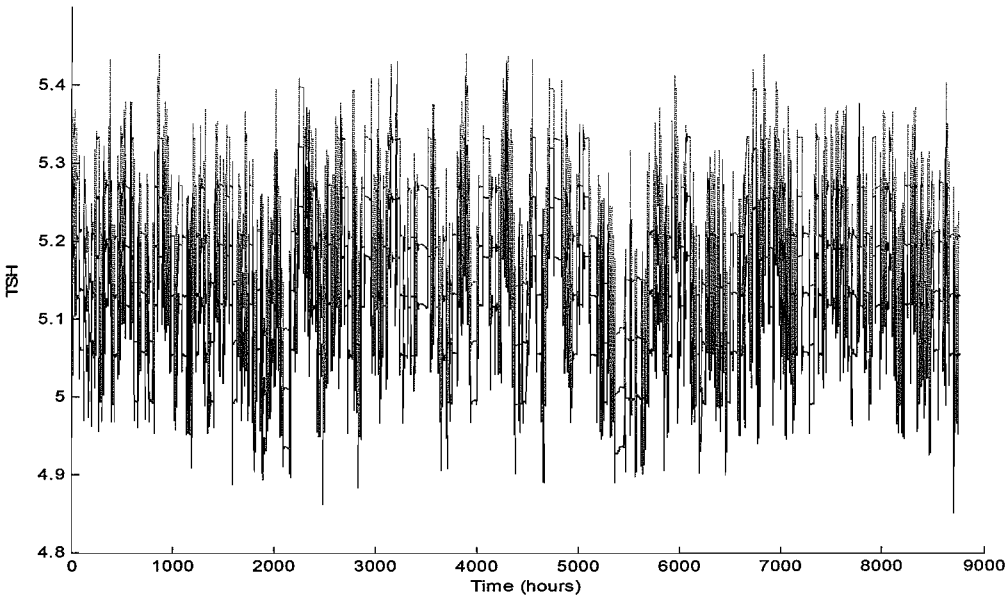

Figure 6. Simulated plasma concentrations of TSH for quail in the Harrison Bayou drainage.

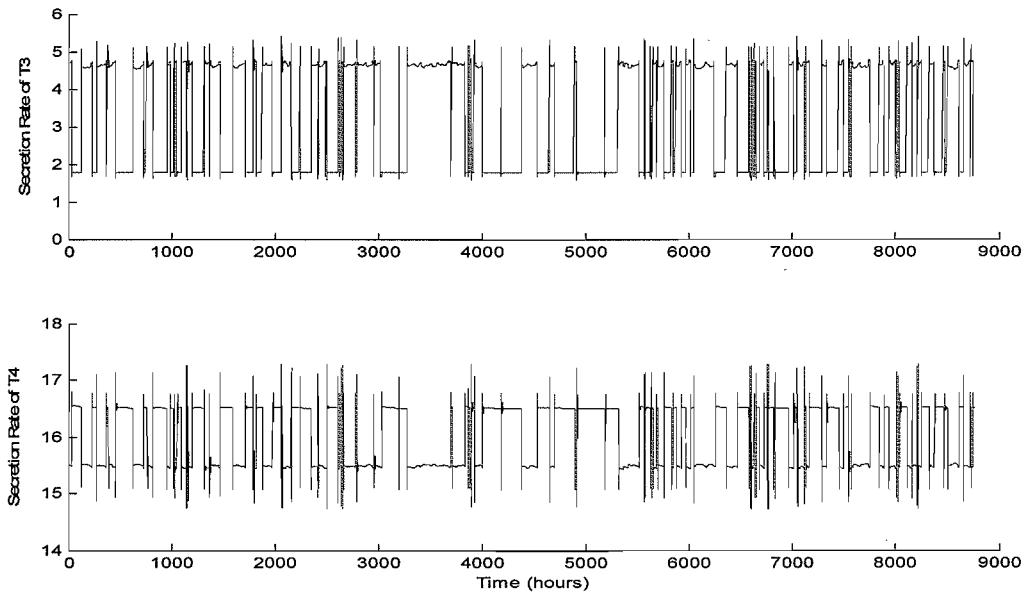

Figure 7. Simulated T3 and T4 secretion rates in quail from the Harrison Bayou drainage.

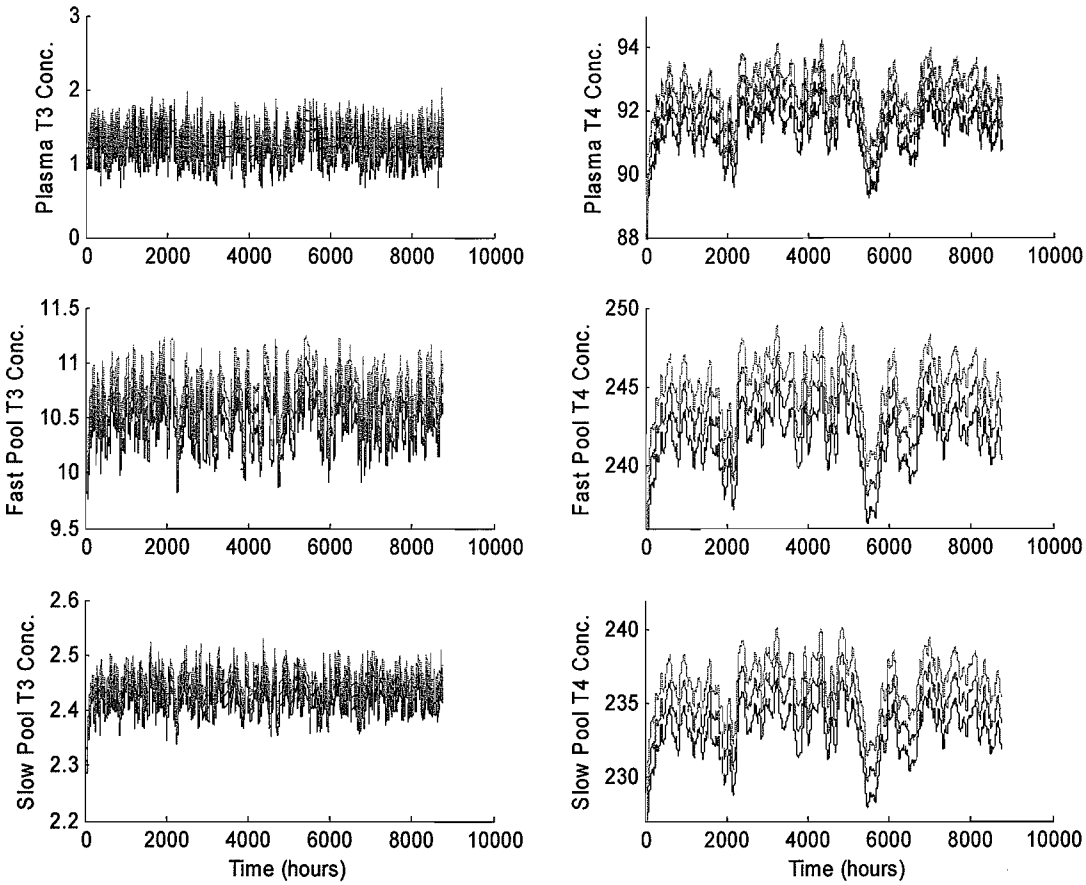

Figure 8. Simulated T3 and T4 concentrations in quail from the Harrison Bayou drainage.

Central Creek

Simulation output for Central Creek drainage included perchlorate concentrations in quail organs and tissues (Figures 9 and 10), quail eggs (Figure 11), the concentrations of TSH in the plasma (Figure 12), the secretion rates of T3 and T4 (Figure13), and the concentration of T3 and T4 in plasma and slow and fast pools (Figure 14).

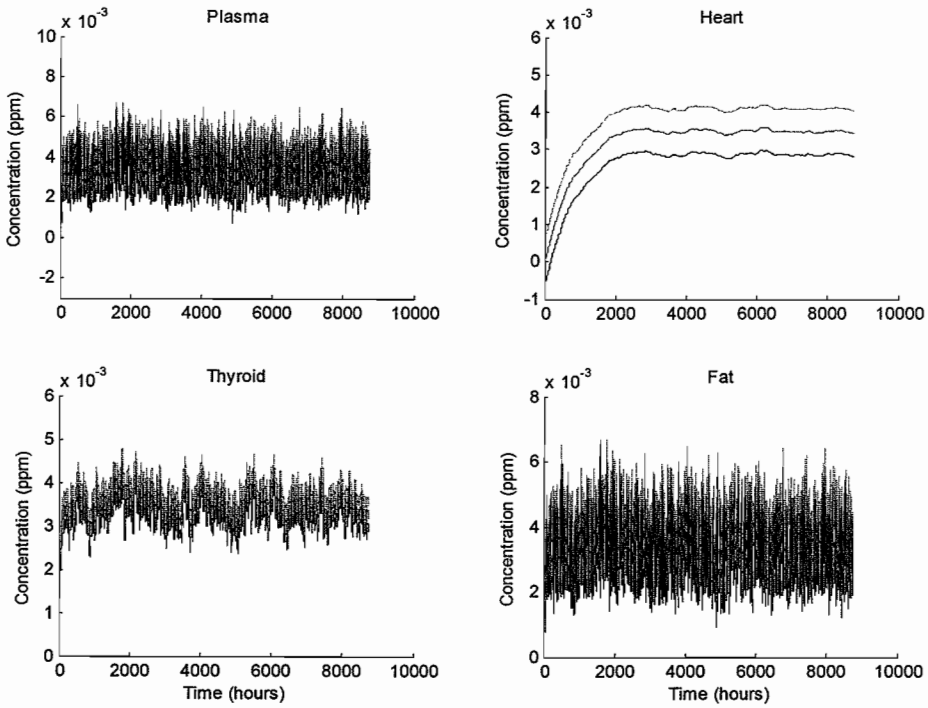

Figure 9. Simulated perchlorate concentrations in quail plasma, heart, thyroid, and fat from Central Creek drainage.

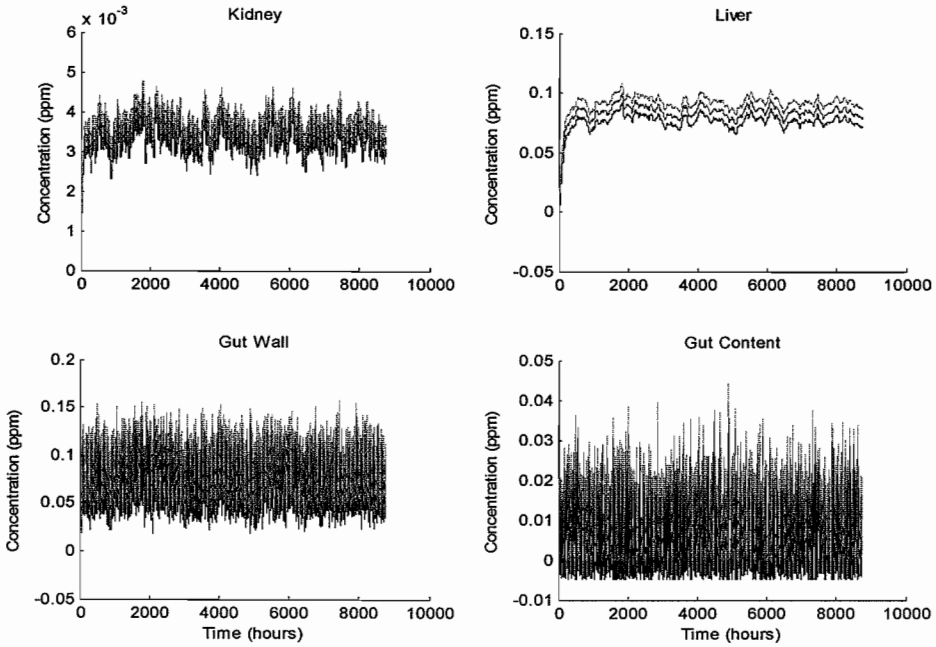

Figure 10. Simulated perchlorate concentrations in quail kidney, liver, gut wall, and gut contents from Central Creek drainage.

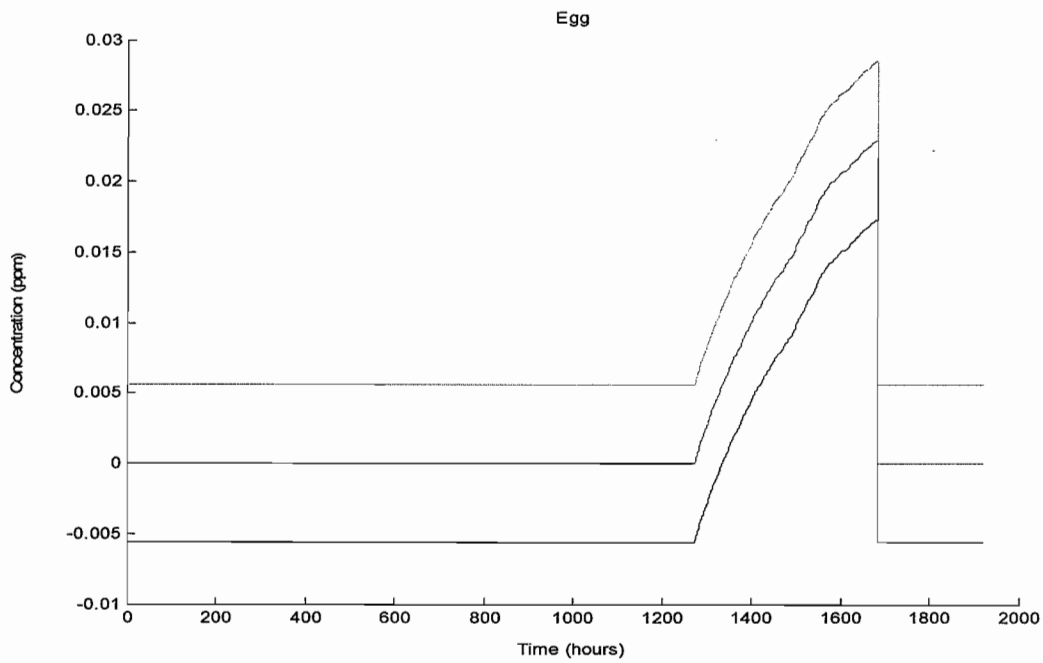

Figure11. Simulated perchlorate concentrations in quail eggs from Central Creek drainage.

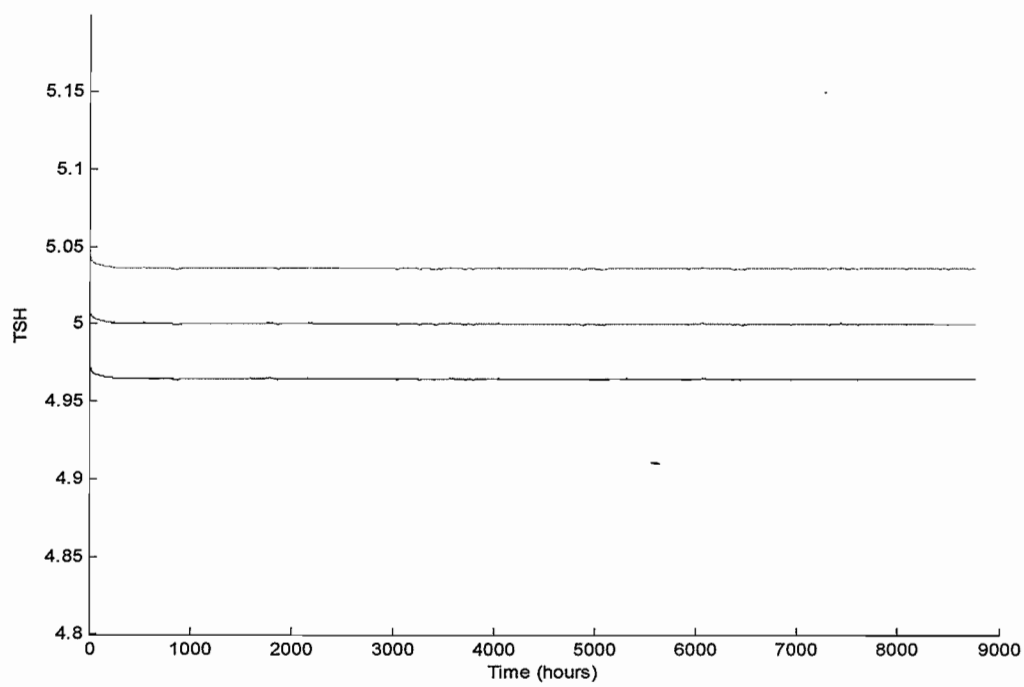

Figure 12. Simulated plasma concentrations of TSH for quail in the Central Creek drainage.

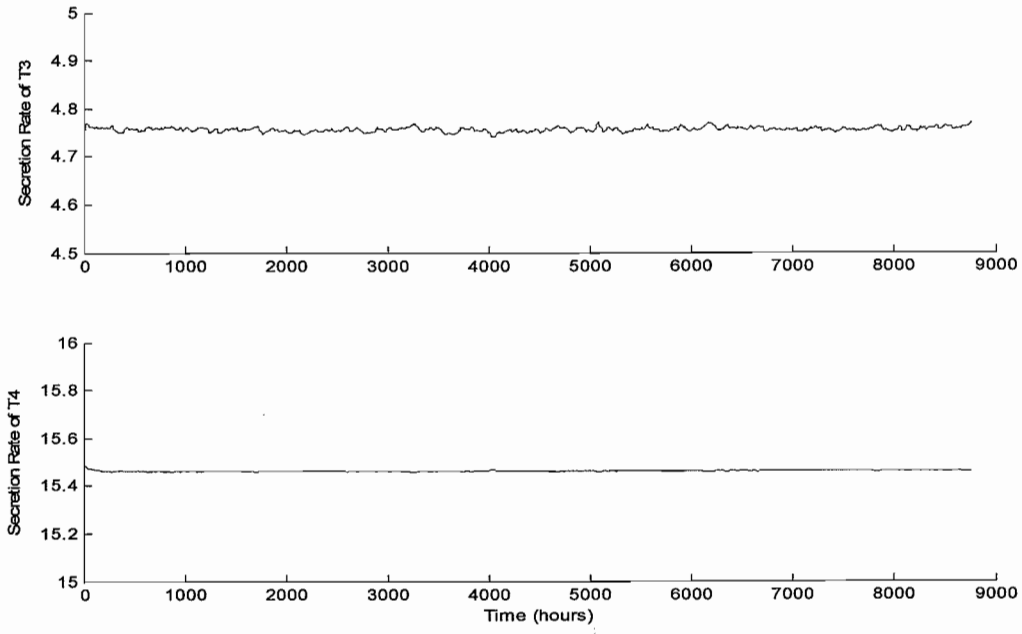

Figure 13. Simulated T3 and T4 secretion rates in quail from the Central Creek drainage.

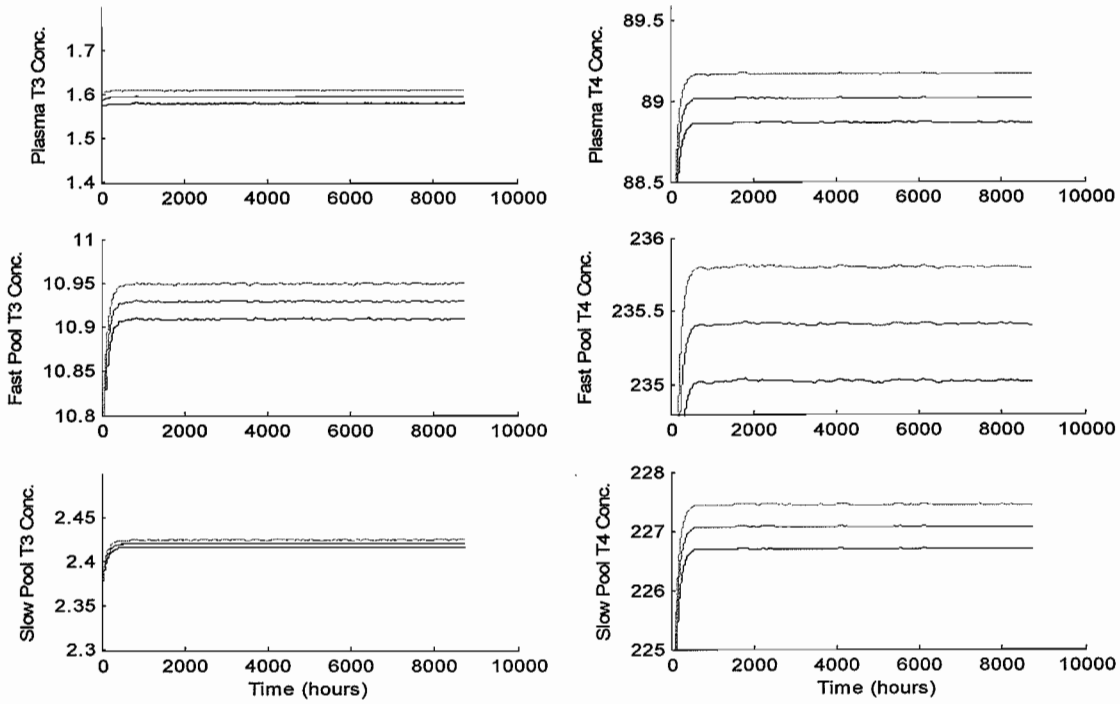

Figure 14. Simulated T3 and T4 concentrations in quail from the Harrison Bayou drainage.

D. Discussion and Conclusion

The model predicts significantly higher perchlorate concentrations in all modeled quail organ and tissue compartments for the Central Creek drainage area compared with the Harrison Bayou drainage area. The different organ and tissue concentrations did not, however, translate to significantly reduced TSH blood concentrations, T3 and T4 secretion rates, or lower T3 and T4 concentrations in model compartments. It was apparent that fluctuating perchlorate concentrations at the thyroid did result in fluctuating TSH blood concentrations, T3 and T4 secretion rates, as well as T3 and T4 concentrations in model compartments.

Caution must be taken in considering the concentration data provided by the model. With the exception of the egg compartment, all other compartment parameter estimates are mostly educated guesses at this point. The parameter estimates were made with conservative assumptions based on: (1) high transport rates from liver to blood, and (2) low elimination rates from the gut and kidney. The egg compartment provides output closer to expected real values, but caution must be utilized here as well. Partitioning coefficient estimation for this compartment was based on a small sample size, which may not fully represent the dynamics of maternal transfer of perchlorate to eggs.

These simulations are only preliminary, but with the current parameter set the simulation experiments showed reduced thyroid function with increased perchlorate concentration in the thyroid. In addition, significantly high amounts of perchlorate were predicted to be transferred from mother to egg. In order to more accurately depict levels of perchlorate in quail tissues and the resultant effect on thyroid function, more laboratory data describing the disposition of perchlorate in avian species is required.

6) Literature Cited

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Saratchandran, P., E. R. Carson, and J. Reeve. 1976. An improved mathematical model of the human thyroid hormone regulation. Clinical Endocrinology 5:473-483.

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7) Associated Personnel:

Randy Apodaca – graduate student

A FINAL REPORT

ENTITLED

RESPONSE OF LARVAL *XENOPUS LAEVIS* TO AMMONIUM PERCHLORATE AT DIFFERENT STAGES OF DEVELOPMENT

STUDY NUMBER: XEN-02-02

SPONSOR: United States Air Force
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2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

CONTRACT ADMINISTRATOR: The Institute of Environmental and Human Health
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RESEARCH INITIATION: March 8, 2002

RESEARCH COMPLETION: October 1, 2002

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GOOD LABORATORIES PRACTICES STATEMENT

Project XEN-02-02, entitled " RESPONSE OF LARVAL *XENOPUS LAEVIS* TO AMMONIUM PERCHLORATE AT DIFFERENT STAGES OF DEVELOPMENT ", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

_____ _____

James A. Carr, Ph.D Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	2-27-02	3-05-02	3-05-02	3-19-02
Facility Inspection	3-08-02	3-08-02	3-08-02	3-26-02
Test System Care and Application	3-15-02	3-15-02	3-25-02	4-04-02
Test Material mixing	3-08-02	3-08-02	3-08-02	3-26-02
Dosing	3-07-02	3-07-02	3-08-02	4-03-02
Final Report and Raw Data Review	2-28-03	3-06-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

03/28/03
Date

1. DESCRIPTIVE STUDY TITLE:
Response Of Larval *Xenopus Laevis* To Ammonium Perchlorate At Different Stages Of Development.

2. STUDY NUMBER: XEN-02-02

3. SPONSOR:
United States Air Force United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. TESTING FACILITY NAME & ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: Feb. 22, 2002
Termination Date: Sept. 15, 2002

6. KEY PERSONNEL:
James A. Carr, Co-Principle Investigator
Dr. Angella Gentles, Study Director
Fang Hu, Graduate Research Assistant
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ken Dixon, Statistical/Modeling support
Ron Kendall, Principal Investigator/ Testing Facility Manager

7. STUDY SUMMARY

Larval *Xenopus laevis* were exposed to ammonium perchlorate (AP, 14,040 ppb), beginning at Nieuwkoop-Faber stages 1-10, stage 49, or stage 55 and continuing for a period of 70-d past fertilization. As expected, exposure to AP significantly reduced the percentage of animals initiating and completing metamorphosis during the exposure period. Exposure to AP beginning at stage 49 had similar effects on metamorphosis, however exposure to AP at stage 55 resulted in greater than 50% of the animals initiating metamorphosis during the exposure period. Similarly exposure to AP at stages 1-10 and stage 49 produced the same effects on incidence of undifferentiated gonads, hindlimb growth, and thyroid histopathology. Exposure to AP at stages 1-10 or stage 49 resulted in a greater percentage of animals with undifferentiated gonads, reduced hindlimb growth, and produced thyrocyte hypertrophy and colloid depletion relative to the FETAX medium controls. In contrast, AP was ineffective in altering these endpoints when exposure began later in development, at Stage 55. In conclusion, these result suggest that tadpoles are more sensitive to perchlorate-disruption of thyroid hormone synthesis early in development, prior to secretion of T4 and T3 by the thyroid gland. Tadpoles that have reached stage 55 are not as sensitive as the younger animals to perchlorate, most likely because thyroid hormone (TH) synthesis in preparation for metamorphosis climax has already begun and alterations in thyroid hormone synthesis at this stage do not alter plasma TH levels. As importantly, our data suggest that tadpoles at stage 49 have a working negative feedback loop, as perchlorate exposure at this stage resulted in hypertrophy of thyrocytes.

8. STUDY OBJECTIVES / PURPOSE:

To determine the response of larval *Xenopus laevis* to ammonium perchlorate when they are exposed at different stages during development.

9. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.
Source: Aldrich Chemical Company
Reference Chemical name: deionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- Xenopus) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: Steam plant condensate water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the

following concentrations (Sunderman et al., 1991): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

10. JUSTIFICATION OF TEST SYSTEM

Perchlorate prevents intake of iodine from water or food and is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by thyroid hormones (TH) in animal development and reproduction, disruption of thyroid function is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health.

Based on morphological characteristics, embryonic and larval development of *X. laevis* can be divided into 66 stages (Nieuwkoop and Faber, 1967): Stage 1 represents fertilization of the egg; embryonic development then proceeds to hatching at stage 35/36. There also are key stages in TH biosynthesis: stage 33/34 is when the thyroid anlagen is first discernible; stage 49 is when thyroid follicle formation begins; stage 50 is when colloid first appears in the thyroid gland. Stage 55 immediately precedes the elevation in TH secretion that accompanies metamorphic climax (Shi, 2000). We propose to treat animals at different developing stages to investigate the stage-dependent sensitivity to AP exposure and their effects on thyroid function and gonadal differentiation in *X. laevis* larvae.

Xenopus are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their aquatic environment. They are also easily and economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of AP on aquatic fauna.

11. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: eggs, larvae, and metamorphosed subadults
Number: Approximately 800
Source: Laboratory colony.

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each tank was labeled as indicated in AQ-1-17, which includes genus and species name, common name, project name and number, date eggs were laid/hatched (if applicable), the group number and the name of the person responsible for animal care.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Approximately 1200 *Xenopus* fertilized eggs were raised in FETAX medium alone or in 14 ppm AP in FETAX medium for 70 d. Treatment began at stages 1-10, stage 49 or stage 55 and continued for 70 d post-fertilization. Treatments were performed in duplicate. Each treatment group contained 50 animals, for a total of approximately 800 (See Table 1). The selected 14ppm AP concentration is within levels reported in AP contaminated surface waters at LHAAP (Smith et al., 2001).

Table 1. Design for XEN-02-02

Experiment	Treatments	Treatment initiation	Replicates	Exposure duration
XEN-02-02-1[a]	FETAX	NF 1-10	2	70 d post fertilization
		NF 49	2	70 d post fertilization
		NF 55	2	70 d post fertilization
	14 ppm AP	NF 1-10	2	70 d post fertilization
		NF 49	2	70 d post fertilization
		NF 55	2	70 d post fertilization
XEN-02-02-2[b]	FETAX	NF 1-10	2	70 d post fertilization
		NF 49	2	70 d post fertilization
		NF 55	2	70 d post fertilization
	14 ppm AP	NF 1-10	2	70 d post fertilization
		NF 49	2	70 d post fertilization
		NF 55	2	70 d post fertilization

[a]experiment performed from 3/8/02-5/17/02. [b]experiment carried out between 7/17/02-10/1/02.

14. METHODS:

14.1 Test System acquisition, quarantine, acclimation

Five adult male and five female *Xenopus laevis* were obtained from our lab colony for each experiment. Refer to SOP AQ-1-06 for details on routine *Xenopus* husbandry. They were maintained in 45-L glass tanks containing 18 L of ultrapure reverse osmosis water for 1-2 d at approximately 22 $\pm$ 2° C on a 12L: 12D light regimen. Male and female *Xenopus* were maintained separately for at least 7 d before breeding. Please refer to SOP AQ-1-04 for details on *Xenopus* breeding.

14.2 Test condition establishment

Naturally fertilized eggs were obtained from five pairs of adults that were artificially induced to spawn (see AQ-1-04). These eggs were collected and examined under a microscope for viability (AQ-1-17). Fertilized eggs were

counted into equal number of eggs from each of three or more pairs for a total of approximately 50 eggs per group. Eggs were held in 250mL beakers. Five-day-old tadpoles were transferred to 21-L glass tanks. Each tank was labeled as indicated in section 5.5 of SOP AQ-1-17, which includes species name, common name, project name and number, date eggs were laid/hatched (if applicable), group number, and the name of the person responsible for animal care. AP or control solution was added to each glass beaker/ tank at NF stages 1-10, 49, or 55, depending upon the experiment (see Table 1). Exposures continued for 70-d post-fertilization at 22 ± 2 °C.

14.2.1 Adults were induced to spawn according to SOP AQ-1-04.

14.2.2 Fertilized eggs were held in 250mL beakers containing 100 mL AP or FETAX solution.

14.2.3 On post-hatch day 5, larvae were transferred to 21-L glass tanks containing 8 L medium. Eggs/larvae were maintained as stated in SOP AQ-1-06.

14.2.4 14 ppm test solution in FETAX medium was added into glass tanks at NF stage 1-10, stage 49 and stage 55, respectively. Fifty-percent of the test and reference solution was changed every 72 h as stated in the SOP for *Xenopus* care (SOP AQ-1-06) and was added back to each aquarium daily as needed to maintain test conditions.

14.3 Test Material Application

Rates/concentrations: 14 ppm

Frequency: Constant exposure from NF stage 1-10, stage 49, stage 55 to70 d.

Route/Method of Application: Eggs and larvae were exposed to AP or FETAX medium alone. To prepare the stock solution, 1.404 g/L of ammonium perchlorate was dissolved in 1 L ultrapure water, and served as a 100x concentration stored in 1 L amber bottle. FETAX stock solution were prepared at a 10x concentration. See SOP AQ-1-13 for FETAX preparation. AP and FETAX are added to the appropriate containers according to the following table:

Table 2. Preparation of stock solutions.

	Aquaria	Beaker
Final Nominal AP Concentration	14 ppm	14 ppm
AP Stock Solution	80 mL	1 mL
FETAX Stock Solution	800 mL	10 mL
Ultrapure Water	7120 mL	89 mL
Total Volume	8000 mL	100 mL

Method of application was immersion. Route of exposure was via dermal, oral, and respiratory exposure as the chemical was in the aquaria medium.

Justification for Exposure Route: *Xenopus* are fully aquatic as larvae and as adults.

Exposure Verification: Samples of test and reference solutions were analyzed for perchlorate content (AC-2-11).

14.4 Test System Observation

Water quality, including salinity, conductivity, pH, dissolved O_2, and ammonia was performed once a week. Temperature, by means of a surrogate tank, was monitored daily. Water samples from each tank and diluted stock solution were removed every 3 d for perchlorate analysis. Tank water samples were collected after the medium has been renewed. Dead animals were removed and preserved in 10% neutral buffered formalin (NBF).

14.5 Animal Sacrifice and Sample Collections

Sample collections were performed at 70 d post-fertilization. All larvae from each tank were euthanized by immersion in MS-222 (1g/L, AQ-1-03), staged, measured for snout-vent length, and weighed. Five larvae will be frozen on dry ice for subsequent determination of whole body thyroid hormone content, with the remaining larvae fixed in Bouin's fixative for subsequent histological assessment of the thyroid gland and gonad.

14.6 Endpoint Analysis

Assays for whole body tetraiodothyronine (T_4) and triiodothyronine (T_3) were performed by RIA (DBS SOP IN-2-04). Gonads of all fixed animals sampled were examined by visual inspection to determine sex. Please see DBS SOP IN-2-03 for gonad determination by light microscope. Gonads and thyroid glands were examined using routine histological methods (Bouin's fixative, paraffin sections) currently operational in Dr. Carr's laboratory. Sections will be stained with hematoxylin and eosin. See DBS SOP IN-2-08 for thyroid gland histopathology

15. RESULTS

Mortality in the these experiments was somewhat higher than expected for 70-d test exposures in *X. laevis* in our lab, ranging from 7-35% over two experiments. Although incidence of edema was generally low, 0-1.85%, incidence of bent tails tended to be higher than normal ranging from 0-8.17% (Table 1). There were no obvious treatment-related effects on incidence of bent tails or incidence of edema. There were clear treatment effects on initiation of metamorphosis. Ammonium perchlorate significantly inhibited forelimb emergence when exposures began at NF stage 1-10 and stage 49 (Table 1). There were no obvious treatment-related effects on sex ratio, although the number of undifferentiated gonads tended to be greater in the larvae treated with AP beginning at fertilization and NF-stage 49 (Table 2). Larvae with undifferentiated gonads were processed for routine paraffin histology and the gonads sectioned histologically to determine the phenotypic gonadal sex. There were no clear affects of AP on snout-vent length or body weight (Table 3), although as predicted AP exposure reduced hindlimb length growth when exposure began at fertilization or at NF-stage 49, but not when exposure began at NF-stage 55. AP exposure increased thyroid follicle cell hypertrophy and colloid depletion relative to their respective controls as determined by two-tailed t-test when larvae were exposed beginning at stages 1-10 or at NF-stage 49 ($p<0.0001$) but not when exposed at NF-stage 55 ($p>0.05$, Table 6).

Analytical results indicate that perchlorate was not detected in the FETAX medium stock solutions or test tanks. Perchlorate concentrations (14,040 ppb nominal) in the perchlorate test tanks (data based on experiment XEN-02-2-2) were 12,268 $\pm$ 428 ppb (n=46, stage 1-10 exposures), 11,471 $\pm$ 748 ppb (n=24, stage 55 exposures) and 10,440 $\pm$ 275 ppb (stage 49 exposures). Although the perchlorate concentrations in the test tanks were significantly different between the stage 1-10 and stage 49 exposures, it is unlikely this affected the outcome of the exposures ($p = 0.0024$, one-way ANOVA), as none of the thyroid-sensitive endpoints were significantly different between these treatment groups. Stock solutions of perchlorate ranged from 11,260 ppb to 13,400 ppb in experiment XEN-02-2-2.

Table 3. Mean Percentage (%) of Development Deformities and Metamorphosis in *Xenopus laevis* tadpoles exposed to FETAX[a] medium and Ammonium Perchlorate (14 ppm) at Different Developmental Stages[b]

Treatment	Exposure Stage	Mortality	Hatching	Bent Tail	Edema	FLE[c]	Tail Absorption
FETAX	1-10	35.2	74.6	2.45	1.31	60.1	45.5
AP		14.9	80.3	8.17	1.85	5.07	0.00
FETAX	49	20.0	NA	3.89	0.00	56.1	1.50
AP		27.3	NA	7.21	0.00	10.5	0.00
FETAX	55	25.2	NA	0.00	0.49	84.1	51.2
AP		6.67	NA	0.00	0.00	52.5	43.3

[a] FETAX, Frog Embryo Teratogenesis Assay-*Xenopus*
[b] Nieuwkoop-Faber (1967), larvae were exposed at these stages
[c] Forelimb emergence, # number with forelimbs divided by # hatching

Table 4. Sex Ratios in *Xenopus laevis* tadpoles exposed to FETAX[a] medium and Ammonium Perchlorate (14 ppm) at Different Developmental Stages[b]

Treatment	Exposure stage	# Male	# Female	Sex Ratio (M/F)	# undifferentiated gonad[c]
FETAX	1-10	45	64	0.70	2
AP		42	59	0.71	35
FETAX	49	79	78	1.01	15
AP		42	58	0.72	47
FETAX	55	73	78	0.93	3
AP		76	80	0.95	4

[a] FETAX, Frog Embryo Teratogenesis Assay-*Xenopus*
[b] Nieuwkoop-Faber (1967), larvae were exposed at these stages.
[c] Gonads cannot be identified based on gross morphology.

Table 5. Mean Body Weight, HLL[a] and SVL[b] in *Xenopus laevis* tadpoles exposed to FETAX[c] and Ammonium Perchlorate (AP) at Different Stages[d]

Treatment	Stage	Body Weight (g)	SVL (mm)	HLL (mm)
FETAX	1-10	0.93 ± 0.03	18.1 ± 0.24	19.2 ± 0.50
AP		1.22 ± 0.06	17.7 ± 0.31	3.60 ± 0.33
FETAX	49	0.68 ± 0.02	15.7 ± 0.16	13.2 ± 0.51
AP		0.79 ± 0.03	16.0 ± 0.81	4.97 ± 0.38
FETAX	55	0.76 ± 0.02	16.3 ± 0.14	17.9 ± 0.29
AP		0.74 ± 0.03	16.2 ± 0.20	13.8 ± 0.48

[a]HLL, hind limb length
[b]SVL, snout vent length
[c]FETAX, Frog Embryo Teratogenesis Assay-*Xenopus*
[d]Nieuwkoop-Faber (1967), larvae were exposed at these stages.

Table 6. Thyroid Histopathology of *Xenopus laevis* tadpoles exposed to FETAX[a] and Ammonium Perchlorate (AP) at Different Stages[b]

Treatment	Stage	Mean Colloid Depletion	Mean Follicular Cell Hypertrophy	Mean Hyperplasia
FETAX	1-10	0.00 ± 0.00	0.12 ± 0.03	0.00 ± 0.00
AP		1.53 ± 0.04	1.45 ± 0.03	0.05 ± 0.01
FETAX	49	0.00 ± 0.00	0.11 ± 0.04	0.00 ± 0.00
AP		1.84 ± 0.02	1.21 ± 0.03	0.59 ± 0.03
FETAX	55	0.71 ± 0.05	0.29 ± 0.05	0.00 ± 0.00
AP		0.07 ± 0.02	0.42 ± 0.03	0.01 ± 0.01

[a] FETAX, Frog Embryo Teratogenesis Assay-*Xenopus*
[b] Nieuwkoop-Faber (1967), larvae were exposed at these stages

16. DISCUSSION

Our data are the first to indicate that the sensitivity of *X. laevis* tadpoles to the antimetamorphic effects of AP change with development. Tadpoles exposed to AP prior to thyroid gland development exhibit much more pronounced disruption of thyroid function than tadpoles exposed at NF stage 55. The effects of AP on tadpoles exposed from stage 1-10 and stage 49 are consistent with previous reports on the effects of AP on development and metamorphosis when exposed from stages 1-10 (< 24 h after fertilization). We (Goleman et al. 2002a,b) have previously shown that 14-ppm AP reduced hindlimb growth but does not affect somatic growth, and inhibits the initiation and completion of metamorphosis in *X. laevis*. Furthermore, exposure of AP to produced profound changes in the histology of the thyroid gland, causing thyrocyte hypertrophy and colloid depletion (Goleman et al., 2002b; Carr et al., 2003). None of these effects were observed in tadpoles exposed to AP beginning at stage 55. The most likely cause for the reduced sensitivity of stage 55 animals to AP is the fact that T4 and T3 secretion are already becoming elevated in plasma (Shi, 2000), and the impact of disrupting TH synthesis is much less noticeable. Simply stated, AP exposure at stage 55 would not be expected to impact T4 and T3 level already established in the blood circulation.

Another novel finding of this study is the fact that tadpoles at stage 49 have an operational negative feedback loop between the thyroid gland and the pituitary. This is the only way to explain the fact that stage 49 tadpoles exhibited hypertrophy of the thyroid follicle epithelium, which is caused by reduced negative feedback on pituitary TSH secretion. As a result of perchlorate disrupting the normal negative feedback pathways, plasma TSH levels become elevated and result in hypertrophy of thyrocytes. The fact that negative feedback is present early in development is significant, as others have proposed that negative feedback is not established until much later during metamorphic climax (Huang et al., 2001).

17. REFERENCES:

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18. APPENDICES:

Study Protocol
Change to Study Documentation
List of Key Standard Operating Procedures

A STUDY PROTOCOL

ENTITLED

RESPONSE OF LARVAL *XENOPUS LAEVIS* TO AMMONIUM PERCHLORATE AT DIFFERENT STAGES OF DEVELOPMENT

STUDY/PROTOCOL NUMBER: XEN-02-02

SPONSOR: United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY

Name/Address: The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. James A. Carr

Study Director: Dr. Angela Gentles

PROPOSED EXPERIMENTAL START DATE FEBRUARY 22, 2002

1. **DESCRIPTIVE STUDY TITLE:** Response of Larval *Xenopus Laevis* to Ammonium Perchlorate at Different Stages of Development.

2. **STUDY NUMBER:** XEN-02-2

3. **SPONSOR:**
United States Air Force United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: Feb. 22, 2002
Termination Date: Sept. 15, 2002

6. **KEY PERSONNEL:**
James A. Carr, Co-Principle Investigator
Dr. Angela Gentles, Study Director
Fang Hu, Graduate Research Assistant
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ken Dixon, Statistical/Modeling support
Ron Kendall, Principal Investigator/ Testing Facility Manager

7. DATED SIGNATURES:

BASGentles	3/5/02.	Dr. Angela Gentles Study Director
	3/14/02	Dr. James Carr Co-Principle Investigator
	3/15/02	Ryan Bounds Quality Assurance Manager
Todd Anderson	3-15-02	Dr. Todd Anderson Analytical Chemist
	3/18/02	Dr. Ken Dixon Statistical/Modeling Support
	19-MAR-02	Dr. Lou Chiodo Assistant Director for Science
	19 March '02	Dr. Ron Kendall Principal Investigator/ Testing Facility Management

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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Dr. James A. Carr
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Lubbock, Texas 79409

9. STUDY OBJECTIVES / PURPOSE:

To determine the response of larval *Xenopus laevis* to ammonium perchlorate when they are exposed at different stages during development

10. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.
Source: Aldrich Chemical Company
Reference Chemical name: deionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- Xenopus) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: Steam plant condensate water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Sunderman et al., 1991): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

11. JUSTIFICATION OF TEST SYSTEM

Perchlorate prevents intake of iodine from water or food and is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by thyroid hormones (TH) in animal development and reproduction, disruption of thyroid function is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health.

Based on morphological characteristics, embryonic and larval development of *X. laevis* can be divided into 66 stages (Nieuwkoop and Faber, 1967): Stage 1 represents fertilization of the egg; embryonic development then proceeds to hatching at stage 35/36. There also are key stages in TH biosynthesis: stage 33/34 is when the thyroid anlagen is first discernible; stage 49 is when thyroid follicle formation begins; stage 50 is when colloid first appears in the thyroid gland. Stage 55 immediately precedes the elevation in TH secretion that accompanies metamorphic climax (Shi, 2000). We propose to treat animals at different developing stages to investigate the stage-dependent sensitivity to AP exposure and their effects on thyroid function and gonadal differentiation in *X. laevis* larvae.

Xenopus are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their aquatic environment. They are also easily and

economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of AP on aquatic fauna.

12. **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: eggs, larvae, and metamorphosed subadults
Number: Approximately 800
Source: Laboratory colony.

13. **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**
Each tank will be labeled as indicated in AQ-1-17, which includes genus and species name, common name, project name and number, date eggs were laid/hatched (if applicable), the group number and the name of the person responsible for animal care.

14. **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**
Approximately 400 *Xenopus* fertilized eggs will be raised in FETAX medium alone or in 14 ppm AP in FETAX medium for 70 d. Treatments will begin at stages 1-10, stage 49 or stage 55 and continue for 70 d post-fertilization. The treatment will be performed in duplicate. Each treatment group will contain 50 animals, for a total of approximately 800 (See Table 1). The selected 14ppm AP concentration is within levels reported in AP contaminated surface waters at LHAAP and referred in Goleman et al. (2002).

Table 1. Design for XEN-02-02

Experiment	Treatments	Treatment initiation	Replicates	Exposure duration
XEN-02-02-1	FETAX	NF 1-10	2	70 d post fertilization
		NF 55	2*	70 d post fertilization
	14 ppm AP	NF 1-10	2	70 d post fertilization
		NF 55	2*	70 d post fertilization
XEN-02-02-2	FETAX	NF 1-10	2	70 d post fertilization
		NF 49	2*	70 d post fertilization
	14 ppm AP	NF 1-10	2	70 d post fertilization
		NF 49	2*	70 d post fertilization

*This is dependent on the number of animals available at each stage. Each experiment may need to be repeated to ensure sufficient replicates for determining sex ratio.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation

Five adult male and five female *Xenopus laevis* will be obtained from our lab colony. Refer to SOP AQ-1-06 for details on routine *Xenopus* husbandry. They will be maintained in 45-L glass tanks containing 18 L of ultrapure reverse osmosis water for 1-2 d at approximately 22 $\pm$ 2° C on a 12L: 12D light regimen. Male and female *Xenopus* will be maintained separately for at least 7 d before breeding. Please refer to SOP AQ-1-04 for details on *Xenopus* breeding.

15.2 Test condition establishment

Naturally fertilized eggs will be obtained from five pairs of adults that have been artificially induced to spawn (see AQ-1-04). These eggs will be collected and examined under a microscope for viability (AQ-1-17). Fertilized eggs will be counted into equal number of eggs from each of three or more pairs for a total of approximately 50 eggs per group. Eggs will be held in 250mL beakers. Five-day-old tadpoles will be transferred to 21-L glass tanks. Each tank will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes species name, common name, project name and number, date eggs were laid/hatched (if applicable), group number, and the name of the person responsible for animal care. AP or control solution will be added to each glass beaker/ tank at NF stages 1-10, 49, or 55, depending upon the experiment (see Table 1). Exposures will continue for 70-d post-fertilization at 22 ± 2 °C.

15.2a Adults will be induced to spawn according to SOP AQ-1-04.

15.2b Fertilized eggs will be held in 250mL beakers containing 100 mL AP or FETAX solution.

15.2c On post-hatch day 5, larvae will be transferred to 21-L glass tanks containing 8 L medium. Maintain eggs/larvae as stated in SOP AQ-1-06.

15.2d 14 ppm test solution in FETAX medium will be added into glass tanks at NF stage 1-10, stage 49 and stage 55, respectively. Fifty-percent of the test and reference solution will be changed every 72 hr as stated in the SOP for *Xenopus* care (SOP AQ-1-06) and will be added back to each aquarium daily as needed to maintain test conditions.

15.3 Test Material Application

Rates/concentrations: 14 ppm

Frequency: Constant exposure from NF stage 1-10, stage 49, stage 55 to70 days.

Route/Method of Application: Eggs and larvae will be exposed to AP or FETAX medium alone. Stock solution (1.404 g/L): dissolve 1.404 g AP in 1 L ultrapure water solution, which is a 100x concentration, and store in 1 L amber bottle. FETAX stock solution will be a 10x concentration. See SOP AQ-1-13 for FETAX preparation. AP and FETAX are added to the appropriate containers according to the following table:

	Aquaria	Beaker
Final Nominal AP Concentration	14 ppm	14 ppm
AP Stock Solution	80 mL	1 mL
FETAX Stock Solution	800 mL	10 mL
Ultrapure Water	7120 mL	89 mL
Total Volume	8000 mL	100 mL

Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the aquaria medium.

Justification for Exposure Route: *Xenopus* are fully aquatic as larvae and as adults.

Exposure Verification: Samples of test and reference solutions will be analyzed for perchlorate content (AC-2-11).

15.4 Test System Observation

Water quality, including salinity, conductivity, pH, dissolved O_2, and ammonia will be performed once a week. Temperature, by means of a surrogate tank, will be monitored daily. Water samples from each tank and diluted stock solution will be removed every 3 d for perchlorate analysis. Tank water samples will be collected after the medium has been renewed. Dead animals will be removed and preserved in 10% neutral buffered formalin (NBF).

15.5 Animal Sacrifice and Sample Collections

Sample collections will be performed at 70 d post-fertilization. All larvae from each tank will be euthanized by immersion in MS-222 (1g/L, AQ-1-03), staged, measured for snout-vent length, and weighed. Five larvae will be frozen on dry ice for subsequent determination of whole body thyroid hormone content, with the remaining larvae fixed in Bouin's fixative for subsequent histological assessment of the thyroid gland and gonad.

15.6 Endpoint Analysis

Assays for whole body tetraiodothyronine (T_4) and triiodothyronine (T_3) will be performed by RIA (DBS SOP IN-2-04). Gonads of all fixed animals sampled will be examined by visual inspection to determine sex. Please see DBS SOP IN-2-03 for gonad

determination by light microscope. Gonads and thyroid glands will be examined using routine histological methods (Bouin's fixative, paraffin sections) currently operational in Dr. Carr's laboratory. Sections will be stained with hematoxylin and eosin. See DBS SOP IN-2-08 for thyroid gland histopathology

16. PROPOSED STATISTICAL METHODS

Time to forelimb emergence will be analyzed by one-way ANOVA. Thyroid follicle cell height, whole body T_3 and T_4, hindlimb length, snout-vent length, and body weight will be analyzed by two-way ANOVA (Treatment x Time). Chi-square analysis will be used to determine significant differences among percentages. Gonadal sex ratios will be analyzed with the chi-square test to determine deviations from expected ratios of 1:1.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include:

- Room temperature and water temperature, salinity, pH, dissolved oxygen content, and ammonia will be collected.
- Date, time, frequencies and amount of feedings per tank will be recorded. Number of expired larvae removed prior to termination of exposure will be recorded, including each date and tank.
- Deformities, abnormal swimming behavior and percent metamorphosed animals (complete tail resorption) will be recorded daily prior to termination of the experiment.

Report content will also include presentation of data, interpretation, and discussion of the following end-points:

- Whole body thyroid hormone content
- Gonadal sex differentiation
- Gonadal and thyroid histopathology
- Discussion of the relevance of the findings
- List of all SOPs used.
- List of all personel.

18. RECORDS TO BE MAINTAINED / LOCATION:

A draft of the final report will be delivered to the Sponsor on or before Dec. 31, 2002. The final report will be delivered to the Sponsor on or before Feb 31, 2003. Copies of all data, documentation, records, protocol information, and the specimens shall be sent to the Sponsor, or designated delivery point, for final archive within six months of study completion. All data, the protocol and a copy of the final report shall be maintained by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

Goleman, W. L., Carr, J. A. and Anderson, T. A (2002). Environmentally relevant concentration of ammonium perchlorate inhibit thyroid function and alter sex ratios in development *X. laevis.* Environ. Toxicol. Chem. 21 (3): in press.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). *Gen. Comp. Endocrinol.* 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. *Biocell* 20: 147-153.

Nieuwkoop, P.D. and Faber, J. (1967). Normal table of *Xenopus laevis (Daudin),* North Holland, Amsterdam.

Shi, Y-B. (2000). Amphibian Metamorphosis. John Wiley & Sons, Inc. N.Y.

Sunderman, F.W., Plowman, M.C. and Hopfer, S.M. (1991). Embryotoxicity and teratogenicity of cadmium chloride in *Xenopus laevis*, assayed by the FETAX procedure. Ann. Clin. Lab. Sci. 21: 381-391.

U.S. Army Corps of Engineers. 1999. Results of a chemical test of water samples, groundwater treatment plant, Longhorn Army Ammunition Plant-Tulsa District. SWF-EV-D report No. 16967-1.

Project No.: T9700
Study No.: XEN-02-02

SOPs Referenced in the Protocol

1. AQ-1-17 Exposure of Amphibian Eggs/Larvae to Test Substance (s)
2. AQ-1-06 Care and Maintenance of Xenopus laevis
3. AQ-1-04 Zebrafish (danio rerio) Husbandry
4. AQ-1-13 Preparation of FETAX Media
5. AQ-1-03 MS-222 Anesthesia and Euthanasia of Amphibians and Fish
6. DBS IN-2-04 Radioimmunoassay for Thyroid Hormones
7. DBS IN-2-03 Sexual Determination of *Xenopus* by Gonadal Inspection
8. DBS IN-2-08 Histopathology of the Thyroid Gland

A FINAL REPORT ENTITLED: ENVIRONMENTAL MODELING

STUDY NUMBER:	MOD-02-02
SPONSOR:	United States Air Force AFIERA/RSE 2413 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
RESEARCH INITIATION:	September 28, 2001
RESEARCH COMPLETION:	December 31, 2002

GOOD LABORATORIES PRACTICES STATEMENT

Project MOD-02-02, entitled "Environmental Modeling ", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

Ken Dixon

Dr. Ken Dixon

3/28/03

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

The final report was reviewed by the Quality Assurance department.

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/03
Date

1) **Sub- Project Title:**
Environmental Modeling

2) **Project Background:**
This research project will address perchlorate exposure to several animal species.

3) **Objective:**
The objectives of this sub-project are: 1) to develop mathematical models of the uptake, distribution and effects of perchlorate, 2) use computer simulation to predict long-term effects of the contaminants on wildlife populations, and 3) generate simulation results that can be used in a probabilistic risk assessment.

4) **Technical Approach:**
The modeling approach was to develop and apply mathematical models, either through modification of off-the-shelf models or *de novo* development, as appropriate, and parameterize the models using data from Phase IV laboratory studies above.

Software tests are intended to challenge the application software and other parts of the overall system functionally and structurally. Functional testing demonstrates only that the system outputs appear to be correct. It does not allow an assessment of whether the software is actually performing according to specifications and requirements. A complete functional test of every combination of inputs may not be feasible except for very small programs. Functional testing is essentially a subset of structural testing.

Structural testing is designed to exercise all modules and branches of the software and their interrelationships with the hardware and peripheral devices. Structural testing is performed to ensure that all relevant functions in the software perform as intended.

Each of the testing types described below was conducted. Performing only one type of test will not prove that the system is working properly.

A. Normal Testing includes cases that test the functional and structural integrity of the computerized system. The input data for these test cases all fall within the range the user considers to be normal. Performing enough test cases can give a reasonable level of confidence that the system behaves as intended under normal conditions.

B. Boundary Testing is performed using values that force the system to discern whether the input is valid or invalid, or to make a decision as to which branch of the program to execute. Boundary test values are set at the edges (i.e., slightly below and above) of valid input ranges. Boundary testing does not mean making the computerized system "crash" or involuntarily stop.

C. Special Case Testing, also known as "exceptional case testing", documents the system's reactions to specific types of data or lack of data and is intended to ensure that the computerized system does not accept unsuitable data. These tests should be designed to document what happens when values that are not included in the ranges defined in the specifications are entered. Use of test cases with no data entry in a field will assist in establishing software system defaults.

D. Parallel Testing is one of the most common types of tests performed by software developers. Parallel testing is performed by running two systems in parallel and comparing the outputs (e.g., two software application versions or software compared with a manual procedure). The comparison of the outputs from the same software release on different systems or different releases on the same system is part of parallel testing plans. Parallel testing can be a valuable tool when it is used in conjunction with other testing types for validation, or to train personnel to use a new computer system.

5) Project Accomplishments:

A. Develop aquatic vegetation models.

These models will provide predicted perchlorate concentrations in vegetation that will provide input to models of perchlorate uptake in fish and aquatic birds. The aquatic vegetation models included an algae model and an aquatic macrophyte model. Both models were programmed in Matlab; the algae model was programmed using differential equations and the aquatic macrophyte model was programmed using difference equations.

The models were tested with each of the structural tests described above and passed each test. The parallel test, comparing the output from the Matlab model with that of the FORTRAN model, showed equivalent output.

Model Description

We used the model for growth and production of algae contained in The Enhanced Stream Water Quality Model QUAL2E (Brown and Barnwell 1987). This model was used because extensive experience using it in a risk assessment of atrazine (Solomon, et al. 1996). The model is expressed as the differential equation:

$$\frac{dA}{dt} = \mu A - \rho A - \frac{\sigma_1}{d} A \qquad (1)$$

where

A	=	algal biomass concentration, $mg \cdot L^{-1}$
t	=	time, hours
μ	=	the local specific growth rate, h^{-1}
ρ	=	the local respiration rate of algae, h^{-1}
σ_1	=	the local settling rate for algae, $m \cdot h^{-1}$
d	=	average depth, m

We changed the original time step of a day to an hour. In the QUAL2E model, the algal specific growth rate is written as a function of light, and the nutrients nitrogen and phosphorus. For the purposes of these simulations, we assumed that nutrients are not limiting. Because no significant effect of perchlorate was observed (TIEHH 2002), we did not include a growth limitation factor for perchlorate. In our growth experiments, we only measured net growth rate and not gross photosynthetic rate and respiration rate independently. Therefore, we used a revised model of algal dynamics in which growth rate, μ, and respiration rate, ρ , are both coupled to the expressions for the limitation factors for light, FL:

$$\mu = \mu_{\max(FL)} \qquad (2)$$

where

μ_{max} = maximum specific algal growth rate constant, h^{-1}

FL = algal growth limitation factor for light

Both μ_{max} and ρ are functions of temperature (Brown and Barnwell 1987, page 51):

$$X_T = X_{20}\theta^{(T-20^\circ)} \qquad (3)$$

where

X_T = the value of the coefficient at the local temperature (T)

X_{20} = the value of the coefficient at the standard temperature (20 C)

Θ = an empirical constant for each reaction coefficient

Of the three options for the light functions in QUAL2E, we selected Smith's function. In this option, the algal growth limitation factor for light is formulated to include second order effects of light intensity:

$$FL_z = \frac{I_z}{\sqrt{K_L^2 + I_z^2}} \qquad (4)$$

where

FL = algal growth attenuation factor for light at intensity I_z

I_z = light intensity at a given depth (z), $cal \cdot cm^{-2} \cdot min^{-1}$

K_L = light intensity corresponding to 71% of the maximum growth rate, $cal \cdot cm^{-2} \cdot min^{-1}$

Z = depth variable, m

In QUAL2E, the light intensity, I_z, varies with depth according to Beer's law:

$$I_z = e^{-Kz} \qquad (5)$$

where

I	=	surface light intensity, cal•cm^{-2}•min^{-1}
K	=	light extinction coefficient, m^{-1}
Z	=	depth variable, m

Uptake of perchlorate from water into algal cells was assumed to follow from passive diffusion:

$$\frac{dC_{cellH2O}}{dt} = \mathrm{d}(C_{H2O} - C_{cellH2O}) - k \cdot C_{cellH2O} \qquad (6)$$

where:

$C_{cellH2O}$	=	soluble perchlorate concentration in algal cells, ppb
C_{H2O}	=	water perchlorate concentration, ppb
d	=	diffusion coefficient, h^{-1}
k	=	degradation constant, h^{-1}

We assumed an active transport mechanism between cellular water concentration and insoluble algal biomass:

$$\frac{dC_{\text{algae}}}{dt} = k_1 \cdot C_{cellH2O} - k_2 C_{\text{algae}} \qquad (7)$$

where:

C_{algae}	=	insoluble algal perchlorate concentration, ppb
k_1	=	transport rate coefficient, h^{-1}
k_2	=	metabolic/degradation coefficient, h^{-1}

Model Calibration

We used data from laboratory studies on uptake of perchlorate into duckweed (*Lemna minor*) (TIEHH 2002) to calibrate the model. Depth and settling rate in the lab experiments were assumed to be zero. Initial algal biomass was set to 0.55g (TIEHH 2002: Figure16). We allowed the simulated algal biomass to increase over the 10 day simulation experiment (Figure 1). Water concentration was set to 100ppb. Predicted perchlorate concentration in duckweed showed dynamics similar to observed values (Figure 2). We concluded that the model

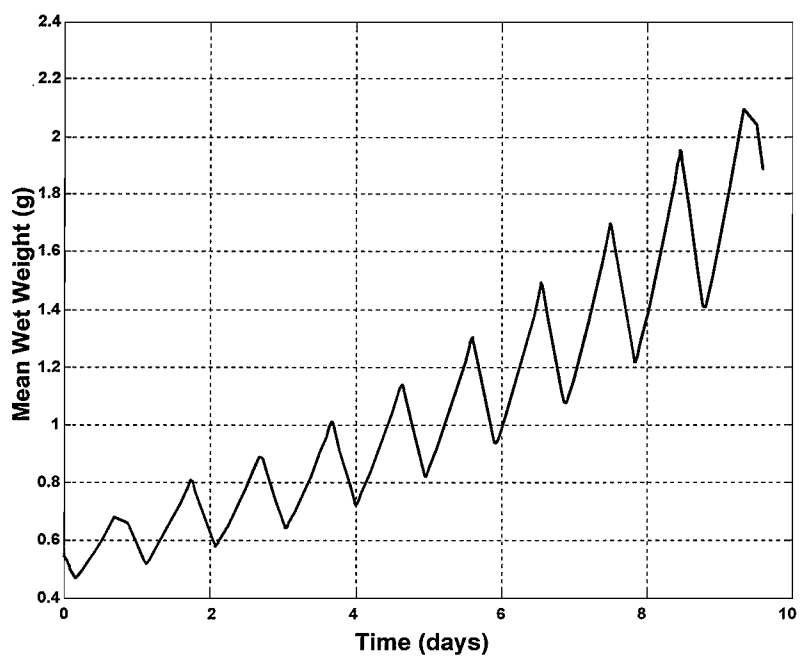

Figure 1. Simulated algal growth in 10 day experiment.

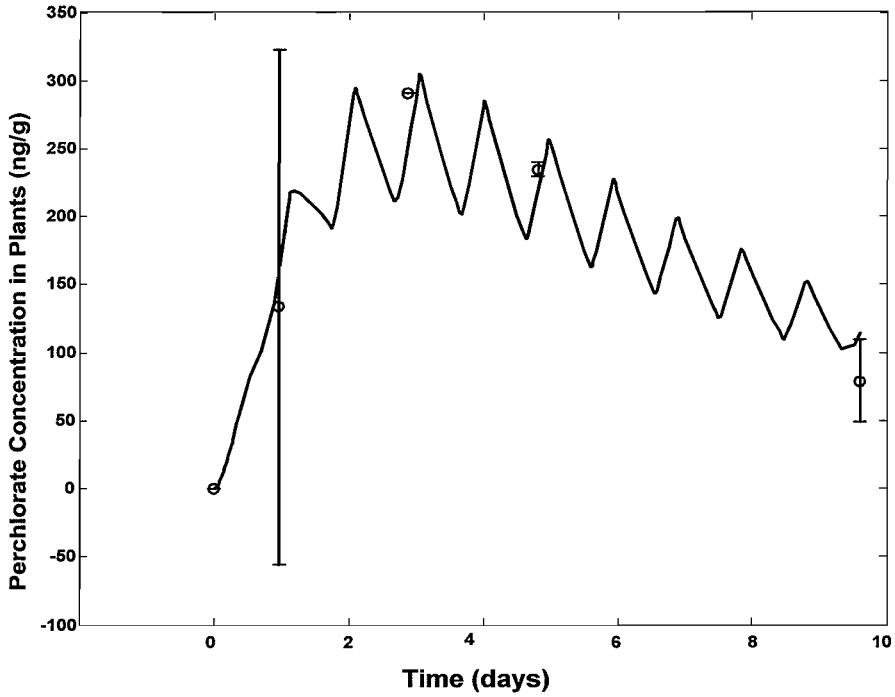

Figure 2. Predicted and observed perchlorate concentration in duckweed in 10 day experiment. Predicted values are in blue. Observed values, in red, are mean ± standard deviation.

Simulation Studies to Assess Remediation Alternatives

To simulate remediation alternatives, simulations were run for algal populations in four drainages of LHAAP (Harrison Bayou, Goose Prairie Creek, Central Creek, and INF Pond). Only output from the Harrison Bayou and INF Pond areas are presented, as they represent the lowest and highest perchlorate concentrations, respectively (Table 1). The simulations for Harrison Bayou represent a site remediated to minimal perchlorate concentrations. Simulations for INF Pond represent little or no remediation of the site.

Table 1. Perchlorate concentrations (ppb) in water used in model simulations.

LHAAP Area	Mean	S.D.
Harrison Bayou	4	N/A
Goose Prairie Creek	70.0	22.6
Central Creek	8	N/A
INF Pond	31,194	391.24

Results for Harrison Bayou

Simulation output included graphs of perchlorate concentrations and algal biomass (Figures 3 and 4).

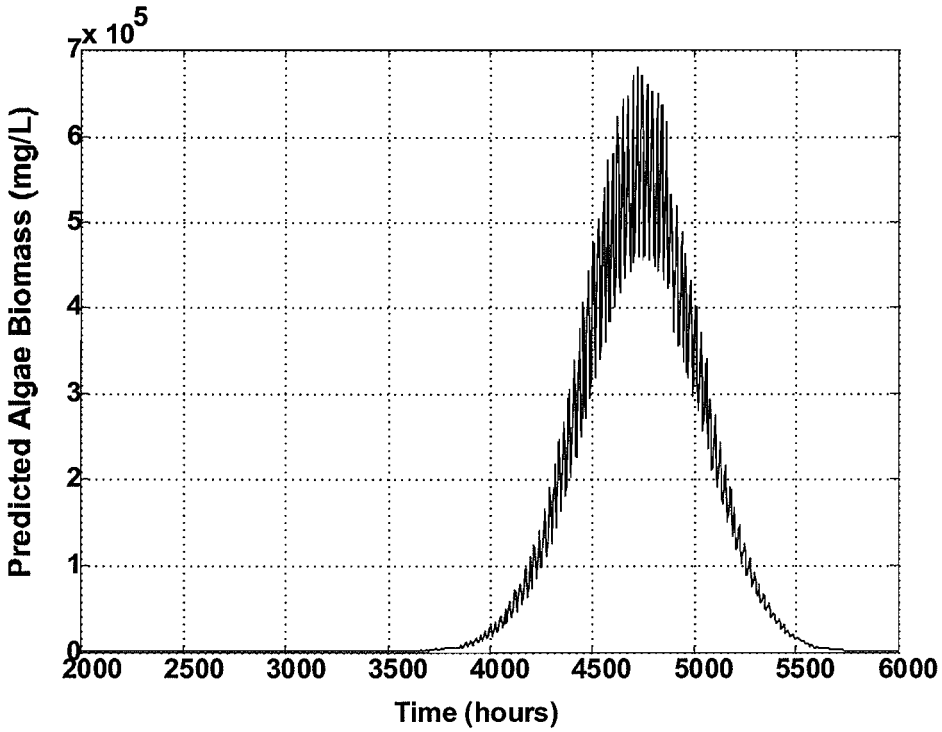

Figure 3. Predicted algal biomass in simulated exposure to perchlorate concentration from Harrison Bayou site.

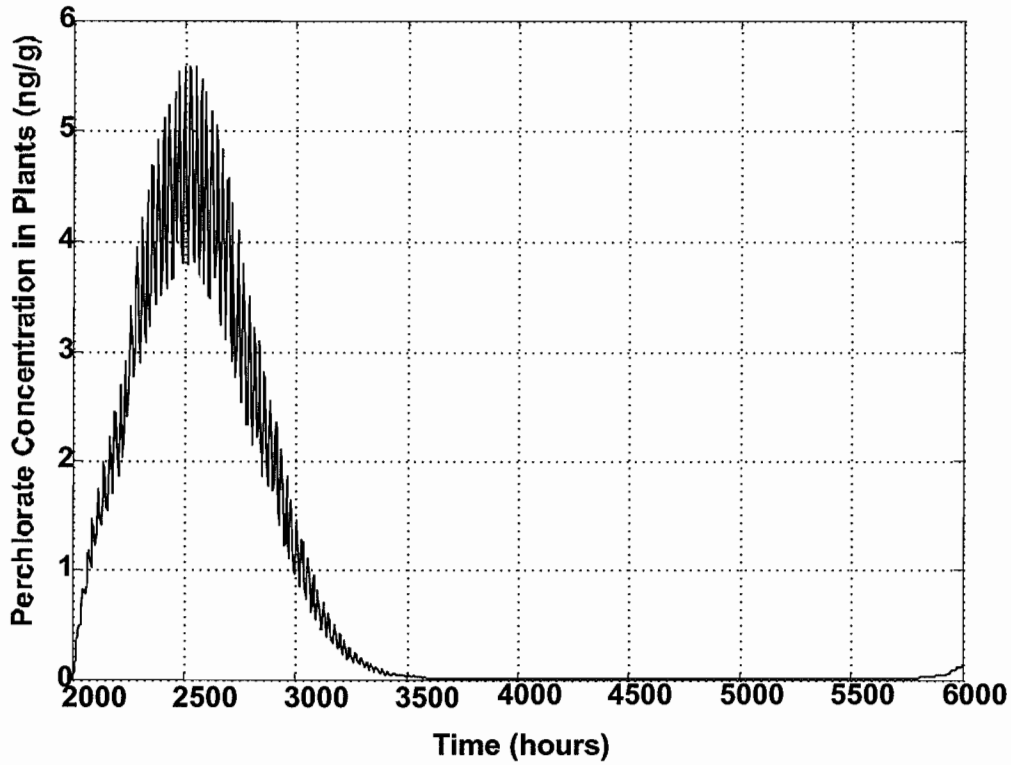

Figure 4. Predicted algal perchlorate concentration for Harrison Bayou simulation.

These simulation results show predicted algal biomass increases seasonally. Perchlorate concentration increases to a peak after about 21 days (500 hours) into the growing season. Because of the low algal biomass density, the perchlorate exposure through the aquatic food chain is low. As biomass increases, the perchlorate concentration decreases, further reducing potential exposure.

Results for INF Pond

Simulation output included graphs of algal biomass and perchlorate concentrations in (Figures 5 and 6).

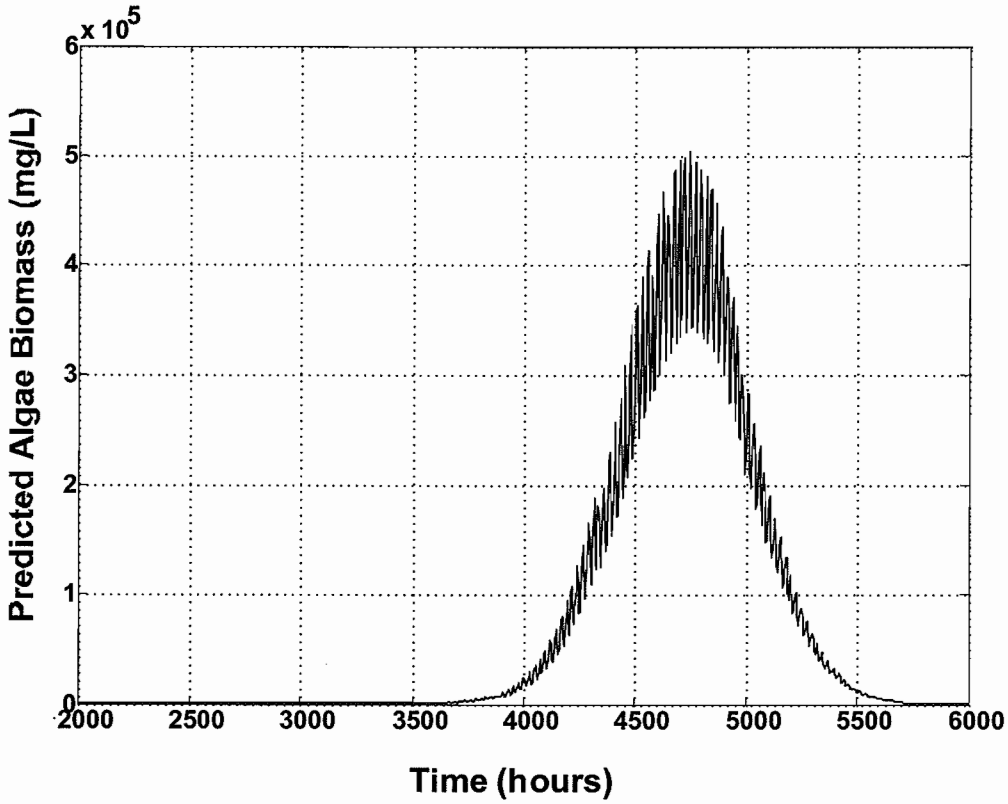

Figure 5. Predicted algal biomass in simulated exposure to perchlorate concentration from INF Pond site

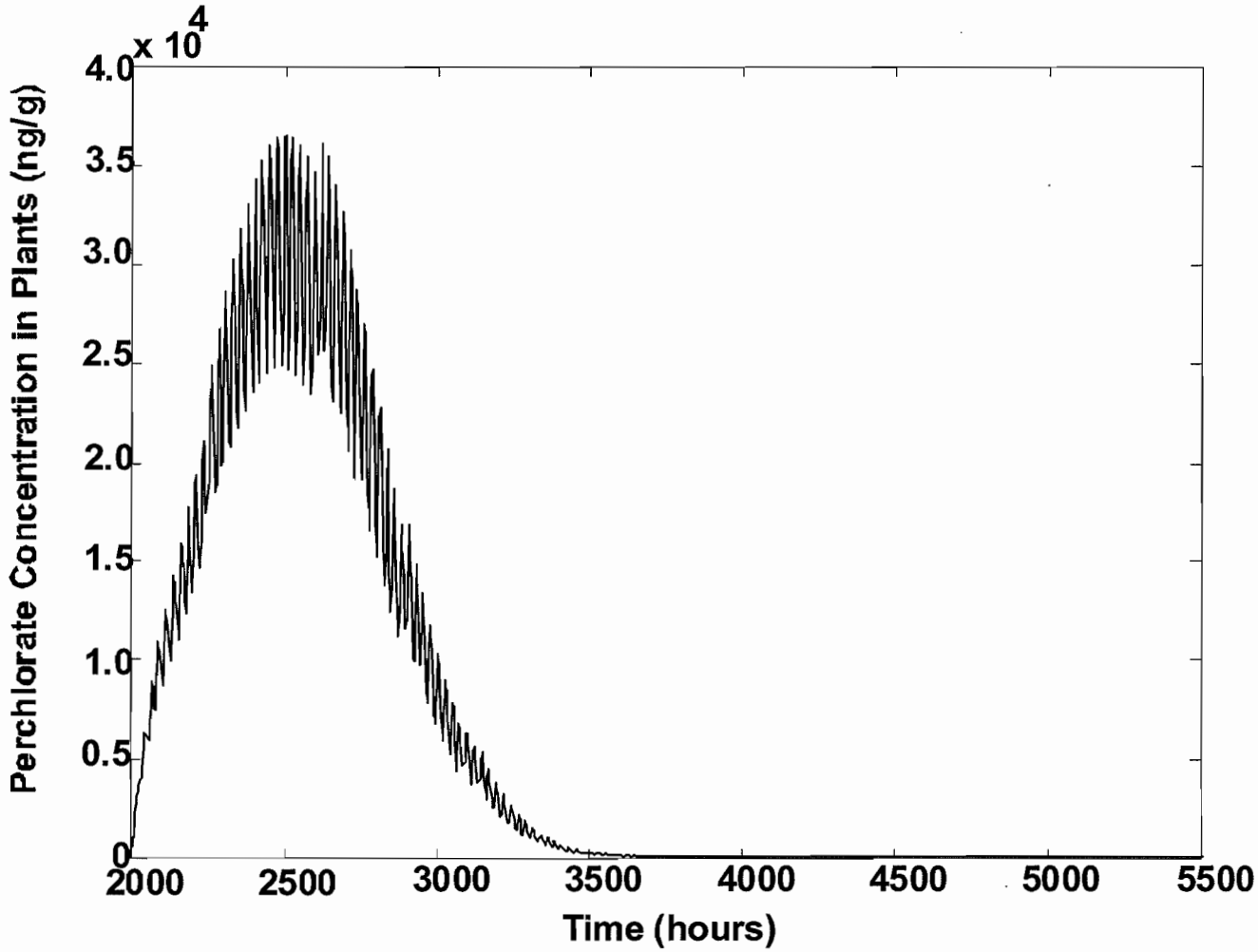

Figure 6. Predicted algal perchlorate concentration for INF Pond simulation.

These simulation results show seasonal algal biomass increases similar to those of Harrison Bayou. Perchlorate concentration, however, increases significantly higher than in Harrison Bayou, because of the higher water concentration. Again, as biomass increases, the perchlorate concentration decreases, reducing potential exposure.

Discussion and Conclusion

There were significantly higher perchlorate concentrations predicted in all model algae populations for the INF Pond area, compared with the Harrison Bayou drainage. Although parameter estimates were based on calibration with lab experimental data, direct parameter estimation may improve the accuracy of the model predictions. These simulations are subject to change, but with the current parameter set, simulation experiments showed four orders-of-magnitude greater algal perchlorate concentrations in the IMF Pond site compared with the Harrison Bayou site.

6) Literature Cited

Brown, L.C., and T.O. Barnwell, Jr.,. 1987. The Enhanced Stream Water Quality Models QUAL2E AND QUAL2E-UNCAS: Documentation and User Model. EPA/600/3-87/007, U. S. Environmental Protection Agency, Environmental Research Laboratory, Athens, GA.

MATLAB. 1992. MATLAB User's Guide. The MathWorks, Inc., Natick, MA.

Solomon, K. R., D. B. Baker, R. P. Richards, K. R. Dixon, S. J. Klaine, T. W. La Point, R. J. Kendall, C. P. Weisskopf, J. M. Giddings, J. P. Giesy, L. W. Hall, Jr., and W. M. Williams. 1996. Ecological risk assessment of atrazine in North American surface waters. Environ. Toxicol. Chem. 15:31-76.

TIEHH (The Institute of Environmental and Human Health). 2002. A final report entitled: uptake of perchlorate into various plant species. Final Report, TIEHH Project N. T7900, Soil Ecotox-Plants Phase III.

7) Associated Personnel:

Randy Apodaca – graduate student
Eric Albers – graduate student

A FINAL REPORT ENTITLED: ENVIRONMENTAL MODELING

STUDY NUMBER:	MOD-02-01
SPONSOR:	United States Air Force AFIERA/RSE 2413 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
RESEARCH INITIATION:	September 28, 2001
RESEARCH COMPLETION:	December 31, 2002

GOOD LABORATORIES PRACTICES STATEMENT

Project MOD-02-01, entitled "Environmental Modeling ", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

Ken Dixon

Dr. Ken Dixon

3/28/03

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

The final report was reviewed by the Quality Assurance department.

Submitted By:

Ryan Bounds
Quality Assurance Manager

03/28/03
Date

1) **Sub- Project Title:**
Environmental Modeling

2) **Project Background:**
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3) **Objective:**
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5) Project Accomplishments:

A. Develop groundwater and surface water models.

We have collected preliminary data for the generation of the groundwater model, including (stratigraphy characterization, pontiometric head data, hydraulic conductivity values, recharge values, three years of monitoring well data, and soil sample data. For the surface water model we have soil texture classifications, channel surveys, and field measurement data.

B. Modification of "Fleshing File"

A subroutine was developed to determine the concentration within a contaminant plume at the individual's exact location. Trilinear interpolation uses the eight nearest known concentrations to determine the unknown concentration at a known point, based on 3-dimensional Euclidean distance. By using an animal's location at each time step to determine the dose we arrive at a more realistic scenario than a maximum dose (using the highest concentration in the plume) or time-lapsed maximum dose (using the highest concentration in the plume for that time step). This routine has been modified to allow for the inclusion of field measurements taken after the plume simulation has been generated, through the use of sparse matrices. These additional field measurements can help increase the precision around regions of concern. Fish velocity (Wendel and Kelsch 1999) and home range size (Fischer 1999) movement parameters were taken from the literature for channel catfish (*Ictalurus punctatus*). Additional parameters such as habitat, dwelling depth, and food prevalence are being researched for potential development into a more refined movement model.

C. Improvement of PBTK model.

A Physiologically Based Toxicokinetic (PBTK) model developed for perchlorate in the channel catfish during 2001/2002 (Albers and Dixon 2002) was modified to allow for the inclusion of new lab data as well as improvements to model structure. The flow diagram

(Figure 1) shows the compartments in the current model. The new version of the model accounts for portal blood flow from muscle and skin to the kidney and from the GI tract to the liver (Nichols et al. 1990). Physiological parameters for blood flow and tissue volumes were obtained from existing literature (Erickson and McKim 1990a, Erickson and McKim 1990b, Hughes 1984, and Nichols et al. 1990). The general equations used in the model were taken from PBTKs developed by Nichols et al. for rainbow trout (1990 and 1991) and channel catfish (1993). Data from a 2 day 100 ppm exposure followed by a 10-day elimination time was used to calibrate the model (Theodorakis et al. 2003). Additional exposure simulations were run until tissues reached approximate equilibrium using water concentrations measured at LHAAP (Smith et al. 2001).

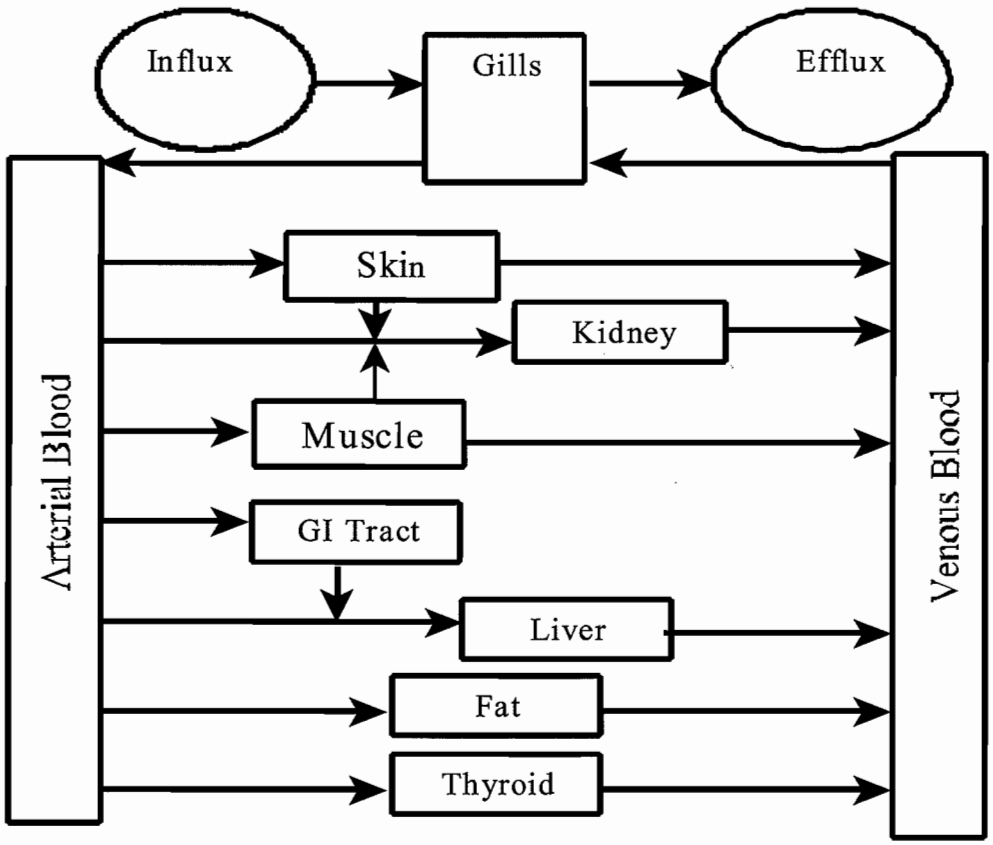

Figure 1 Flow Diagram of the PBTK model for perchlorate inhalation in fish.

Model Description

The new PBTK model adds a fat compartment, replaces poorly perfused tissue with separate skin and muscle compartments and richly perfused tissue was replaced with GI tract. Portal blood flow to the kidney was set as 60% of blood flow to skin and muscle

compartments, with portal flow to the liver equal to blood flow to the GI tract (Nichols et al. 1990).

Table 1. Abbreviations and Symbols Used.

F^G = flux of perchlorate across the gills, mg·kg^{-1}·h^{-1}
k_X^G = exchange coefficient
f_W = ratio of free chemical in exposure water to total concentration
f_B = ratio of free to total perchlorate in blood
$C_W^{aff,G}$ = total concentration of perchlorate in exposure water, mg·kg^{-1}
$C_B^{aff,G}$ = concentration of perchlorate in the blood afferent to the gills, mg·kg^{-1}
$C_B^{eff,G}$ = concentration of perchlorate in the blood efferent to the gills, mg·kg^{-1}
$C_B^{eff,i}$ = concentration of perchlorate in the blood efferent to the tissue compartment, mg·kg^{-1}
Q_B^i = blood flow rate from the tissue, L·h^{-1}
Q_B^G = total cardiac output, L·h^{-1}·kg
A^i = amount of perchlorate in the tissue, mg
V^i = volume the tissue compartment, kg
K_B^i = tissue/blood partitioning coefficient
$\frac{dA^i}{dt}$ = rate of change in perchlorate concentration in the tissue compartment, mg·kg^{-1}·h^{-1}

Water intake (dose) is governed by the gill flux equation below :

$$F^G = k_X^G\left(f_W C_W^{aff,G} - f_B C_B^{aff,G}\right)$$

The concentration of perchlorate in the blood afferent to the gills, also known as the venous blood, was calculated as follows:

$$C_B^{aff,G} = \frac{\sum\left(C_B^{eff,i} * Q_B^i\right)}{Q_B^G}$$

The concentration of perchlorate in blood efferent to each tissue compartment was calculated as follows:

$$C_B^{eff,i} = \frac{\left(\frac{A^i}{V^i}\right)}{K_B^i}$$

The rate of change in perchlorate concentration for each tissue compartment is defined by the differential equation:

$$\frac{dA^i}{dt} = Q_B^i (C_B^{eff,G} - C_B^{eff,i})$$

Finally the concentration of perchlorate in the blood efferent to the gills was calculated as follows:

$$C_B^{eff,G} = C_B^{aff,G} + \frac{F^G}{Q_B^G}$$

Calibration Run using lab data

To ensure the PBTK model accurately simulated the transport and fate of perchlorate in the channel catfish, it was necessary to calibrate the model using laboratory data. This was done by fitting the model to data from a 10-day elimination study (Theodarakis et al. 2003) by altering partitioning coefficients. The model provided a good fit to the measured data (figure 2-3). Skin and kidney compartments were partially fitted (Bradford 2003), however this data was not robust enough for calibration purposes.

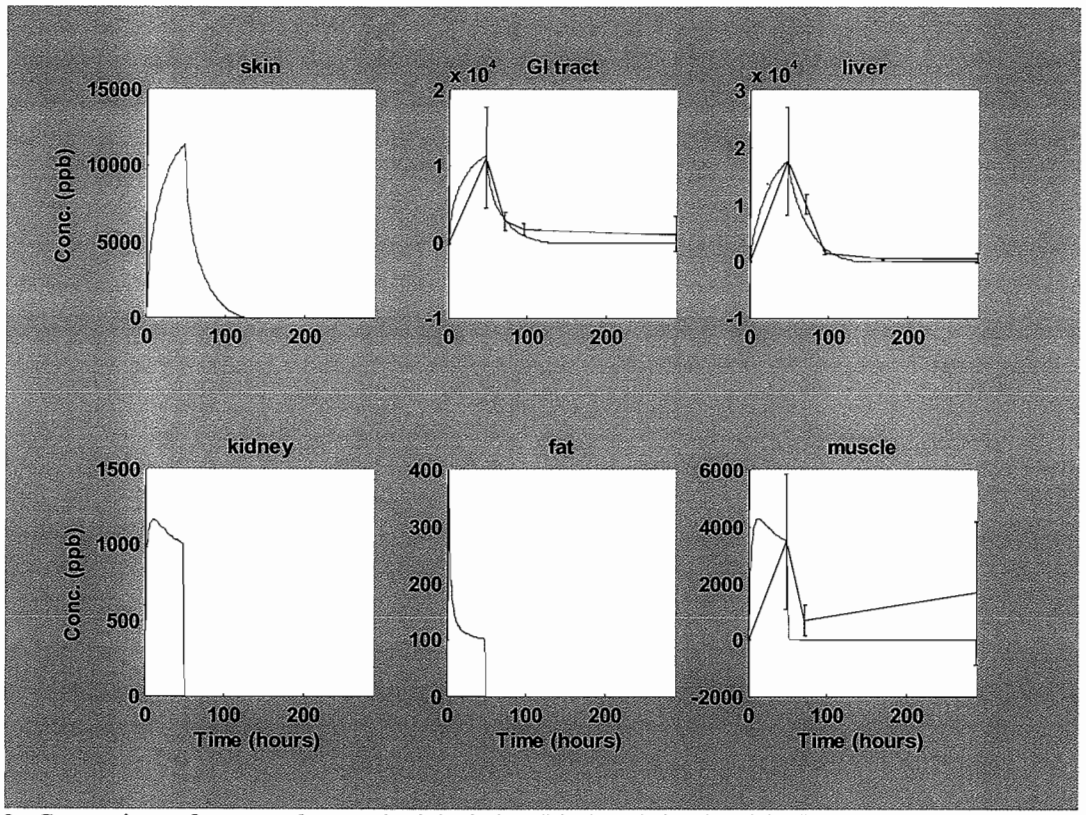

Figure 2. Comparison of measured ± standard deviation (blue) and simulated (red) perchlorate concentrations for a 10-day elimination (post 100 ppm dose).

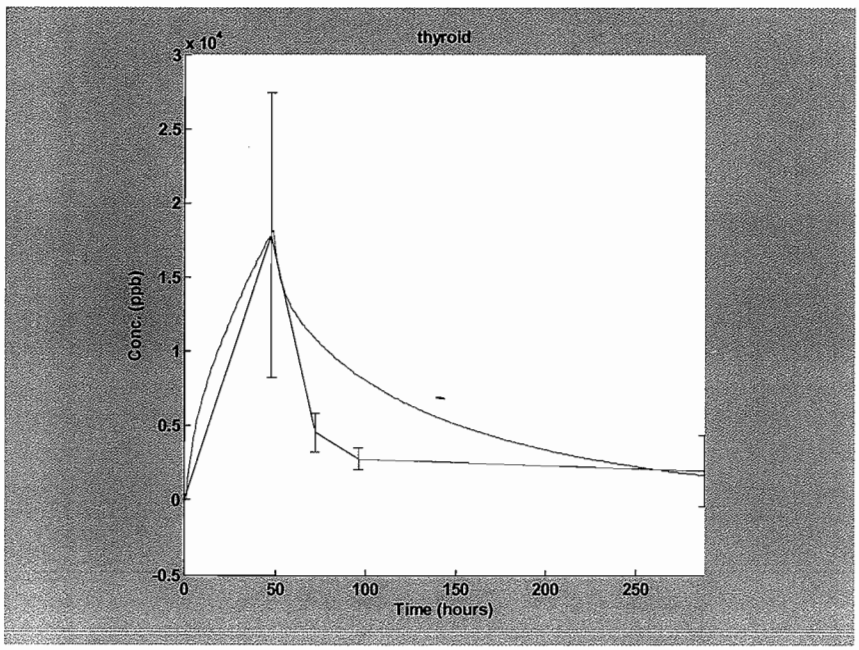

Figure 3. Comparison of measured ± standard deviation (blue) and simulated ± standard deviation (red) concentrations for a 10-day elimination (post 100 ppm dose).

Simulation Studies to Assess Remediation Alternatives

Remediation alternatives were simulated for Harrison Bayou - 4 ppb (Figure 4,5) and INF Pond - 776 ppb (Figure 6,7) as done for phase III to give real world low and high dose scenarios.

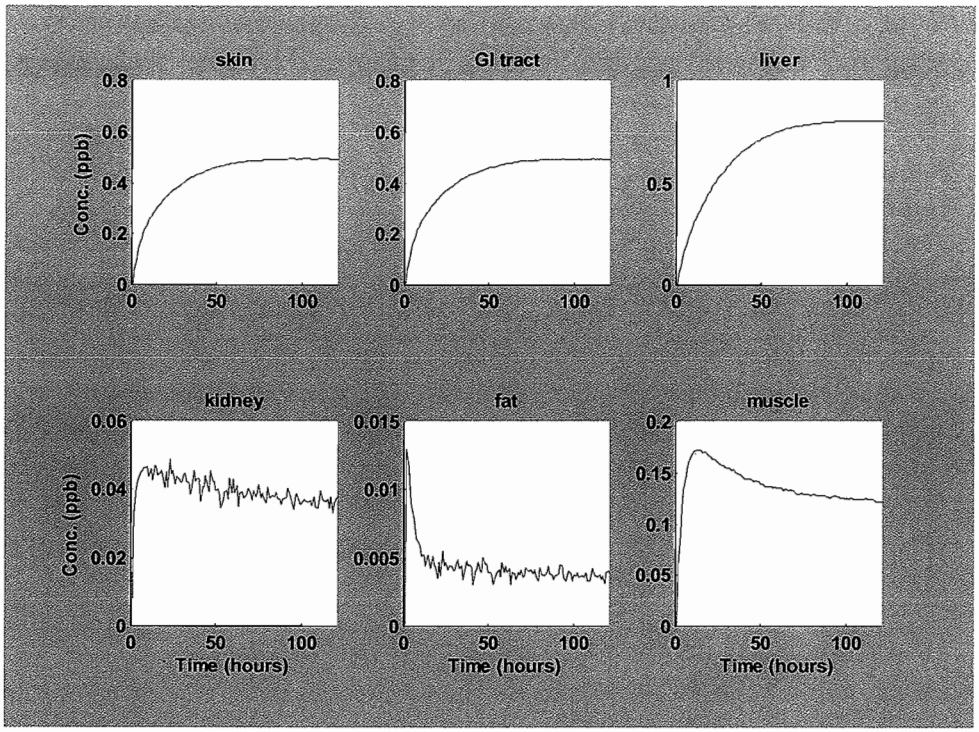

Figure 4. Simulated perchlorate concentrations to equilibrium at the Harrison Bayou site (4 ppb).

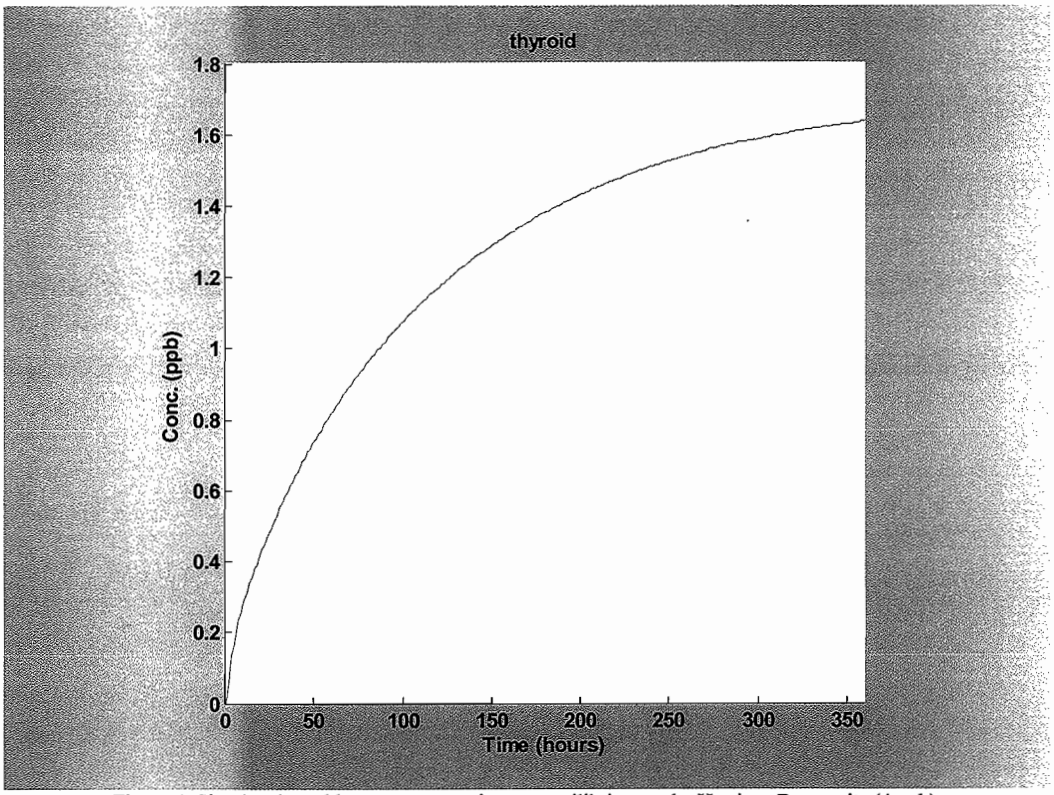

Figure 5. Simulated perchlorate concentrations to equilibrium at the Harrison Bayou site (4 ppb).

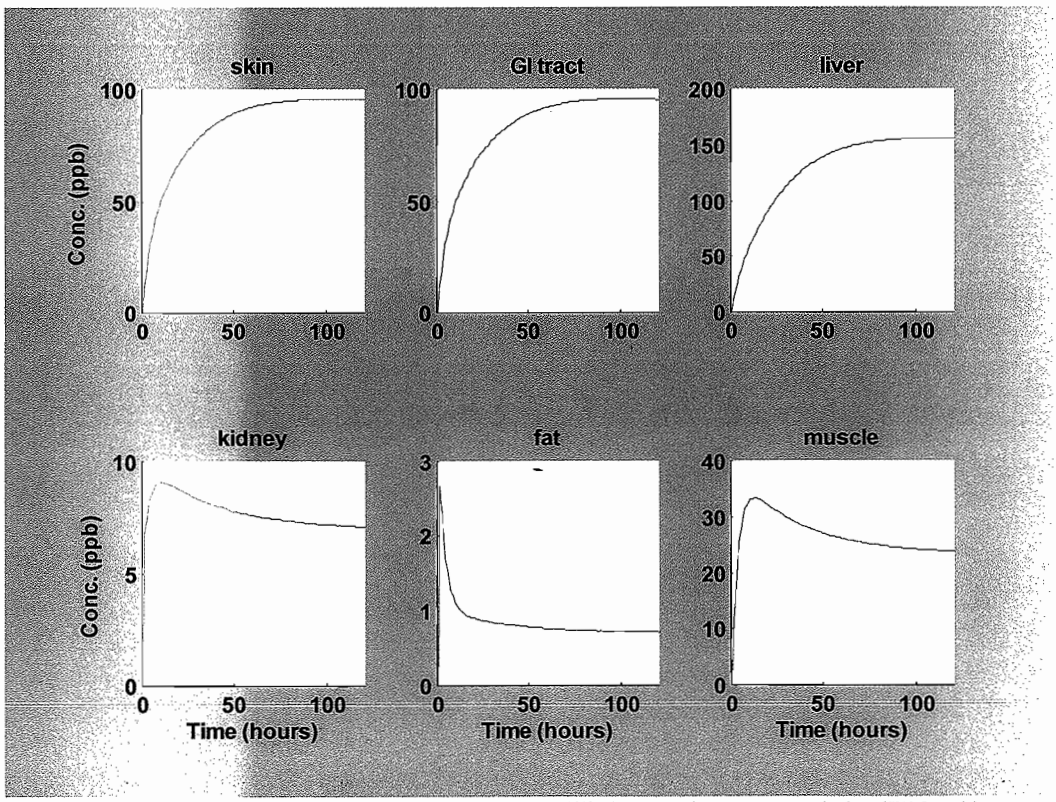

Figure 6. Simulated perchlorate concentrations to equilibrium at the INF Pond site (776 ppb).

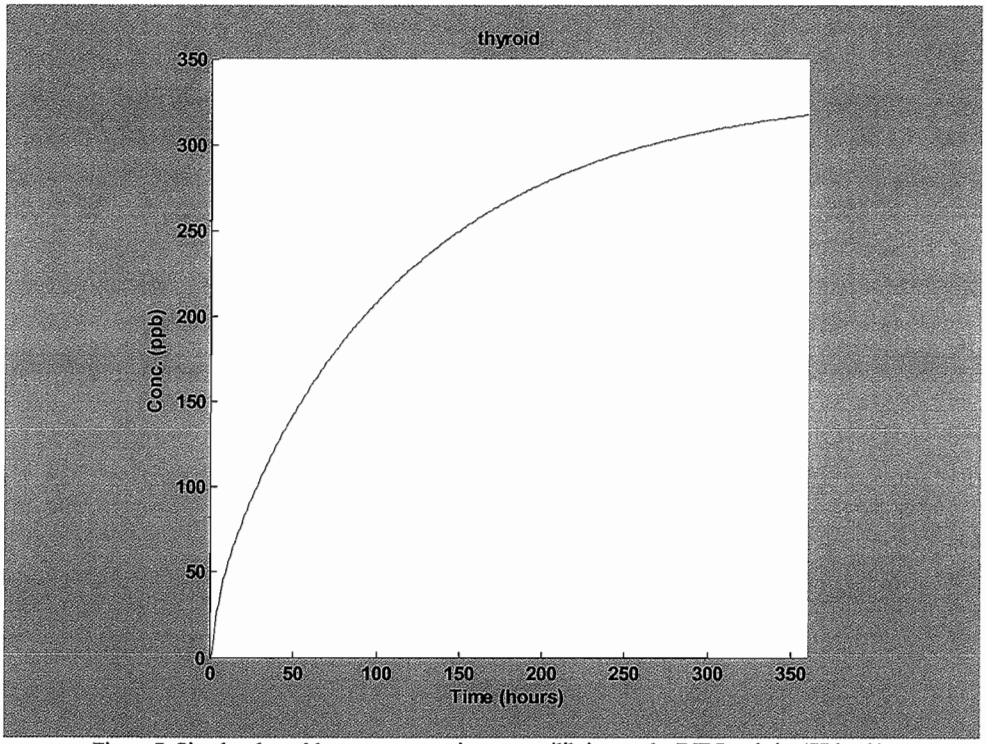

Figure 7. Simulated perchlorate concentrations to equilibrium at the INF Pond site (776 ppb).

Table 2. Comparison of Harrison Bayou and INF Pond simulations.

	LHAAP Study Site	
	Harrison Bayou (4 ppb)	*INF Pond (776 ppb)*
Compartment	**Perchlorate Concentration (ppb)**	**Perchlorate Concentration (ppb)**
Skin	0.4719	91.58
Muscle	0.117	22.72
GI tract	0.4719	91.59
Liver	0.7720	149.8
Kidney	0.0570	10.94
Fat	0.0037	0.6877
Thyroid	1.509	292.83

D. Thyroid Model

This model was discussed in detail in the Phase II report, where it was attached to a raccoon PBTK model to show the effect of perchlorate levels on hormone secretion. This same model has been linked to the PBTK for the catfish. Due to a lack of baseline and calibration data we can only make qualitative not quantitative assessments of hormone levels (figure 7,8). Harrison Bayou's concentration was too low to have an impact on hormone secretion. Additional model structures are being investigated for possible use with the PBTK (Eales 1986 and Sefkow et al. 1996). Literature searches are being used to

identify potential baseline data for these models as well as dosing data in channel catfish and possible surrogate species, i.e. rainbow trout (*Salmo gairdneri*).

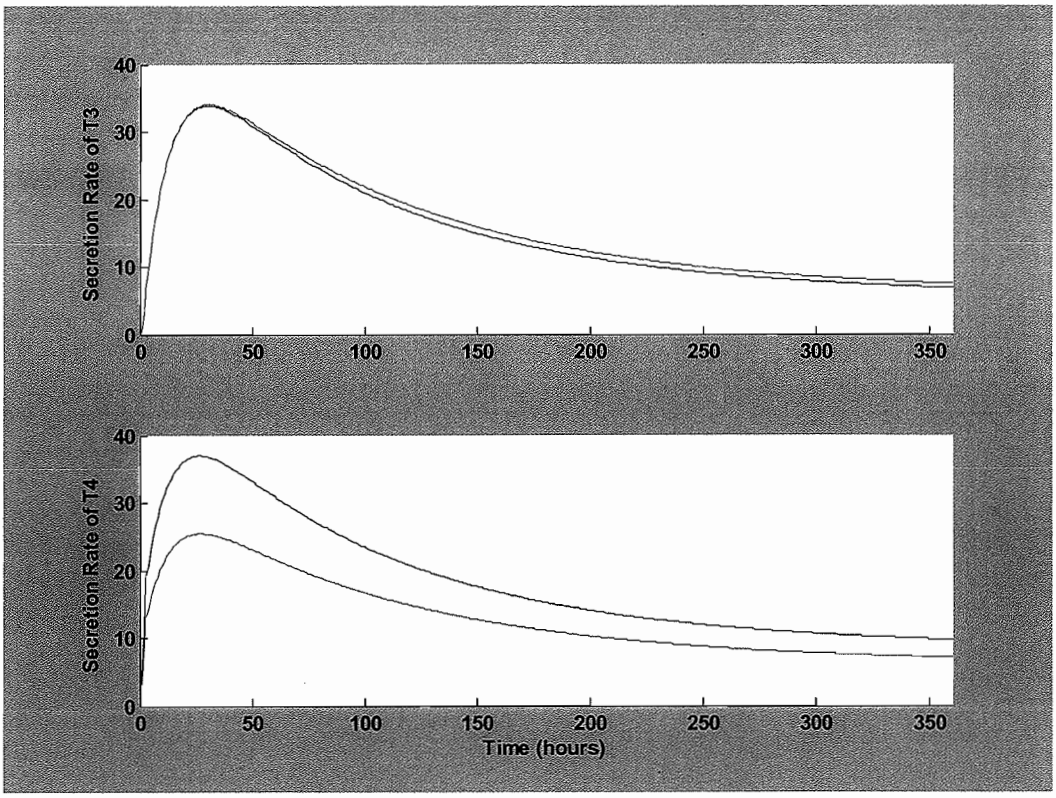

Figure 7. T3 and T4 secretion rates (blue-baseline & Harrison Bayou, red-INF pond)

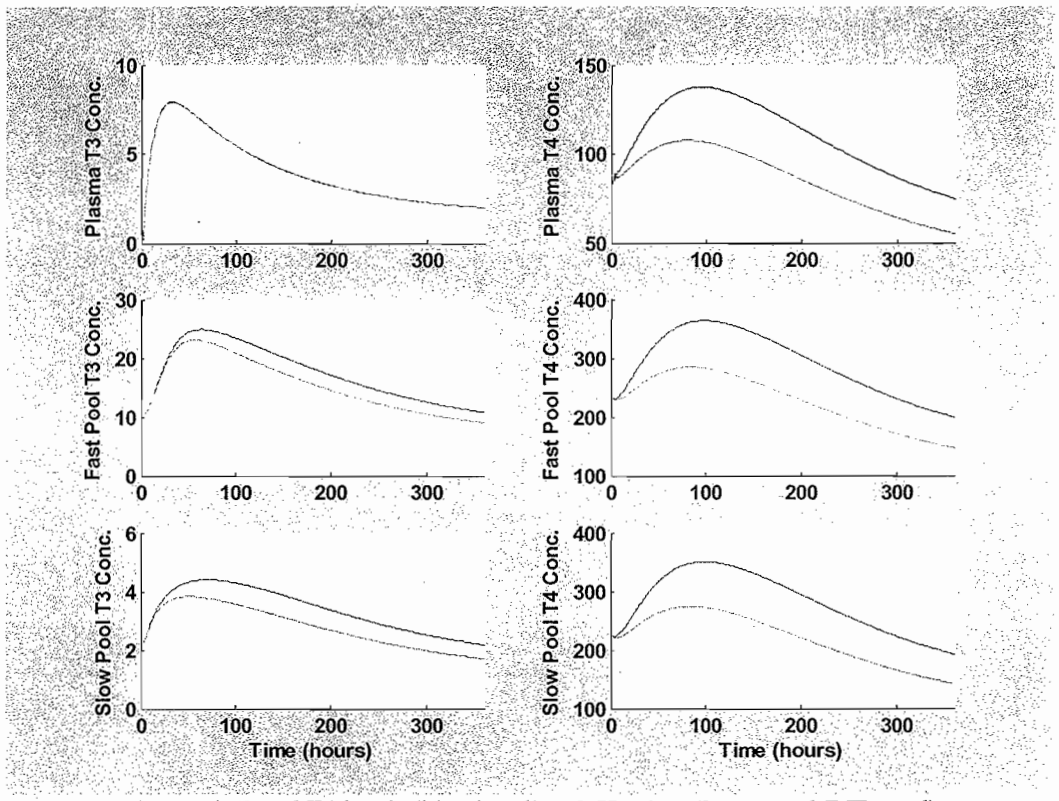

Figure 8. T3 and T4 levels (blue-baseline & Harrison Bayou, red-INF pond)

E. 3-Dimensional PBTK Model

A 3-dimensional channel catfish was created using Alias Wavefront's MAYA®. This model includes complete fish organs (liver, kidney, intestines, pyloric caeca, gonads, swim bladder, heart, and brain) as well as a representative outer shell (figures 9-11). These models allow us to show contaminant movement through a specific organ, relative or time-lapsed organ concentrations (figure 12), as well as hormone secretion rates. The catfish has also been animated along the movement path generated from our movement model, allowing us to visualize it within a lake or river.

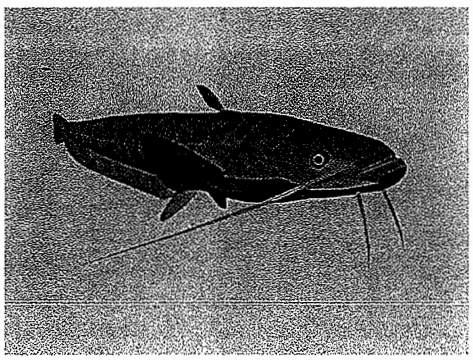

Figure 9. 3-D channel catfish

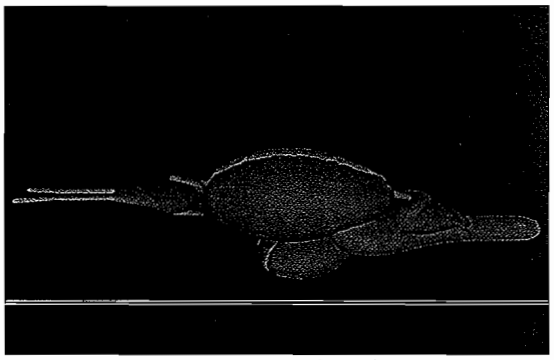

Figure 10. 3-D view of the fish brain

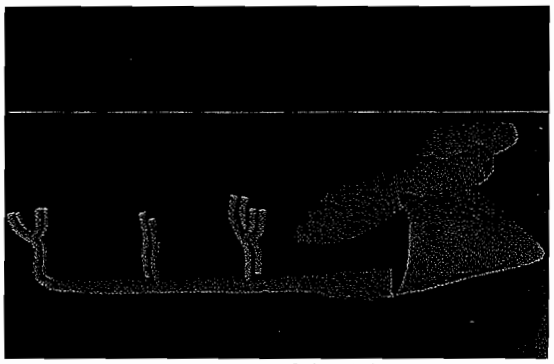

Figure 11. 3-D view of the fish heart

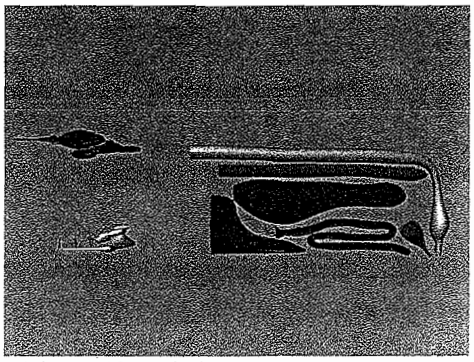

Figure 12. Relative organ concentrations (Red-high, yellow-moderate, green-low) note:brain is representative of thyroid tissue

Discussion and Conclusion

There were significantly higher perchlorate concentrations predicted in all model catfish organ and tissue compartments for the INF Pond area, compared with the Harrison Bayou drainage. It is important to note however, that until we obtain more robust data we cannot have complete confidence in the results. The PBTK model would be greatly aided by lab determination of partitioning coefficients, which will allow for a more refined calibration. T3 and T4 hormone level outputs indicate a dose-dependent relationship, with the largest impact seen on T4. The minimal impact on T3 is explained by the lack of an inhibition term for T3 in channel catfish. In addition thyroid hormone regulation is different between fish and humans, requiring modifications in the current model structure. Our inability to make quantitative assessments for hormone inhibition is due to the previous reasons as well as limited data that exhibited very low recovery rates. The use of literature data and new model structures will fix these problems.

Upon completion of the groundwater and surface water models we will be able to fully utilize the fleshing file. The modifications to the fleshing file allow us to model more complex systems more accurately by using the locational dose received by the channel catfish and the subsequent response, allowing for more informed decision-making. Through the inclusion of sparse matrices we will be able to rapidly update the locational risk to individuals without generating new environmental models.

The development of a 3-dimensional PBTK model will aid scientists in the expression of complex systems using easy to understand visual queues. While 3-D organ representations are nothing new in the field of medicine, modern computer software allows us to achieve similar results without the use of expensive MRI type equipment. This work is the beginning step in generating a complete virtual risk assessment to aid regulators, politicians, and the public to make informed decisions.

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7) Associated Personnel:

Eric Albers – graduate student

TITLE

Response of Quail to Perchlorate Contaminated Water Following Oral Exposure

STUDY NUMBER:	AELS-02-01
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	Texas Tech University Human Sciences Building Box 42002 Lubbock, TX 79409-2002
RESEARCH INITIATION:	4/28/02
RESEARCH COMPLETION:	12/31/02

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GOOD LABORATORIES PRACTICES STATEMENT

Study AELS-02-01, "Response of Quail to Perchlorate Contaminated Water Following Oral Exposure", was conducted in accordance with established Quality Assurance Program guidelines and whenever possible, in the spirit of the Good Laboratory Practices. (40 CFR Part 160, August 19, 1989).

Submitted By:

Ernest E. Smith, Ph.D

3/28/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Officer notified the Study Director of all findings in writing following each audit.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	5-17-02	5-20-02	5-20-02	6-6-02
Euthanasia	7-11-02	7-11-02	7-15-02	7-24-02
Final Report and Raw Data Review	3-3-03	3-24-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/03
Date

1.0 DESCRIPTIVE STUDY TITLE:

Response of Quail to Perchlorate Contaminated Water Following Oral Exposure

2.0 STUDY NUMBER: AELS-02-01

3.0 SPONSOR:

United States Air Force
AFIERA/RSRE
2513 Kennedy Cir
Brooks AFB, TX 7235-5123

4.0 TESTING FACILITY NAME & ADDRESS:

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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start Date: April 28, 2002
Termination Date: December 31, 2002

6.0 KEY PERSONNEL:

Ernest E. Smith, Co-Principal Investigator
Lisa Perlmutter, Study Director
Lance Williams, Histopathologist
Ryan Bounds, Quality Assurance Manager
James Surles, Statistical Support
Ron Kendall, Principal Investigator and Testing Facility Management

7.0 STUDY OBJECTIVES / PURPOSE:

The study objective is to determine the response of quail to perchlorate by assessing general toxicity and histological end points of selected organs.

8.0 STUDY SUMMARY:

This study consisted of exposing birds to water containing ammonium perchlorate and comparing egg production, egg and liver accumulation, and perchlorate-related endpoints. This experiment provides a realistic assessment of exposures that are likely to occur where environmental contamination with perchlorate occurs. We observed alteration of thyroid gland morphology and significant alteration in total thyroid hormone following expose to perchlorate. We also observed accumulation of perchlorate in the eggs of exposed birds. However, perchlorate did not affect any phyiscal parameters of egg weight, diameter or length.

9.0 TEST MATERIALS:

Test Chemical:	Ammonium Perchlorate 99.999% pure
Source:	Sigma-Aldrich
Characterization:	Oxidizer; explodes when heated
Test Medium:	Deionized Water

10.0 JUSTIFICATION OF TEST SYSTEM

This project is intended to evaluate risks of perchlorate exposure among organisms consuming perchlorate-contaminated water. Birds were used as surrogates to livestock (the most likely exposed organisms) because they are easily handled, permit large sample sizes, and are more economically feasible in a study of this nature. Nothern Bobwhite Quail (*Colinus viginianus)* provide a useful avian model of exposure and toxicity. There are no other suitable models for assessing exposure risks in an ecological environmental setting. Culture and computer models cannot simulate changes in general homeostasis and thyroid hormone alteration. In addition it would not provide pertinent scientific data for furture use in risk assessment.

11.0 TEST ANIMALS:

Species:	Northern Bobwhite Quail (*Colinus viginianus)*
Strain:	Wild type
Age:	Adults
Sex:	Females
Number:	100 quail
Source:	Purchased from Routhwood game bird farm in San Angelo, TX

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each cage was labeled as indicated in TIEHH SOP **IN-3-06**, which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. Each cage was also labeled to include sex of the individuals (if appropriate), date of exposure, the name of the test substance and its concentration. Birds were leg banded with unique identification numbers.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Perchlorate Treated Water Dosing Only for quail:
23 females (quail) X 4 treatment groups (perchlorate contaminated water; control (0 ppm), 0.01 mM (1.1749 ppm), 0.1 mM (11.749 ppm), and millimolar (117.49 ppm) = 92 quail.

Table 1 Number of quail for experimental exposure.

Treatment	Quail Female
Control	23
0.117 ppb	23
117 ppb	23
117 ppm	23
Total	92

Grand total for quail experimental exposure = 92

14.0 METHODS:

Prior to any studies and pending approval all personnel were required to read their appropriate material safety data sheets including, but not limited to ammonium perchlorate. All laboratory personnel were required to wear gloves, lab coat, and hair restraints at all times when working with birds and/or dosing solutions.

Quail were randomly assigned to treatment groups. Initial body weights were recorded for all birds prior to initiation of the study. There were 2 to 3 quail per cage, housed in stacked cages (91 x 71 x 27 cm) in test facility conditions of 72°F and 18:6 hours of light:dark.

Water and food (Bluebonnet poultry feed) were available *ad libitum* throughout the experiment. Daily observations were made on all quail. Animals showing signs of distress or disease were treated according to the instructions of the University Veternarian.

All avian handling was done according to Standard Operating Procedures.

14.1 Test System acquisition, quarantine, and acclimation.

Quail were obtained from Routhwood game bird farm in San Angelo, TX. They were housed in stacked cages and kept on an 18L: 6D light regimen. Quail were given a thirty day acclimation period.

14.2 Assignment of Animals to Study Group and Identification

Animals were assigned randomly to treatment groups upon selection from the pool. The quail were identified by their unique leg band number.

14.3 Test Material Application

Ammonium perchlorate was dissolved in the drinking water (SOP IN 3-05) and given to the quail at concentrations of control (0 ppm), 0.01 mM (1.17 ppm), 0.1 mM (11.75 ppm), and millimolar (117.49 ppm). Birds in the control group were given deionized water not treated with ammonium perchlorate. Weights were

recorded three times over the exposure period. Once at the beginning, the mid-point of exposure and at the end of the study to evaluate weight gain or loss.

Rates/concentrations: ammonium perchlorate0.01mM (1.17 ppm), 0.1 mM (11.75 ppm), and millimolar (117.49 ppm) or no treatment.

Frequency: Test substances were supplied *ad libitum* and renewed every day.

Route/Method of Application: Quails were exposed to water contaminated with ammonium perchlorate for 30 days. After 30 day Phase II, all dosing was stopped except for quail being dosed with 117.49 ppm and this regimen lasted for 21 days. Route of exposure was oral.

14.4 Daily Observations

Animals were monitored daily for changes in general health. Observed changes in animal health was reported immediately to the University Veterinarian and treated according to his instructions.

14.5 Animal Euthanasia and Sample Collections

At the end of exposure all animals were weighed using an aluminum animal weighing pan with an even distribution of holes and a lid. After the quail were weighed, they were rendered unconscious by carbon dioxide, bled, and euthanized by exsanguination (AF 1-03) and the following tissues were collected: livers, gizzards, kidneys, hearts, and thyroid glands. Blood samples were placed in EDTA tubes, centrifuged, and the resulting plasma was used in steroid and thyroid hormone assays.

14.6 Evaluations

Selected organs and blood samples were collected according to SOP ET-3-19 for chemical and histological analyses.
Quails from Phase I of this study were analyzed using one-way ANOVA.

15.0 RESULTS

Several one-way analyses of variance were used to determine overall levels of significance and Tukey's multi-comparison test was used to separate means. No males were available from the commercial supplier during the planned time of this experiment; therefore no data was collected for males. Female laying cycles were induced by lighting changes. Consequently the egg data in this study represents non-fertilized eggs. We believe that this would not alter the expected results for egg parameters.

Body and organ weights and serological endpoints were used as response variables, and the Treatment as the factor. There were no significant differences for any of the endpoints evaluated

In Phase II two ANOVA's were executed identical to Phase I. We compared the 1 mM-group to the control group using a one-way ANOVA. There was a borderline difference in the kidney means for the two groups ($p = 0.053$). The mean kidney weight for the control group was 1.61 and 1.89 for Milli group; indicating potential evidence of an increase in kidney weight. There were no other significant results.

A second set of analyses for Phase II, a one-way ANOVA was used to compare the control, 0.01 mM, and 0.1 mM groups. Additional significant and borderline results were observed. Total protein showed a significant difference (p-value = 0.01). The means were 4.27 for the control, 4.79 g/dl for the 0.01 mM group, and 3.86 g/dl for the 0.1 mM group. Tukey's multiple comparisons procedure resulted in the identification of a significant difference between the 0.1 and 0.01 mM perchlorate groups. This does not follow a logical ordering of either an increasing or decreasing total protein as the concentration increases.

Lactate dehydrogenase was borderline ($p = 0.053$). The means were 335.1 IU/L for the control, 201.4 IU/L for the 0.01 mM, and 371.2 IU/L for the 0.1 mM. Running Tukey's, the only significant difference was between the 0.1 and the 0.01 mM groups. The ordering is the reverse of what we observed for total protein.

Free T_4 had a significant $p = 0.01$. The means in this case are 21.29 pmol/Ll for control, 30.66 pmol/Ll for 0.01mM and 16.35 pmol/Ll for 0.1 mM. Tukey's shows the only significant difference to be between the 0.1 and the 0.01 mM groups. The ordering here is similar to total protein.

There were no other significant differences for any of the other endpoints.

The following data (Tables 1-3) represent quail data for mean body and organ weights following exposure to perchlorate. These endpoints were not significantly different from the control groups. The data are represented as mean ± SD of the mean.

Table 2 Showing Average Organ Weights of Quail Exposed to Perchlorate

Perchlorate Concentration	Organ Weights (g)			
	Gizzard	Heart	Kidney	Liver
Control	7.3 ± 1.0	0.99 ± 0.12	1.6 ± 0.3	5.7 ± 1.5
0.01mM	7.7 ± 1.2	1.02 ± 0.2	1.8 ± 0.2	5.9 ± 0.4
0.1mM	7.7 ± 1.0	1.10 ± 0.2	1.8 ± 0.3	5.4 ± 1.1
1.0mM	7.2 ± 1.0	1.03 ± 0.2	1.9 ± 0.3	5.6 ± 0.9
p-value	p = 0.53	p = 0.0.76	P = 0.12	p = 0.63

Values represent the mean ±SD

Table 3 Showing Serological endpoints from Quails Exposed to Perchlorate

Perchlorate Concentration	Serological Parameters					
	Glucose	Total Serum Protein	SGOT	Uric Acid	T3 (ng/dl)	T4 (ug/dl)
Control	336 ± 98	4.4 ± 0.9	878 ± 748	8.1 ± 4	61.1 ± 34	0.85 ± 0.5
0.01mM	333 ± 33	4.5 ± 0.7	917 ± 612	7.02 ± 2	6.2 ± 36	1.09 ± 0.5
0.1mM	340 ± 37	4.2 ± 0.8	782 ± 510	7.6 ± 2	53.4 ± 28	0.85 ± 0.4
1.0mM	333 ± 65	4.2 ± 0.9	872 ± 553	7.3 ± 3	63.4 ± 49	0.67 ± 0.5
p-value	p = 0.39	p = 0.45	p = 0..89	p = 0.65	p = 0.85	p = 0.063

SGOT-Serum glutamic oxalacetic transaminase
Values represent the mean ±SD

Table 4 Showing Average Body Weights Quails Exposed to Perchlorate in Food

Perchlorate Concentration	Body Weights (g)		
	Week 2	Week 3	Week 4
Control	240 ± 12	238 ± 21	233 ± 18
0.01mM	230 ± 15	234 ± 17	233 ± 16
0.1mM	221 ± 20	217 ± 19	217 ± 34
1.0mM	232 ± 12	224 ± 13	228 ± 21
p-value	p = 0.08	p = 0.033	p = 0.33

Values represent the mean ±SD

Thyroid Histological Analysis

Histological slides of the thyroid gland of three quail from the highest dose group treated with perchlorate and three quail from the control group were assessed qualitatively for the effect of perchlorate. Three sections from the beginning, the middle and the end of the thyroid were assessed in each of the quail. The sections were observed at 40X. The quail were arbitrarily chosen from each group.

In the control animals the thyroid glands had large follicles that were filled with colloid. The follicular cells in these thyroid glands were squamous to cuboidal in shape. No evidence of hyperplasia was observed in any section from the control thyroid glands in these animals. These section contained few inactive follicles (Figure 1).

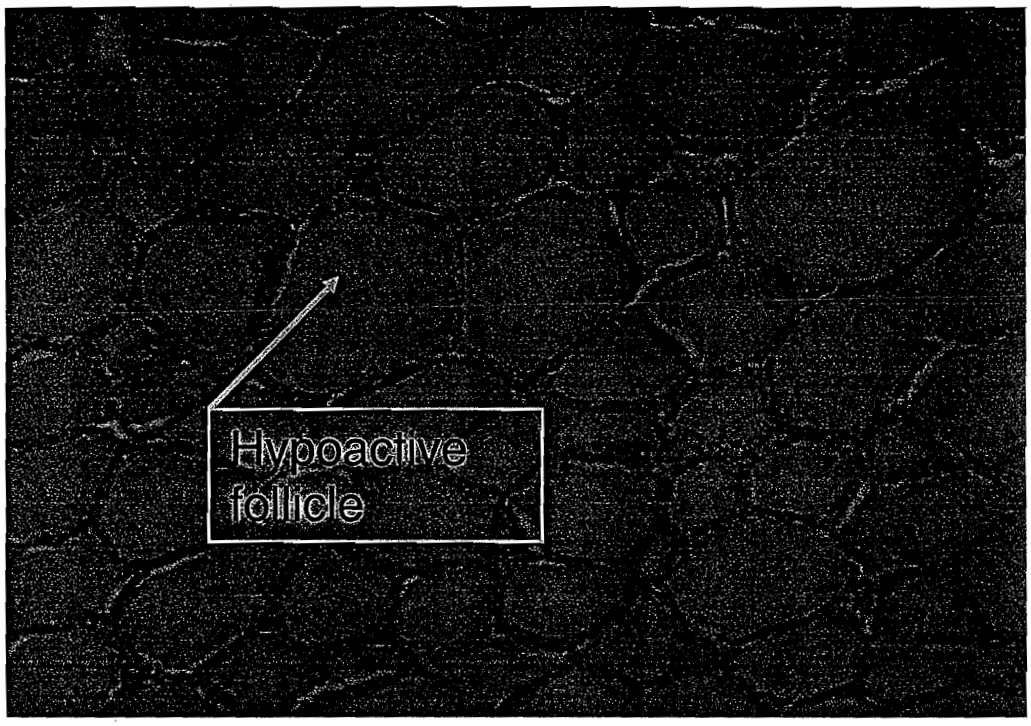

Figure 1 Showing a photomicrograph of a normal thyroid gland of a quail from the control group.

Generally, the thyroid glands of quail exposed to 1mM perchlorate showed hyperplastic regions throughout the sections observed. The lumen of several of the follicles in these sections had a lacy appearance and some were void of colloid. Also, many microfollicles were observed in these tissues. The follicular cells were columnar in shape and in many instances the cells appeared to be hypertrophic (Figure 2).

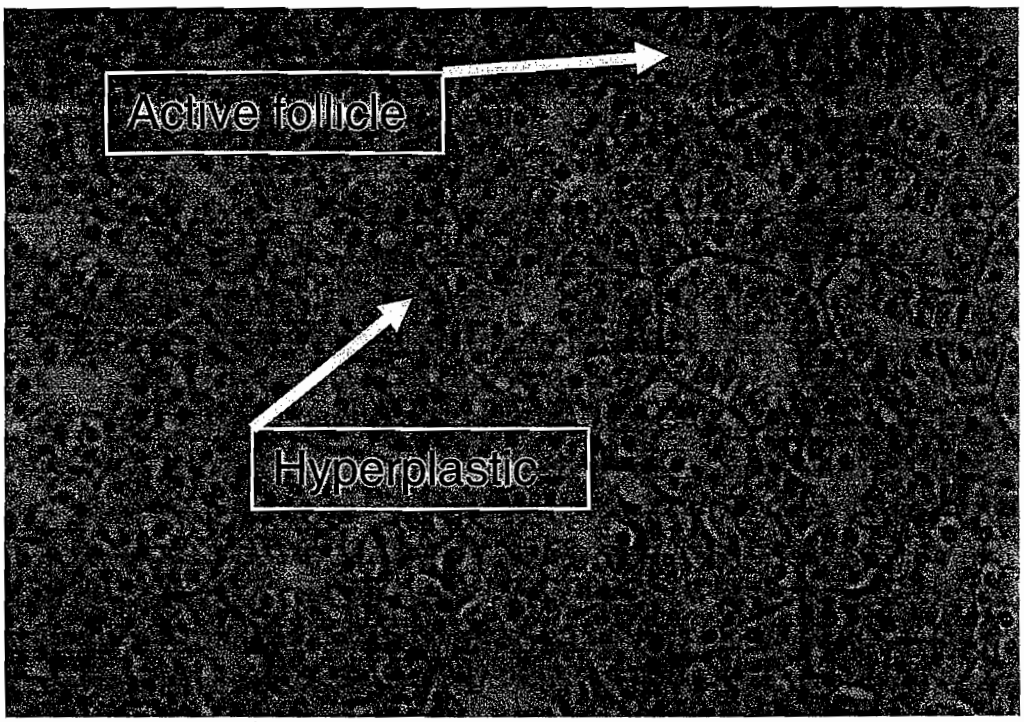

Figure 2 showing a photomicrograph of the thyroid gland from a quail exposed to 1 mM perchlorate.

Effect of Perchlorate on Quail Eggs

The endpoints used in the analysis to determine if ammonium perchlorate had any effect on the physical characteristics of the eggs were the egg weight, average egg length (hereafter denoted length), and the average egg diameter (hereafter denoted diameter). Both averages were computed from three independent measurements. The factors included in the model are the dose, the cage the eggs were taken from, and the date the eggs were taken. There were 36 different cages in use, each housing 2 or 3 birds, and there were 29 different dates. There were a total of 1937 eggs that were obtained and measured.

The analysis was carried out using standard analysis of variance procedures in SAS. Three different analyses were performed, one for each endpoint. The model used in each was to have the dose as a fixed factor, the date as a random factor, and the cage as a random factor nested within the dose factor. The nesting is necessary since, for example, cage #1 was used for only the control level for dose. The cage and date are considered random factors since they represent a random sampling from a larger population of cages and dates that could have been used to conduct the study. No interactions were included in the model since none were seen to be significant.

One thing that complicated the analysis was the presence of outliers. There were 16 outliers (defined as having a standardized residual – in absolute value – greater than 3

in any of the three analyses). Having this number of outliers for a data set of this size is not unexpected. The magnitude of the residuals, on the other hand, differed greatly from what are predicted assuming normally distributed errors. These 16 points were examined to determine if there was any systematic cause. There were no apparent relationships between them except that 8 were from the control dose level and the other 8 were from the 0.1 mM dose level. These outliers were removed from the data set, but no impact was seen on the conclusions drawn from the analyses whether they were present or not.

In every analysis, there is a significant date effect and cage effect. The eggs tended to increase in size as the experiment progressed. This is obvious in Figure 1, which plots the average weight as a function of time grouped by treatment. The cage effect is most likely due to bird-to-bird differences, which is to be expected. There are no differences in the means (for weight, length, or diameter) that can be attributed to the dose. The p-values are 0.5605 for weight, 0.3995 for length, and 0.7400 for diameter. Figure 1 also illustrates the difficulty in picking out differences due to the dose in the presence of other extraneous sources of variation (such as the date and cage).

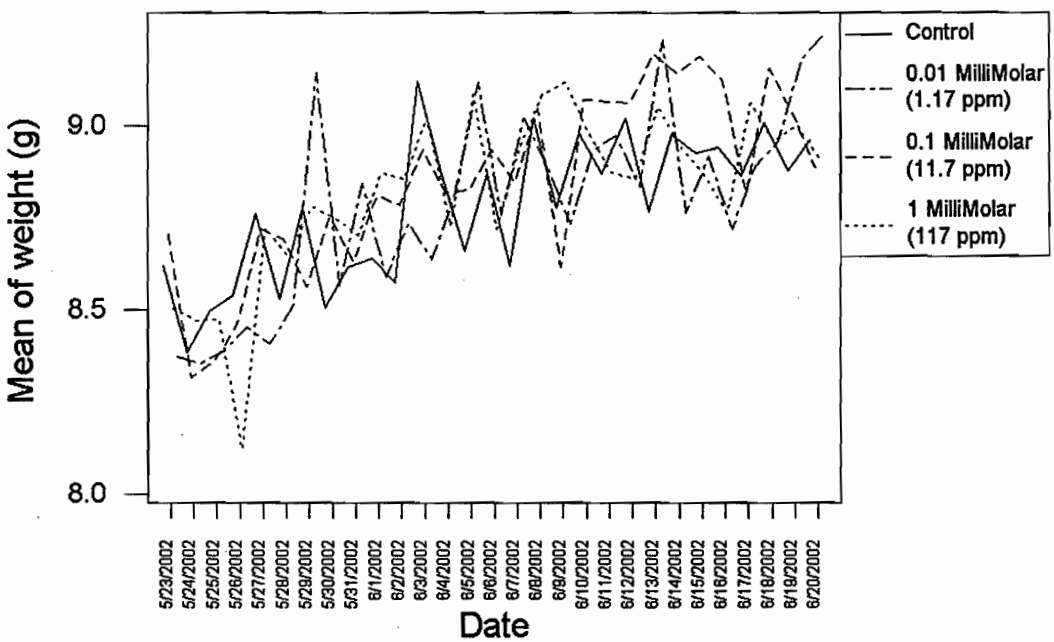

Figure 3. Mean weight versus date grouped by dose level.

Another analysis concentrated on the numbers of eggs laid by the birds exposed to different levels of dose. Since there were not equal numbers of birds in each cage, for each cage and date, the number of eggs per bird was computed, and this was the endpoint used. The model used here is the same as that described above. There were no significant interactions. As before, both date and cage were highly significant, but there

was no significant dose effect (p = 0.5208). Figure 2 shows the eggs per bird plotted as a function of date and grouped by dose level. As can be seen from Figure 2, the significant date effect is not due to some systematic increasing or decreasing over time, but due to some unusual fluctuations in egg production.

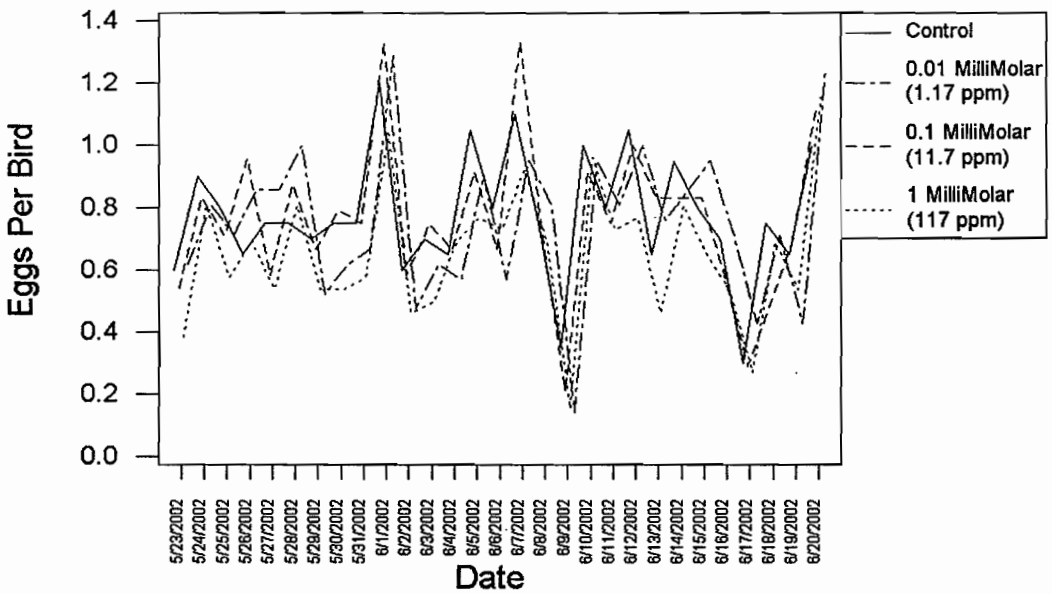

Figure 4. Eggs per bird versus date grouped by dose level.

Quail Egg and Liver Perchlorate Concentration Following Exposure in Water
Analytical evaluation of perchlorate concentration in quail eggs was carried out by the analytical core research team. Exposure to 0.01 mM exposure resulted in detection of perchlorate in quail eggs ranging from 0 to 58 ng/gm. The average was 21.3 ng/gm. At 0.1 mM, perchlorate in quail eggs ranged from 11 to 236 ng/gm, while at 1 mM the range was 1165 to 3415 ng/gm with an average of 50 and 1802 ng/g respectively.

No perchlorate was detected in quail livers from the control and 0.01 mM groups. At 0.1 mM, perchlorate concentration ranged from 0 to 294 ng/gm liver dry weight, while at 1 mM perchlorate ranged from 0 to 878 ng/gm liver dry weight

16. DISCUSSION

There is little information on the potential effects of perchlorate in birds. We evaluated the effects of perchlorate in bobwhite quail using gross toxicology, thyroid hormone, serological endpoint and egg production. Previously we reported that perchlorate induced alterations of thyroid hormone and reduced the number of active follicle in a rodent species (Thuett, K. A. *et al.* (2002a);Thuett, K. A. *et al.* (2002b). Recently, it was reported that perchlorate affects bobwhites and the order of thyroid variables from most to least sensitive was stated as: Thyroid gland –thyroid hormone content >thyroid gland weight >plasma T4. They reported also, that low doses and short exposure times decrease thyroid gland-thyroid hormone content and high doses and longer exposures decrease plasma thyroid hormone. They stated also that plasma thyroid hormones, the most frequently used measure of avian thyroid function, is the least sensitive while thyroid gland-hormone content is the most sensitive measure for assessing thyroid disruption. Thyroid glands can maintain extracellular stores of thyroid hormone, so circulating thyroid hormone and organismal thyroid status, can be maintained for some time despite decreases in or cessation of thyroid hormone synthesis (McNabb, et al., 2002a). In another study, McNabb et al., (2002b) investigated the effects of ammonium perchlorate (AP) on thyroid function in embryos, chicks and adult bobwhite quail. They reported a rank order of thyroid variables from most to least sensitive, for all three life stages were similar to the order stated in the first study above. Thyroid gland-thyroid hormone content > thyroid gland weight >plasma T4. Perchlorate is a competitive inhibitor of iodide uptake into the thyroid gland, and so exposure to perchlorate has the potential to impair the synthesis of thyroid hormone

In this study control animals the thyroid glands had large follicles that were filled with colloid. The follicular cells in these thyroid glands were squamous to cuboidal in shape. No evidence of hyperplasia was observed in any section from the control thyroid glands in these animals. Generally, the thyroid glands of quail treated with 1mM perchlorate in food, showed hyperplastic regions throughout the sections observed. The lumen of several of the follicles in these sections had a lacy appearance and some were void of colloid. Also, many microfollicles were observed in these tissues. The follicular cells were columnar in shape and in many instances the cells appeared to be hypertrophic. The results of the somatic indices in this study support the idea that perchlorate is a developmental toxicant and those toxic responses seen in more mature animals are secondary to perchlorate thyrotoxicosis. This concept is supported by limited published data by Thuett et al., (2002a and b) and McNabb et al., (2002a and b).

Evaluations of the egg data revealed no significant difference for treatment group versus experimental groups. However, with the demonstrated effect of perchlorate in developing avian species (McNabb, et al., 2002b), it is worthy to

note that we believe the effect of perchlorate could alter physiological endpoints to a similar degree as observed in the other studies

17. STUDY RECORDS AND ARCHIVE:

Study records will be maintained at The Institute of Environmental and Human Health Archive for a minimum of one year from study completion date.

18. REFERENCES

McNabb, F.M.Anne, Maher, R.D., Jones, J.E. and Hood, S.A. (2002a). The effects of perchlorate on thyroid function in developing and adult bobwhite quail. SETAC Annual National Meeting, Utah.

McNabb, F.M.Anne[1], Fox, G.A.[2] and Grasman, K.A. (2002b). Comparison of variables for evaluating pollutant effects on thyroid function in birds. . SETAC Annual National Meeting, Utah.

Thuett, K. A., Roots, E. H., Mitchell, L. P., Gentles, B. A., Anderson, T., Kendall, R. J., and Smith, E. E. (2002a). Effects of in utero and lactational ammonium perchlorate exposure on thyroid gland histology and thyroid and sex hormones in developing deer mice (peromyscus maniculatus) through postnatal day 21. J Toxicol Environ Health A **65**, 2119-30.

Thuett, K. A., Roots, E. H., Mitchell, L. P., Gentles, B. A., Anderson, T. A., and Smith, E. E. (2002b). In utero and lactational exposure to ammonium perchlorate in drinking water: effects on developing deer mice at postnatal day 21. J Toxicol Environ Health A **65**, 1061-76.

Project No.: T9700

A STUDY PROTOCOL

ENTITLED

Avian Exposure Laboratory Studies

STUDY NUMBER: AELS-02-01

SPONSOR: United States Air Force
AFIERA/RSRE
2513 Kennedy Cir
Brooks AFB, TX 7235-5123

TESTING FACILITY
Name/Address: The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. Ronald J. Kendall

Study Director: Ms. Lisa Perlmutter

PROPOSED EXPERIMENTAL START DATE: APRIL 28, 2002

1. **DESCRIPTIVE STUDY TITLE:**
Response of Quail to Perchlorate Contaminated Water Following Oral Exposure

2. **STUDY NUMBER: AELS-02-01**

3. **SPONSOR:**
United States Air Force
AFIERA/RSRE
2513 Kennedy Cir
Brooks AFB, TX 7235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: April 28, 2002
Termination Date: December 31, 2002

6. **KEY PERSONNEL:**
Ernest E. Smith, Co-Principal Investigator
Lisa Perlmutter, Study Director
Lance Williams, Histopathologist
Ryan Bounds, Quality Assurance Manager
James Surles, Statistical Support
Ron Kendall, Principal Investigator and Testing Facility Management

7. **DATED SIGNATURES:**

_____	5/17/02	Dr. Ernest Smith Co-Principal Investigator
Lisa Perlmutter	5/17/02	Ms. Lisa Perlmutter Study Director
_____	5/20/02	Mr. Ryan Bounds Quality Assurance Manager

	5/17/02	Dr. James Surles Statistical Support
	3-JUN-02	Dr. Lou Chiodo Assistant Director for Science
	6/6/02	Dr. Ron Kendall Testing Facility Management

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

9. STUDY OBJECTIVES / PURPOSE:

The study objective is to determine the response of quail to perchlorate by assessing general toxicity and histological end points of selected organs.

10. STUDY SUMMARY:

This study will consist of exposing birds to water containing ammonium perchlorate and comparing those accumulation and perchlorate-related endpoints. This experiment provides a realistic assessment of exposures that are likely to occur where environmental contamination with perchlorate occurs.

11. TEST MATERIALS:

Test Chemical:	Ammonium Perchlorate 99.999% pure
Source:	Sigma-Aldrich
Characterization:	Oxidizer; explodes when heated
Test Medium:	Deionized Water

12. JUSTIFICATION OF TEST SYSTEM

This project is intended to evaluate risks of perchlorate exposure among organisms consuming perchlorate-contaminated water. Birds will be used as surrogates to livestock (the most likely exposed organisms) because they are easily handled, permit large sample sizes, and are more economically feasible in a study of this nature. Nothern Bobwhite Quail (*Colinus viginianus)* provide a useful avian model of exposure and toxicity. There are no other suitable models for assessing exposure risks in an ecological environmental setting.

This study represents the second phase of an ongoing project and requires live animals for each experiment and cannot be substituted with culture or computer generated models. Culture and computer models cannot simulate changes in general homeostasis and thyroid

hormone alteration. In addition it would not provide pertinent scientific data for furture use in risk assessment.

13. TEST ANIMALS:

Species: Northern Bobwhite Quail
Strain: Wild type
Age: Adults
Sex: Females
Number: Approximately 100 quail
Source: Purchased from Routhwood game bird farm in San Angelo, TX

14. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each cage will be labeled as indicated in TIEHH SOP **IN-3-06**, which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. Each cage will also be labeled to include sex of the individuals (if appropriate), date of exposure, the name of the test substance and its concentration. Birds will be leg banded with unique identification numbers.

15. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Perchlorate Treated Water Dosing Only for quail:
23 females (quail) X 4 treatment groups (perchlorate contaminated water; control (0 ppm), nanomolar (0.117 ppb), micromolar (117 ppb), and millimolar (117 ppm) = 92 quail.

Table 1. Number of quail for experimental exposure.

Treatment	Quail Female
Control	23
0.117 ppb	23
117 ppb	23
117 ppm	23
Total	92

Grand total for quail experimental exposure = 92

16. METHODS:

Prior to any studies and pending approval all personnel will be required to read ammonium perchlorate material safety data sheet and must wear gloves, lab coat, and hair restraints at all times when working with birds and/or dosing solutions.

Quail will be randomly assigned to treatment groups. Initial body weights will be recorded for all birds prior to initiation of the study. There will be 2 to 3 quail per cage, housed in stacked cages (91 x 71 x 27 cm) in test facility conditions of 72°F and 18:6 hours of light:dark.

Water and food (Bluebonnet poultry feed) will be available *ad libitum* throughout the experiment. Daily observations will be made on all quail. Animals showing signs of distress or disease will be treated according to the instructions of the University Veternarian.

All avian handling will be done according to Standard Operating Procedures.

16.1 Test System acquisition, quarantine, and acclimation.
Quail will be obtained from Routhwood game bird farm in San Angelo, TX. They will be maintained in stacked cages and kept on an 18L: 6D light regimen. Quail will be given a minimum of a one-week acclimation period.

16.2 Assignment of Animals to Study Group and Identification
Animals will be assigned randomly to treatment groups upon selection from the pool. The quail will be identified by their unique leg band number.

16.3 Test Material Application
Ammonium perchlorate will be dissolved in the drinking water (SOP IN 3-05) and given to the quail at concentrations of control (0 ppm), nanomolar (0.117 ppb), micromolar (117 ppb), and millimolar (117 ppm). Birds in the control group will be given deionized water not treated with ammonium perchlorate. Quail weights will be recorded on the same day of each week during the study to evaluate weight gain or loss.

Rates/concentrations: perchlorate (0.117 ppb, 117 ppb, and 117 ppm), or no treatment.

Frequency: Test substances will be supplied *ad libitum* and renewed every day.

Route/Method of Application: Quail will be exposed to water contaminated with ammonium perchlorate for 30 days. Route of exposure will be via oral.

16.4 Daily Observations
Animals will be monitored daily for changes in general health. If any changes are observed, animals will be treated according to University Veterinarian.

16.5 Animal Euthanasia and Sample Collections
At dosing day 31, all animals will be weighed using an aluminum animal weighing pan with an even distribution of holes and a lid. After the quail are weighed, they will be rendered unconscious by carbon dioxide, bled, and euthanized by exsanguination (AF 1-03) and the following tissues will be collected: livers, kidneys, hearts, muscle, ovaries, adrenal glands and thyroid glands. Blood samples will be placed in EDTA tubes, centrifuged, and the resulting plasma will be used in steroid and thyroid hormone assays.

16.6 Evaluations

Selected organs and blood samples will be collected according to SOP ET-3-19 for chemical and histological analyses.

17. PROPOSED STATISTICAL METHODS

Organ weight and hormone concentration will be subjected to ANOVA and multi-comparison analysis.

18. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include animal receipt, animal care, test material preparation and application, animal observations, and facility records for personnel, equipment, etc.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

Study Methods
Survival of treatment animals
Chemical analysis results
Gonadal morphology summary
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used

19. RECORDS TO BE MAINTAINED LOCATION:

A final report containing the results of the dosing studies will be delivered to the Sponsor on or before **December 31, 2002.** Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility for up to 3 years.

20. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to affect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

21. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

22. REFERENCES:

Thuett et al., 2002. Journal of Environmental Toxicology and Health

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Lubbock, TX 79409-1163
(806) 885-4567
qa@tiehh.ttu.edu

Form No. 014 Rev. 3.06/00
Project No.: T9700 AELS-02-01
*Change No: 1
Page: 1 of 1

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ✓ Amendment ____ Deviation ____ Addendums

Document Reference Information
Check One: ✓ Protocol ____ SOP ____ Other ____
Title: Avian Exposure Laboratory Studies
Dated: 12/05/02
Document # (if appropriate): ____
Page #(s): 6
Section #: 8
Text to reference: Regulatory Compliance Statement: "in compliance with Good Laboratory Practice Standards."

Change in Document: in compliance, where appropriate and possible, with Good Laboratory Practice Standards.

Justification and Impact on Study: To comply with TIEHH quality assurance plan. No impact on study.

Submitted by: Signature: Lisa Perlmutter Date: 12/05/02
Authorized by: Study Director: Lisa Perlmutter Date: 12/05/02
Received by: Quality Assurance Unit: Brian Bridwell Date: 12/05/02

* Sequentially numbered in order of the date that the change is effective

TIEHH
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Form No. 014 Rev. 3.06/00
Project No.: T9700 AELS-02-01
*Change No: 2
Page: 1 of 1

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment ✓ Deviation ____ Addendums

Document Reference Information
Check One: ✓ Protocol ____ SOP ____ Other ____
Title: Avian Exposure Laboratory Studies
Dated: 12/09/02
Document # (if appropriate): ____
Page #(s): 6
Section #: 16.5
Text to reference: The following tissues will be collected: livers, kidneys, hearts, muscle, ovaries, adrenal glands, and thyroid glands.

Change in Document: The following tissues will be collected: livers, kidneys, hearts, gizzards, and thyroid glands.

Justification and Impact on Study: Space and time constraints would not allow for the collection of these tissues. No impact on study is expected.

Submitted by: Signature: Lisa Perlmutter Date: 12/09/02
Authorized by: Study Director: Lisa Perlmutter Date: 12/09/02
Received by: Quality Assurance Unit: Brian Bidwell Date: 12-9-02

* Sequentially numbered in order of the date that the change is effective

A FINAL REPORT

ENTITLED

COMBINED EFFECTS OF PERCHLORATE AND UV ON DNA DAMAGE AND DEVELOPMENT IN *Xenopus laevis*

STUDY NUMBER:	LARV-02-01
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	02/28/2002
RESEARCH COMPLETION:	12/31/2002

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GOOD LABORATORIES PRACTICES STATEMENT

Project LARV-02-01, entitled " Combined Effects of Perchlorate and UV on DNA Damage and Development in *Xenopus laevis* ", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

3/27/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase/Activity	Audit Dates Start	End	Date written report submitted to Study Director	Date written report submitted to Management
Protocol Review	6-28-02	6-28-02	6-28-02	7-2-02
Final Report and Raw Data Review	2-24-03	2-26-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

03/27/03
Date

1. DESCRIPTIVE STUDY TITLE:
Combined Effects of Perchlorate and UV on DNA Damage and Development in *Xenopus laevis*

2. STUDY NUMBER:
LARV-02-01

3. SPONSOR:
United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: (date of chemical application) July 1, 2002
Termination Date: (date of last data collected) September 31, 2002

6. KEY PERSONNEL:
Ron Kendall, Principal Investigator
Christopher Theodorakis, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Brian Birdwell, Quality Assurance Officer
Jacques Rinchard, Postdoctoral research Associate
June-Woo Park, Graduate Student
Les McDaniel, Graduate Student
Fujun Liu, Graduate Student

7. STUDY SUMMARY:
Two groups of laboratory reared *Xenopus laevis* larvae were housed in 1.0L beakers the sides of which were wrapped in aluminum tape to prevent lateral ultraviolet light exposure: One group was exposed to low dosages of ultraviolet-b (uv-b) radiation through the tops of the beakers via styrene covers, while the second group was protected from uv-b exposure through polycarbonate plastic top covers. The two groups were further subdivided into 3 replicates each of 3 different sodium perchlorate concentrations: 0.0, 0.05, and 10.0 ppm.

Ten stage 53 (NF) larvae were randomly selected for each of the 18 beakers and were housed in a low temperature incubator outfitted with the fluorescent lights that were used to deliver uv dose. Larvae remained in the exposure chamber until they developed

to NF stage 60, and the exposure was terminated after 80% of the control animals reached NF stage 60. Larvae were frozen and temporarily stored in liquid nitrogen prior to storage at –80° C. Tail tissue from 3 larvae from each replicate of the 6 exposure regimes was collected and pooled for DNA extraction and purification. Alkaline gel electrophoresis was then used to detect thymine dimers in samples, if present.

8. STUDY OBJECTIVES / PURPOSE:

To determine if combined exposure of perchlorate and uv increased the incidence of DNA damage in larval *Xenopus laevis*.

9. TEST MATERIALS:

Test Chemical name: Sodium perchlorate
CAS number: 7601-89-0
Characterization: Determination of concentration in water samples.
Source: Aldrich Chemical Company
Reference Chemical name: (list any standards, positive or negative control materials) ultrapure water with added sea salts ("Instant Ocean®" or any other brand of sea salts with identical or nearly identical composition).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to Convert it to ultrapure water. 60 mg/L salts will be added.

10. JUSTIFICATION OF TEST SYSTEM:

Ionic perchlorate alters thyroid homeostasis in larvae and amphibians as well as other vertebrates. The interaction between perchlorate exposure and UV is important because perchlorate exposure may elicit indirect effects on wild animals, such as the ability to cope with natural stressors (e.g. solar UV). Amphibians are currently a major focus of research because of worldwide population declines and occurrence of deformities, and UV radiation has been implicated as a potential contributor to both of these effects. Also, recent evidence has suggested that perchlorate exposure increases amphibian's susceptibility to UV-induced effects. One suggested mechanism behind this is that thyroid hormone controls development of melanocytes and their responsiveness to UV exposure, and melanin is a principal protectant against UV damage. Anecdotal evidence from LHAAP has suggested that tadpoles collected from a perchlorate-contaminated pond were lighter in color than those from a reference site. One of the main types of UV damage is DNA damage, which has been associated with developmental deformities. This could affect management of perchlorate-contaminated sites, because environmental perchlorate exposure could increase the susceptibility of amphibians to even current ambient levels of solar UV radiation.

11. TEST ANIMALS: (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: African clawed frog (*Xenopus laevis*)

Strain: Outbred

Age: Larvae.

Number: 180

Source: Bred from captive stocks currently maintained in the laboratory.

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Aquaria were labeled with the beaker number, species name, animal use protocol number, project number, test system, and date of hatch. Experimental beakers were labeled with beaker number, project number, date of exposure and date of collection, concentration, and person responsible.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Xenopus larvae were exposed to two concentrations of sodium perchlorate plus zero control. They were allowed to develop while being exposed to UV light while in beakers covered with either UV-transparent beaker cover or UV-opaque beaker cover. Larvae were placed into precleaned aquaria or beakers. They were exposed to a total UV dose of 58mJ/cm^2/d. Larvae were allowed to develop to NK stage 53 before being placed in the UV exposure system. Aquaria and beakers were cleaned by washing according to SOP AQ-1-23 "Cleaning Glassware for Use with Xenopus laevis". For exposures, aquaria or beakers were located on shelves capable of supporting such weight. The experimental design consisted of a randomized block design, with each shelf constituting a block. The arrangement of the beakers within each block were randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. Determination of the arrangement of the beakers within each block was via a random number generator. Each block contained at least 1 beaker of each treatment. Larvae were placed in the beakers in random order, within blocks, using the procedure described below.

For the larvae, each block consisted of 9 beakers: 3 replicates per block, 3 perchlorate concentration per replicate, 2 beaker cover treatments per concentration (UV transparent or UV opaque). There were 2 blocks for a total of 18 beakers. Each beaker contained 10 larvae. Within each block, each beaker was assigned a number from 1-18. A random number generator was used to randomly order the numbers 1-18, and this was repeated 15 times. This was performed for both blocks in the experiment.

14. METHODS

14.1. Test System acquisition, quarantine, acclimation

Larvae were bred from captive adult stocks according to SOP AQ-1-06 "Care and Maintenance of *Xenopus laevis*". Larvae were maintained in FETAX (Frog Embryo

Teratogenesis Assay—Xenopus) media. Every other day, debris at the bottom of the beaker were cleaned by suction and 50% of the media exchanged with fresh; all beakers received continuous gentle aeration. Animal husbandry was maintained according to SOP AQ-1-06. Staging of larvae was according to SOP AQ-1-07 "Developmental Staging and Measurements for *Xenopus laevis* larvae".

14.2. Test Material Application

Rates/concentrations: Larvae were exposed to 0.0, 50.0 or 10 ppm sodium perchlorate in FETAX solution, and were exposed to UV at a dose of 0.0 or 58.0 $mJ/cm^2/d$.

Frequency: Larvae were maintained in exposure conditions from NF stage 53 until stage 60.

Route/Method of Application: Route was via dermal, oral and respiratory exposure as the chemical will be in the beaker media.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because larvae respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification: A sample of treated water was tested by chemical analysis once per week during the exposure.

14.3. Test System Observation

Tanks or beakers were observed on a daily basis. The number of individuals that expire each day was recorded for each perchlorate concentration. In addition, ammonia, pH, dissolved oxygen, conductivity, temperature, and salinity were to be determined at least 3 times per week.

14.4. Animal Sacrifice and Sample Collections

At the end of the exposure, all larvae were collected, euthanized, wrapped in aluminum foil, uniquely labeled, and frozen in liquid nitrogen. Individuals were then removed from storage, and a 1 cm section from the tip of the tail will be collected for DNA damage analysis. The remainder of the carcass was examined for limb/spinal deformities. Labeling: samples were labeled with a unique ID number according to the following scheme: LPUV (laboratory perchlorate UV)– (exposure regime)(beaker number)-(sample number)(analysis). E.g., LPUV-A17-8D. Exposure regimes were indicated by A = exposure from hatching to stage 60; B = exposure from stage 40-60. Designations for analysis were: D = sample frozen for DNA analysis; H = sample preserved in Bouin's fixative for histological analysis; M = sample preserved in formalin for observation of malformations. For example, LPUV-A14-8D is a tail sample frozen for DNA analysis that was taken from larvae # 8 from beaker #14 that was exposed to UV light from hatching to stage 60 (exposure regime A), while LPUV-B17-9M is a sample preserved in formalin for observation of abnormalities that was taken from larvae # 9 from beaker #17

that was exposed to UV light from stage 40 to stage 60 (exposure regime B). For each beaker, the block number (block 1 or 2), concentration of perchlorate, and beaker cover will be recorded in a bound laboratory notebook.

If samples are batched, batches will be labeled with a unique ID according to the following scheme: LPUV-BX###, where X = D, H, or M, as described above, e.g., LPUV-BD001 is batch #1 frozen for DNA damage analysis. Minimum information on batch labels included study number and batch ID. Additional information included type of sample (tissue, ethanol precipitated DNA, DNA dissolved in TE, etc.) or treatment group. If batches were divided into smaller batches, then they were labeled according to LPUV-BX###.#, e.g., LPUV-BD001.1 is a batch that was derived from a subsample of LPUV-BD001. If batches were composed subsamples from 2 or more batches, they were given a new ID.

Procedures for adding or removing samples from batches, tracking samples, tracking batches, and recording information for all samples on forms or in bound notebooks followed SOP IN-3-02 "Sample Labeling/Logging Proceedure", SOP IN-3-10 "Batching of Samples for Storage and/or Transportation", and SOP IN-1-05 "Notebook/Data Form/Label Documentation".

Minimum information included on the labels were project number and unique ID (SOP IN-03-02 Sample Labeling/Logging Procedure). Additional information included concentration of perchlorate, beaker cover and date collected, in decreasing order of importance. All of this information was recorded in the bound laboratory notebook.

14.5. Endpoint Analysis

Analysis of water and of tissue extracts was performed according to SOP AC-2-11 "Analysis of Perchlorate by IC". The endpoint will be tissue/whole body concentration of perchlorate. Analysis of malformations was performed visually, and any individuals with limb, spinal, or facial malformations were recorded for each beaker in a bound laboratory notebook. DNA extraction was performed according to SOP AQ-2-07 "DNA Extraction from Fish or Amphibian Tissues". Analysis of DNA for thymine dimers was performed according to SOP AQ-2-06 "DNA Damage Assays Using Alkaline Electrophoresis". This endpoint was number of thymine dimers /10^5 base pairs.

15. STATISTICAL METHODS

To statistically determine differences between treatments in terms of perchlorate body concentrations, 1-way ANOVA was used to determine effects of concentration. .

16. PROTOCOL CHANGES/REVISIONS:

PROTOCOL LARV-02-01

16.1

Page 1
Text to reference: Food Chain Transfer of Perchlorate in Larvae

Change in Document: Combined Effects of Perchlorate and UV on DNA Damage and Development in *Xenopus laevis*.

16.2
Page 2:
Text to reference: Food Chain Transfer of Perchlorate in Larvae

Change in Document: Combined Effects of Perchlorate and UV on DNA Damage and Development in *Xenopus laevis*.

16.3
Page 3:
Text to reference: To determine contribution of food vs. water in uptake of perchlorate in larvae

Change in Document: To determine the combined effects of UV and perchlorate exposure on DNA damage and development in *Xenopus laevis* larvae.

16.4
Page 4:
Text to reference: There will be 2 different exposure regimes: in one regime, the larvae will develop continuously from hatching to metamorphosis within the UV exposure chamber. In the second regime, they will be allowed to develop to NK stage 50 before being placed in the UV exposure system.

Change in Document: Larvae will be allowed to develop to NK stage 53 before being placed in the UV exposure system.

16.5
Page 5:
Text to reference: Each of the two exposure regimes will be performed separately (i.e., all beakers in the UV exposure chamber will contain either larvae that have 1) developed continuously under UV exposure or 2) developed in the laboratory until NF stage 50, and then placed in the UV exposure chamber). For the larvae exposed to UV under exposure regime #2 (allowed to develop to NF stage 50 before UV exposure), while the larvae are developing outside of the exposure chamber, the beakers will be placed on two shelves designed to hold aquaria, with each shelf being considered a block. The arrangement of beakers within blocks will be the same as described for the arrangement of beakers within the UV chamber. Larvae will be placed in the beakers in random order, within blocks, using the procedure described below.

Change in Document: Text deleted

16.6
Page 5
Text to reference: For the larvae, each block will consist of 18 beakers: 3 replicates per block, 3 perchlorate concentration per replicate, 2 beaker cover treatment per concentration (UV transparent or UV opaque). There will be 2 blocks for a total of 36 beakers. Each beaker will contain 15 larvae. Within each block, each beaker will be assigned a number from 1-18.

Change in Document: For the larvae, each block will consist of 9 beakers: 3 replicates per block, 3 perchlorate concentration per replicate, There will be 2 blocks (block 1 will contain beakers with UV transparent plastic covers, block 2 will contain beakers with UV opaque plastic covers). for a total of 18 beakers. Each beaker will contain 10 larvae. Each beaker will be assigned a number from 1-18.

16.7
Page 5
Text to reference: Larvae will be bred from captive adult stocks according to SOP,. Every other day, debris at the bottom of the beaker will be cleaned by suction. Water will be continuously aerated and filtered using mechanical and biological filtration. Water will consist of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts or other brands of identical composition. Animal husbandry will be according to SOP AQ-1-08.

Change in Document: Larvae will be bred from captive adult stocks according to SOP AQ-1-06 "Care and Maintenance of *Xenopus laevis*". Larvae will be maintained in FETAX (Frog Embryo Teratogenesis Assay—Xenopus) solution. Every other day, debris at the bottom of the beaker will be cleaned by suction and 50% exchanged with fresh solution. Exposure beakers and any aquaria will receive continuous aeration Animal husbandry will be according to SOP AQ-1-06. Staging of larvae will be according to SOP AQ-1-07 "Developmental Staging and Measurements for *Xenopus laevis* larvae".

16.8
Page 5:
Text to reference: Exposures will begin after larvae have hatched.

Change in Document: Perchlorate exposures will begin after larvae have reached NF stage 53.

16.9
Pages 5-6:

Text to reference:

Test Material Application
The two beaker cover treatments will consist of: 1) beakers covered with square glass plates and 2) beakers covered squares of acetate. Both glass or acetate covers 10 cm x 10 cm. Larvae will be exposed to UV until after the emergence of forelimbs, but before complete tail resporbtion has occurred (NF stage xx).

Rates/concentrations: Larvae will be exposed to 10 and 100 ppb sodium perchlorate in water, and will be exposed to UV at a dose of 1 $J/m^2/s$.

Frequency: Larvae will be exposed to perchlorate in water until NF stage xx, and to UV from stage 40 to state xx.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker water.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because larvae respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification:
A sample of treated water will be tested by chemical analysis at least twice per week during the exposure.

Change in Document:
Test Material Application
The two beaker cover treatments will consist of: 1) beakers covered with square tyrene plates and 2) beakers covered squares of polycarbonate. Both styrene or polycarbonate covers will measure 12 cm x 12 cm.

Rates/concentrations: Larvae will be exposed to 0.050 and 10 ppm sodium perchlorate in water, and will be exposed to UV at a dose of 58 $mJ/cm^2/d$.

Frequency: Larvae will be exposed to perchlorate in water from NF stage 53 until stage 60. Larvae will be exposed to UV from NF stage 53 to stage 60.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker water.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because larvae respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification:
A sample of treated water will be tested by chemical analysis once per week during the exposure.

16.10
Page 6:
Text to reference: At the end of the exposure, all larvae will be collected and placed in 3.8 L plastic ziplock bags, and placed on ice in a lightproof container. They will then be removed from the container, and a 1 cm section from the tip of the tail will be collected and frozen in liquid nitrogen. A 0.5 cm section of the tail will then be collected and placed in Bouin's fixative for histological analysis. The remainder of the carcass will preserved in 10% formalin for examination of limb/spinal deformities.

Change in Document: At the end of the exposure, all larvae will be collected and placed in 3.8 L plastic ziplock bags, and placed on ice in a lightproof container. They will then be removed from the container, and a 1 cm section from the tip of the tail will be collected and frozen in liquid nitrogen. The remainder of the carcass will be preserved in 10% formalin for examination of limb/spinal deformities.

16.11
Page 6:
Text to reference: Samples preserved in Bouin's fixative will be prepared according to SOP AQ-2-08 "General Histological Processing of Skin in *Xenopus* Tadpoles". Histological endpoints will include qualitative observations and descriptions of the relative amount of melanocytes, skin thickness, and occurrence of edema recorded in a bound laboratory notebook.

Change in Document: Delete text.

17. RESULTS

No significant differences were observed between UV dose groups; values for thymine dimers per 10^5 base pairs between UV treatment blocks were very similar (Table 1). No developmental abnormalities were discovered in any of the treatments.

Table 1: Thymine dimers per 10^5 base pairs (mean ± SD; n=3) for Xenopus larvae exposed to differing doses of UV-B and different concentrations (0, 0.05, and 10.0 ppm) perchlorate.

	Mean number of Dimers		
	0.00 ppm	0.05 ppm	10.0 ppm
UV-B dose (mJ/cm2/d)			
0.00	2.09 ± 3.63	1.59 ± 0.92	6.27 ± 3.12
58.0	1.75 ± 2.56	0.56 ± 0.97	6.88 ± 7.00

18. DISCUSSION

The results of this study indicate that there are no effects of perchlorate-induced thyroid disruption on susceptibility to UV-induced DNA damage or developmental abnormalities.

19. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20. REFERENCES:

Smith, P.N., Theodorakis, C.W., Anderson, T.A., and Kendall, R.J., 2001. Preliminary assessment of pechlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. Ecotoxicology 10: 305-313.

21. APPENDICES:

Study Protocol
Changes to Study Documentation
List of Key Standard Operating Procedures

Project No. T9700

A STUDY PROTOCOL

ENTITLED

Combined Effects of Perchlorate and UV on DNA Damage, Development, and Skin Histology in *Xenopus laevis*

STUDY NUMBER: LARV-02-01

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

ADMINISTRATOR: The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

TESTING FACILITY

Name/Address:

The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

PROPOSED EXPERIMENTAL START DATE: July 8, 2002

DESCRIPTIVE STUDY TITLE: Combined Effects of Perchlorate and UV on DNA Damage, Development, and Skin Histology in *Xenopus laevis*

1 **STUDY NUMBER:** LARV-02-01

2 **SPONSOR**: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

3 **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

4 **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: (date of chemical application) July 8, 2002
Termination Date: (date of last data collected) September 31, 2002

5 **KEY PERSONNEL:**
Christopher Theodorakis, Study Director
Ronald Kendall, Testing Facility Management
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager

6 **DATED SIGNATURES:**

	6/28/02	Dr. Christopher Theodorakis Study Director
	7/2/02	Dr. Ronald Kendall Testing Facility Management
	6/28/02	Mr. Ryan Bounds Quality Assurance Manager
	6-28-02	Dr. Todd Anderson Analytical Chemist

7 REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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Dr. Ronald Kendall
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

8 STUDY OBJECTIVES / PURPOSE:

To determine contribution of food vs. water in uptake of perchlorate in larvae.

9 TEST MATERIALS:

Test Chemical name: Sodium perchlorate
CAS number: 7601-89-0
Characterization: Determination of concentration in water samples.
Source: Aldrich Chemical Company

Reference Chemical name: (list any standards, positive or negative control materials) ultrapure water with added sea salts ("Instant Ocean®" or any other brand of sea salts with identical or nearly identical composition).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water. 60 mg/L salts will be added.

10 JUSTIFICATION OF TEST SYSTEM

Ionic perchlorate alters thyroid homeostasis in larvae and amphibians as well as other vertebrates. The interaction between perchlorate exposure and UV is important because perchlorate exposure may elicit indirect effects on wild animals, such as the ability to cope with natural stressors (e.g. solar UV). Amphibians are currently a major focus of research

because of worldwide population declines and occurrence of deformities, and UV radiation has been implicated as a potential contributor to both of these effects. Also, recent evidence has suggested that perchlorate exposure increases amphibian's susceptibility to UV-induced effects. One suggested mechanism behind this is that thyroid hormone controls development of melanocytes and their responsiveness to UV exposure, and melanin is a principal protectant against UV damage. Anecdotal evidence from LHAAP has suggested that tadpoles collected from a perchlorate-contaminated pond were lighter in color than those from a reference site. One of the main types of UV damage is DNA damage, which has been associated with developmental deformities. This could affect management of perchlorate-contaminated sites, because environmental perchlorate exposure could increase the susceptibility of amphibians to even current ambient levels of solar UV radiation.

11 TEST ANIMALS:

Species: African clawed frog (*Xenopus laevis*)

Strain: Outbred

Age: Larvae.

Number: Approximately 900

Source: Bred from captive stocks currently maintained in the laboratory.

12 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system will consist of laboratory exposures constructed according to the experimental design described below. Beakers will be labeled with the beaker number, species name, animal use protocol number, project number, test system, and date of hatch. Experimental beakers will be labeled with beaker number, project number, date of exposure and date of collection, concentration, and person responsible.

13 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Xenopus larvae will be exposed to two concentrations of sodium perchlorate plus zero control. They will be allowed to develop while being exposed to UV light while in beakers covered with either UV-transparent beaker cover or UV-opaque beaker cover. Larvae will be placed into precleaned aquaria or beakers. They will be exposed to a total UV dose of 1 $J/m^2/s$. There will be 2 different exposure regimes: in one regime, the larvae will develop continuously from hatching to metamorphosis within the UV exposure chamber. In the second regime, they will be allowed to develop to NK stage 40 before being placed in the UV exposure system. Aquaria and beakers will be cleaned by washing according to SOP AQ-1-23 "Cleaning Glassware for Use with Xenopus laevis".

For exposures, aquaria or beakers will be located on shelves capable of supporting such weight. Each shelf will hold all the 1 L beakers. The experimental design will consist of a randomized block design, with each shelf constituting a block. The arrangement of the beakers within each block will be randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. A random number generator will make determination of the arrangement of the aquaria or beakers within each block. Each block will contain at least 1 beaker of each treatment. Each of the two exposure regimes will be performed separately (i.e., all beakers in the UV exposure chamber will contain either larvae that have 1) developed continuously under UV exposure or 2) developed in the laboratory until NF stage 40, and then placed in the UV exposure chamber). For the larvae exposed to UV under exposure regime #2 (allowed to develop to NF stage 40 before UV exposure), while the larvae are developing outside of the exposure chamber, the beakers will be placed on two shelves designed to hold aquaria, with each shelf being considered a block. The arrangement of beakers within blocks will be the same as described for the arrangement of beakers within the UV chamber. Larvae will be placed in the beakers in random order, within blocks, using the procedure described below.

For the larvae, each block will consist of 18 beakers: 3 replicates per block, 3 perchlorate concentrations per replicate, 2 beaker cover treatment per concentration (UV transparent or UV opaque). There will be 2 blocks for a total of 36 beakers. Each beaker will contain 15 larvae. Within each block, each beaker will be assigned a number from 1-18. A random number generator will be used to randomly order the numbers 1-18, and this will be done 15 times. Dice, a random number table, or a computerized random number generator can be used for this purpose. This will then be done for both blocks in the experiment.

METHODS:

15.1 Test System acquisition, quarantine, acclimation

Larvae will be bred from captive adult stocks according to SOP AQ-1-06 "Care and Maintenance of *Xenopus laevis*". Every other day, debris at the bottom of the beaker will be cleaned by suction. Water will be continuously aerated and filtered using mechanical and biological filtration. Water will consist of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts or other brands of identical composition. Animal husbandry will be according to SOP AQ-1-06. Staging of larvae will be according to SOP AQ-1-07 "Developmental Staging and Measurements for *Xenopus laevis* larvae".

15.2 Test Condition Establishment

Perchlorate exposures will begin after larvae have hatched.

15.3 Test Material Application

The two beaker cover treatments will consist of: 1) beakers covered with square glass plates and 2) beakers covered squares of acetate. Both glass or acetate covers 10 cm x 10 cm.

Rates/concentrations: Larvae will be exposed to 0.050 and 10 ppm sodium perchlorate in water, and will be exposed to UV at a dose of 1 $J/m^2/s$.

Frequency: Larvae will be exposed to perchlorate in water from hatching until stage 60. Larvae will be exposed to UV either 1) from hatching until NF stage 60 or 2) from NF stage 40 to stage 60.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker water.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because larvae respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification:

A sample of treated water will be tested by chemical analysis at least twice per week during the exposure.

15.4 Test System Observation

Tanks or beakers will be observed on a daily basis. The number of individuals that expire each day will be recorded for each perchlorate concentration. In addition, pH, dissolved oxygen, conductivity, temperature, and any other water chemistry parameters deemed appropriate by the project manager will be determined at least 3 times per week.

15.5 Animal Sacrifice and Sample Collections

At the end of the exposure, all larvae will be collected and placed in 3.8 L plastic Ziploc bags, and placed on ice in a lightproof container. They will then be removed from the container, and a 1 cm section from the tip of the tail will be collected and frozen in liquid nitrogen. A 0.5 cm section of the tail will then be collected and placed in Bouin's fixative for histological analysis. The remainder of the carcass will be preserved in 10% formalin for examination of limb/spinal deformities.

Labeling: samples will be labeled with a unique ID number according to the following scheme: LPUV (laboratory perchlorate UV)– (exposure regime)(beaker number)- (sample number)(analysis). E.g., LPUV-A17-8D. Exposure regimes will be indicated by A = exposure from hatching to stage 60; B = exposure from stage 40-60. Designations for

analysis will be: D = sample frozen for DNA analysis; H = sample preserved in Bouin's fixative for histological analysis; M = sample preserved in formalin for observation of malformations. For example, LPUV-A14-8D is a tail sample frozen for DNA analysis that was taken from larvae # 8 from beaker #14 that was exposed to UV light from hatching to stage 60 (exposure regime A), while LPUV-B17-9M is a sample preserved in formalin for observation of abnormalities that was taken from larvae # 9 from beaker #17 that was exposed to UV light from stage 40 to stage 60 (exposure regime B). For each beaker, the block number (block 1 or 2), concentration of perchlorate, and beaker cover will be recorded in a bound laboratory notebook.

If samples are to be batched, batches should be labeled with a unique ID according to the following scheme: LPUV-BX###, where X = D, H, or M, as described above, e.g., LPUV-BD001 is batch #1 frozen for DNA damage analysis. Minimum information on batch labels should include study number and batch ID. Additional information may include type of sample (tissue, ethanol precipitated DNA, DNA dissolved in TE, etc.) or treatment group. If batches are to be divided into smaller batches, then they may be labeled according to LPUV-BX###.#, e.g., LPUV-BD001.1 is a batch that was derived from a subsample of LPUV-BD001. If batches are composed subsamples from of 2 or more batches, they should be given a new ID.

Procedures for adding or removing samples from batches, tracking samples, tracking batches, and recording information for all samples on forms or in bound notebooks should follow SOP IN-3-02 "Sample Labeling/Logging Procedure", SOP IN-3-10 "Batching of Samples for Storage and/or Transportation", and SOP IN-1-05 "Notebook/Data Form/Label Documentation".

Minimum information to be included on the label is project number and unique ID (SOP IN-03-02 Sample Labeling/Logging Procedure). Additional information can include concentration of perchlorate, beaker cover and date collected, in decreasing order of importance. All of this information should be recorded in the bound laboratory notebook.

15.6 Endpoint Analysis

Analysis of water and of tissue extracts will be performed according to SOP AC-2-11 "Analysis of Perchlorate by IC". The endpoint will be tissue/whole body concentration of perchlorate. Analysis of malformations will be done visually, and the percent of individuals with limb, spinal, or facial malformations will be recorded for each beaker in a bound laboratory notebook. DNA extraction will be performed according to SOP AQ-2-07 "DNA Extraction from Fish or Amphibian Tissues". Analysis of DNA for single-strand breaks, thymine dimers and oxidative damage will be performed according to SOP AQ-2-06 "DNA Damage Assays Using Alkaline Electrophoresis". These endpoints will include number of single strand breaks, number of thymine dimers, and number of

oxidized bases/10^5 base pairs. Samples preserved in Bouin's fixative will be prepared according to SOP AQ-2-08 "General Histological Processing of Skin in *Xenopus* Tadpoles". Histological endpoints will include qualitative observations and descriptions of the relative amount of melanocytes, skin thickness, and occurrence of edema recorded in a bound laboratory notebook.

14 PROPOSED STATISTICAL METHODS

To statistically determine differences between treatments in terms of perchlorate body concentrations, 1-way ANOVA will be used to determine effects of concentration. If warranted by lack of normality and/or homogeneity of variances of the data, nonparametric 2-way (e.g. Friedman test) and correlation/regression will be performed.

15 REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include: Room temperature and water temperature, dissolved oxygen, salinity, and pH will be collected. Date, time, and amount of feedings per tank will be recorded. Number of single strand breaks, thymine dimers, and oxidized bases, as well as average percent abnormal larvae, will be included in the report.
Report content will include presentation of data, interpretation, and discussion of the following endpoints:

List individual endpoints and analyses.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

16 RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before December 31, 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point, for final archive within six months of study completion (upon request). All data, the protocol, and a copy of the final report shall be maintained by the testing facility.

17 QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention

of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

18 PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and Test Facility Manager and maintained with the protocol and the Quality Assurance Unit.

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Form No. 014 Rev. 3.06/00
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*Change No: ___1_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: __X__ Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE-02-03~~ LARV-02-01 WE MAB 2-24-03
Page #(s): _____1_____
Section #: _____Title _____
Text to reference: Food Chain Transfer of Perchlorate in Larvae

---

Change in Document: Combined Effects of Perchlorate and UV on DNA Damage and Development in *Xenopus laevis*.

Justification and Impact on Study: An inappropriate title was listed, the title was changed to reflect the true nature of the study

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Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/21/03

Authorized by: Chris Theodorakis Study Director: Chris Theodorakis Date: 2/21/03

Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 2-25-03

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*Change No: ___2_____
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Change In Study Documentation Form

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Check One: __X__ Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE-02-03~~ LARV-02-01 (WE) MAB 2-24-03
Page #(s): _____2_____
Section #: _____1 _____

Text to reference: Food Chain Transfer of Perchlorate in Larvae

---

Change in Document: Combined Effects of Perchlorate and UV on DNA Damage and Development in *Xenopus laevis*.

Justification and Impact on Study: An inappropriate title was listed, the title was changed to reflect the true nature of the study

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Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/21/03

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Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 2-25-03

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*Change No: ___3_____
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The following documents changes in the above referenced study:

Check One: __X__ Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE-02-03~~ LARV-02-01 WE MBB 2-24-03
Page #(s): _____2_____
Section #: _____~~5~~ 4 WE MBB 2-25-03

Text to reference: Termination Date: (date of last data collected) September 31, 2002

Change in Document: Termination Date: (date of last data collected) December 31, 2002

Justification and Impact on Study: _Because of a 4-month delay delivery of necessary equipment by the supplier, the period of data collection had to be extended

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Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/21/03

Authorized by: Chris Theodorakis - Study Director: Chris Theodorakis Date: 2/21/03

Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 2-25-03

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*Change No: ___4_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: __X__ Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE 02-03~~ LARV-02-01 WE MBB 2-24-03
Page #(s): _____3_____
Section #: _____~~9~~ 8 WE MBB 2-25-03

Text to reference: To determine contribution of food vs. water in uptake of perchlorate in larvae

---

Change in Document: To determine the combined effects of UV and perchlorate exposure on DNA damage and development in *Xenopus laevis* larvae.

Justification and Impact on Study: An inappropriate objective was listed, the objective was changed to reflect the true nature of the study

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Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/21/03

Authorized by: Chris Theodorakis Study Director: Chris Theodorakis Date: 2/21/03

Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 2-25-03

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Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE-02-03~~ LARV-02-01 WE MBB 2-24-03
Page #(s): _____4_____
Section #: _____~~14~~ 13 WE MBB 2-25-03

Text to reference: There will be 2 different exposure regimes: in one regime, the larvae will develop continuously from hatching to metamorphosis within the UV exposure chamber. In the second regime, they will be allowed to develop to NK stage 50 before being placed in the UV exposure system.

Change in Document: Larvae will be allowed to develop to NK stage 53 before being placed in the UV exposure system.

Justification and Impact on Study: Because *Xenopus* do not develop thyroid hormones until long after hatching, it was determined by the study director that an exposure beginning at hatch was unnecessary, and would have a higher probability of failure due to increased likelihood of death of the study subjects before termination of the experiment. This resulted in no significant impact on the study. Exposure of larvae to UV at a later stage of development (53 vs. 50) resulted in a higher survival during UV exposure, but did not impact effects on thyoid hormone response because both stages occur before onset of thyroid hormone production.

Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/21/03

Authorized by: Chris Theodorakis Study Director: Chris Theodorakis Date: 2/21/03

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*Change No: ___6_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE-02-03~~ LARV-02-01 WE MBB 2-24-03
Page #(s): _____5_____
Section #: _____~~14~~ 13 WE MBB 2-25-03

Text to reference: Each of the two exposure regimes will be performed separately (i.e., all beakers in the UV exposure chamber will contain either larvae that have 1) developed continuously under UV exposure or 2) developed in the laboratory until NF stage 50, and then placed in the UV exposure chamber). For the larvae exposed to UV under exposure regime #2 (allowed to develop to NF stage 50 before UV exposure), while the larvae are developing outside of the exposure chamber, the beakers will be placed on two shelves designed to hold aquaria, with each shelf being considered a block. The arrangement of beakers within blocks will be the same as described for the arrangement of beakers within the UV chamber. Larvae will be placed in the beakers in random order, within blocks, using the procedure described below.

Change in Document: Text deleted

Justification and Impact on Study: There was only one exposure regime, as described in change #5.

Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/21/03

Authorized by: Chris Theodorakis Study Director: Chris Theodorakis Date: 2/21/03

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*Change No: ___7_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE-02-03~~ LARV-02-01 WE mbb 2-24-03
Page #(s): _____5_____
Section #: _____~~14~~ 13 WE mbb 2-25-03

Text to reference: For the larvae, each block will consist of 18 beakers: 3 replicates per block, 3 perchlorate concentration per replicate, 2 beaker cover treatment per concentration (UV transparent or UV opaque). There will be 2 blocks for a total of 36 beakers. Each beaker will contain 15 larvae. Within each block, each beaker will be assigned a number from 1-18.

Change in Document: For the larvae, each block will consist of 9 beakers: 3 replicates per block, 3 perchlorate concentration per replicate, There will be 2 blocks (block 1 will contain beakers with UV transparent plastic covers, block 2 will contain beakers with UV opaque plastic covers). for a total of 18 beakers. Each beaker will contain 10 larvae. Each beaker will be assigned a number from 1-18.

Justification and Impact on Study: Preliminary studies indicated that it was physically and logistically not possible to perform the experiment with 36 beakers, and that 15 tadpoles per beaker produced overcrowding of the animals. Therefore, the number of beakers was reduced by ½ and the number of animals per beaker was reduced to 10. There was no significant impact on the outcome of the study.

Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/21/03

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Project No.: _____T9700_____
*Change No: ___8_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE 02-03~~ LARV-02-01 WE mAB 2-24-03
Page #(s): _____5_____
Section #: _____15.1_____

Text to reference: Larvae will be bred from captive adult stocks according to SOP,. Every other day, debris at the bottom of the beaker will be cleaned by suction. Water will be continuously aerated and filtered using mechanical and biological filtration. Water will consist of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts or other brands of identical composition. Animal husbandry will be according to SOP AQ-1-08.

Change in Document: Larvae will be bred from captive adult stocks according to SOP AQ-1-06 "Care and Maintenance of *Xenopus laevis*". Larvae will be maintained in FETAX (Frog Embryo Teratogenesis Assay—Xenopus) solution. Every other day, debris at the bottom of the beaker will be cleaned by suction and 50% exchanged with fresh solution. Exposure beakers and any aquaria will receive continuous aeration Animal husbandry will be according to SOP AQ-1-06. Staging of larvae will be according to SOP AQ-1-07 "Developmental Staging and Measurements for *Xenopus laevis* larvae".

Justification and Impact on Study: Preliminary studies indicated that these changes were more condusive to maintaining health and proper development of the animals. There was no significant impact on the outcome of the study.

Submitted by: Chris Theodorakis ~~Signature:~~ Chris Theodorakis Date: 2/21/03

Authorized by: Chris Theodorakis Study Director: Chris Theodorakis Date: 2/21/03

Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 2-25-03

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Project No.: _____T9700_____
*Change No: ___9_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE-02-03~~ LARV-02-01 WE mBB 2-24-03
Page #(s): _____5_____
Section #: _____15.2_____

Text to reference: Exposures will begin after larvae have hatched.

Change in Document: Perchlorate exposures will begin after larvae have reached NF stage 53.

Justification and Impact on Study: See change #5.

Submitted by: Chris Theodorakis Signature: Date: 2/21/03

Authorized by: Chris Theodorakis Study Director: Date: 2/21/03

Received by: Brian Birdwell Quality Assurance Unit: Date: 2-25-03

Brian Birdwell

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Project No.: _____T9700_____
*Change No: ___10_____
Page: __1___ of __2___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: __X__ Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE-02-03~~ LARV-02-01 WE MAB 2-24-03
Page #(s): _____5-6_____
Section #: _____15.3_____

Text to reference:

Test Material Application
The two beaker cover treatments will consist of: 1) beakers covered with square glass plates and 2) beakers covered squares of acetate. Both glass or acetate covers 10 cm x 10 cm. Larvae will be exposed to UV until after the emergence of forelimbs, but before complete tail resporbtion has occurred (NF stage xx).

Rates/concentrations: Larvae will be exposed to 10 and 100 ppb sodium perchlorate in water, and will be exposed to UV at a dose of 1 $J/m^2/s$.

Frequency: Larvae will be exposed to perchlorate in water until NF stage xx, and to UV from stage 40 to state xx.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker water.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because larvae respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification:
A sample of treated water will be tested by chemical analysis at least twice per week during the exposure.

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Change in Document:

Test Material Application

The two beaker cover treatments will consist of: 1) beakers covered with square tyrene plates and 2) beakers covered squares of polycarbonate. Both styrene or polycarbonate covers will measure 12 cm x 12 cm.

Rates/concentrations: Larvae will be exposed to 0.050 and 10 ppm sodium perchlorate in water, and will be exposed to UV at a dose of 58 $mJ/cm^2/d$.

Frequency: Larvae will be exposed to perchlorate in water from NF stage 53 until stage 60. Larvae will be exposed to UV from NF stage 53 to stage 60.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker water.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because larvae respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification:
A sample of treated water will be tested by chemical analysis once per week during the exposure.

Justification and Impact on Study: _ Preliminary experiments suggested that the new experimental procedures would work better. No exposure verification of perchlorate concentrations were conducted because 1) long backlogs for the use of analytical equipment would considerably delay the completion of this project and 2) previous experience with perchlorate exposures in fish and Xenopus have shown that acutal concentrations were very close to nominal. Therefore it was felt that delaying the completion of the study solely for

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Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/21/03

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Check One: ____ Amendment ___X_Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE-02-03~~ LARV-02-01 mab 2-24-03
Page #(s): _____6_____
Section #: _____15.5_____

Text to reference: At the end of the exposure, all larvae will be collected and placed in 3.8 L plastic ziplock bags, and placed on ice in a lightproof container. They will then be removed from the container, and a 1 cm section from the tip of the tail will be collected and frozen in liquid nitrogen. A 0.5 cm section of the tail will then be collected and placed in Bouin's fixative for histological analysis. The remainder of the carcass will preserved in 10% formalin for examination of limb/spinal deformities.

Change in Document: At the end of the exposure, all larvae will be collected and placed in 3.8 L plastic ziplock bags, and placed on ice in a lightproof container. They will then be removed from the container, and a 1 cm section from the tip of the tail will be collected and frozen in liquid nitrogen. The remainder of the carcass will preserved in 10% formalin for examination of limb/spinal deformities.

Justification and Impact on Study: The focus of this study is effects of perchlorate-induced thyroid disruption on UV-induced DNA damage and developmental abnormalities. Histological alterations of the skin were proposed as a mechanism whereby increased UV effects may occur. Because there was minimal or no effects of perchlorate on UV-induced DNA damage or developmental abnormalities, looking for mechanisms for such effects was not necessary. This is particularly true in light of the fact that such histological examinations are cost and labor intensive, so the return on such investments would be minimal. Thus, the resources originally allocated for histology of the skin in Xenopus were reallocated to other subprojects examining uptake of perchlorate and effects of perchlorate on reproduction in fish.

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Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 2-25-03

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*Change No: ___12_____
Page: __1___ of __1___

Change In Study Documentation Form

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Check One: ____ Amendment ___X_Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Larvae

Dated: 2/21/03
Document # (if appropriate): ~~LARVAE-02-03~~ LARV-02-01 (WE) MBB 2-24-03
Page #(s): _____6_____
Section #: _____15.5_____

Text to reference: Samples preserved in Bouin's fixative will be prepared according to SOP AQ-2-08 "Genera Histological Processing of Skin in *Xenopus* Tadpoles". Histological endpoints will include qualitative observations and descriptions of the relative amount of melanocytes, skin thickness, and occurrence of edema recorded in a bound laboratory notebook.

Change in Document: Delete text.

Justification and Impact on Study: _Histology of skin not done, see change 11.

Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/21/03

Authorized by: Chris Theodorakis Study Director: Chris Theodorakis Date: 2/21/03

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Project No.: T9700
Study No.: LARV-02-01

SOPs Referenced in the Protocol

1. AQ-1-23 Cleaning Glassware for use with Xenopus laevis
2. AQ-1-06 Care and Maintenance of Xenopus laevis
3. AQ-1-07 Exposure of Xenopus Laevis Eggs/Larvae to Test Substance(s)
4. IN-3-02 Sample Labeling/Logging Procedure
5. IN-3-10 Batching of Samples for Storage And/or Transportation
6. IN-1-05 Notebook/Data Form/Label Documentation
7. AC-2-11 Analysis of Perchlorate by Ion Chromatography (IC)
8. AQ-2-08 General Histological Processing of Skin in Xenopus Tadpoles
9. AQ-2-07 DNA Extraction from Fish or Amphibian Tissues
10. AQ-2-06 DNA Damage Assays Using Alkaline Electrophoresis

A FINAL REPORT

ENTITLED

FOOD CHAIN TRANSFER OF PERCHLORATE IN FISH

STUDY NUMBER:	FISH 02-03
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	04/15/2002
RESEARCH COMPLETION:	12/31/2002

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Table 7. Concentrations of perchlorate (mean ± SD) in water throughout the experiment in regards to the treatment and time of exposure. 15

GOOD LABORATORIES PRACTICES STATEMENT

Project FISH 02-03, entitled "Food Chain Transfer of Perchlorate in Fish", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

3/27/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase/Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	4-1-02	4-23-02	4-23-02	4-25-02
Final Report and Raw Data Review	1-15-03	1-31-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

03/27/03
Date

1. DESCRIPTIVE STUDY TITLE:
Food Chain Transfer of Perchlorate in Fish

2. STUDY NUMBER:
FISH 02-03

3. SPONSOR:
United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start: 04/15/2002
Termination: 12/31/2002

6. KEY PERSONNEL:
Ron Kendall, Principal Investigator
Christopher Theodorakis, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Brian Birdwell, Quality Assurance Officer
Jacques Rinchard, Postdoctoral research Associate
June-Woo Park, Graduate Student

7. STUDY SUMMARY:
Two experiments were conducted to evaluate the food chain transfer of perchlorate in fish. In experiment 1, bluegill sunfish were exposed to 100 ppm sodium perchlorate for 2 days via different routes: food, water, or by a combination of food and water. The food consisted of mosquitofish exposed to 100 ppm sodium perchlorate in the water for 2 days. Our results indicated that bluegill sunfish fed mosquitofish exposed to perchlorate (food only) did not accumulate perchlorate in their tissues. In contrast, perchlorate was detected in bluegill sunfish exposed to sodium perchlorate in the water (100 ppm) and in the combination exposure (water and food). We hypothesized that the feeding period was too short and the exposure concentration was too low to allow transfer of perchlorate through the food chain. In experiment 2, the feeding period was extended (up to 30 days) and the exposure concentration increased (1000 ppm for fathead minnows and 500 ppm for largemouth bass). Fathead minnows were exposed to 1000 ppm sodium perchlorate for 2 days and then were fed every other day to largemouth bass. Perchlorate body burden concentrations in fathead

minnows throughout the experiment averaged 51.5 ± 12.2 ppm. The body burden concentrations of perchlorate in largemouth bass were measured after 1, 10 and 30 days of feeding. Our results indicated that food chain transfer of perchlorate occurred already after 1 day of feeding. Moreover, fish exposed to perchlorate via food and water presented higher concentration of perchlorate than fish exposed to water only.

8. STUDY OBJECTIVES / PURPOSE:

To determine contribution of food vs. water in uptake of perchlorate in fish.

9. TEST MATERIALS:

Test Chemical: Sodium Perchlorate
CAS Number: 7601-89-0
Characterization: Determination of concentration in environmental samples
Source: Aldrich Chemical Science

Reference Chemical: Ultra-pure water with added sea salts ("Instant Ocean®")
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultra-pure water. 60 mg/L sea salts ("Instant Ocean®") were added.

10. JUSTIFICATION OF TEST SYSTEM:

Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, fish and wildlife population stability as well as human health. However, the transfer of perchlorate through the food chain in this species has not been investigated. Bluegill sunfish and largemouth bass were used as test species because they are native Texas species. Mosquitofish and fathead minnows were used as food item for bluegill, because they are natural prey items of bluegill and bass. After review of the data from an initial experiment using bluegill sunfish as the predator and mosquitofish as the prey, it was decided that the species used would be switched to largemouth bass and fathead minnows. The minnows were chosen because they were more readily available than mosquitofish, and bass were chosen because the minnows were larger than the mosquitofish, so a larger predator was needed. It was also decided to increase the concentration of perchlorate to which predator and prey were exposed in order to ensure detectable levels of perchlorate in fish tissues. Furthermore, the exposure period of the bass was increased to allow more time for the bass to assimilate perchlorate from their food.

11. TEST ANIMALS:

Species: *Gambusia spp.*, mosquitofish; *Lepomis macrochirus* bluegill sunfish; *Pimephales promelas*, fathead minnows; *Micropterus salmoides*, largemouth bass
Strain: Wild type
Age: Juveniles

Number: 30 bluegill, 800 mosquitofish, 40 largemouth bass, 2000 fathead minnows
Source: Purchased from hatcheries

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Aquaria were labeled with the aquaria number, species name, animal use protocol number, project number, test system, date of exposure and date of collection, concentration, and person responsible.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

13.1. Bluegill and mosquitofish

Bluegill were exposed to 100 ppm sodium perchlorate for 2 days. There were 3 treatments for the bluegill: exposed to perchlorate via the food, via the water, or by a combination of food and water. The food consisted of mosquitofish exposed to 100 ppm sodium perchlorate in the water for 2 days prior to feeding to the fish. Fish were placed into precleaned aquaria (1 bluegill per aquarium). Aquaria were cleaned by washing each aquarium according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays". For exposures, aquaria were located on shelves capable of supporting such weight. Each shelf held five 20L aquaria. A random number generator determined arrangement of the aquaria.

13.2. Largemouth bass and fathead minnows

Bass were exposed to 500 ppm sodium perchlorate for 1, 10, or 30 days. There were 3 treatments for the bass: exposed to perchlorate via the food, via the water, or by a combination of food and water. The food consisted of fathead minnows exposed to 1000 ppm sodium perchlorate in the water for 2 days prior to feeding to the bass. Bass were placed into precleaned aquaria (1 bass per aquarium). Aquaria were cleaned by washing each aquarium according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays". For exposures, aquaria were located on shelves capable of supporting such weight. Each shelf held five 20L aquaria. A random number generator determined arrangement of the aquaria.

14. METHODS

14.1. Test System acquisition, quarantine, acclimation

Fish were obtained from fish hatcheries. Upon arrival to the lab, they were treated with commercially available antibiotics for 5 days, as instructed by the manufacturer. After five days, any debris at the bottom of the tank and 1/3 of the tank water were cleaned and replaced with our fish water. Every other day, debris at the bottom of the tank were cleaned by suction. Water was continuously aerated and filtered using mechanical and biological filtration. Water consisted of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean® salts. Animal husbandry was conducted according to SOPs AQ-1-08, "General Fish Husbandry" and AQ-01-09 "Mosquitofish *Gambusia Spp.*Husbandry". Total acclimatization period was one week. Once acclimated, fish were exposed to sodium perchlorate via the water or via the food for 2 days.

14.2. Test Condition Establishment

Exposures were begun after fish had become acclimatized.

14.3. Test Material Application

14.3.1. Bluegill and mosquitofish

Bluegill were weighed in a tared beaker filled with water prior to being placed in the aquaria. The weights were recorded in laboratory notebooks. The three treatments were as follows: 1) Water only: Bluegill were placed into aquaria containing 100 ppm sodium perchlorate for 2 days. 2) Food only: bluegill were placed in aquaria with clean water, and fed mosquitofish (in the amount of 5% of the body weight of the bluegill per day) once per day. The bluegill were fed by placing the mosquitofish in the aquaria with the bluegill and allowing the bluegill to feed on them. Mosquitofish were weighed in tared weighboats filled with water prior to being fed to the bluegill. All mosquitofish to be used in one day of the study (at least 200) were placed in a single 80L aquarium and exposed to sodium perchlorate for 2 days before being fed to the bluegill. Because this was a 2-day exposure, the mosquitofish were exposed in 2 different tanks on consecutive days. I.e. for the first group of mosquitofish (at least 200 individuals) exposure to perchlorate started on day 1 and they were fed to the bluegill on day 3. For the second group of mosquitofish (at least 200 individuals), fish were exposed to perchlorate in a second aquarium starting on day 2 and were fed to the same bluegill on day 4. 3) Food and water: Bluegill were exposed to perchlorate via the water and fed perchlorate-exposed mosquitofish for 2 days following the methods outlined above. At the end of the exposure, all bluegill were collected for analysis of perchlorate. In addition, a composite sample of mosquitofish weighing at least 5 g were taken from each tank after being exposed for 2 days, and these fish were analyzed for perchlorate. Perchlorate was analyzed in whole bodies. Because this was only a 2-day exposure, the water was not changed and the mosquitofish were not fed for the duration of the exposure. However, water samples in the bluegill tanks and mosquitofish tanks were collected and analyzed for perchlorate.

Rates/concentrations: Fish were exposed to 100 ppm sodium perchlorate in water or in contaminated food.

Frequency: Bluegill were exposed to perchlorate in food, water, or food plus water for 2 days.

Route/Method of Application: Route was via dermal, dietary, and respiratory exposure as the chemical was in the aquaria water.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live, and absorb chemicals from their food.

Exposure Verification: A sample of treated water was tested by chemical analysis during

each day of the exposure.

14.3.2 Largemouth bass and fathead minnows

Bass were weighed in a tared beaker filled with water prior to being placed in the aquaria. The weights were recorded in laboratory notebooks. The three treatments were as follows: 1) Water only: bass were placed into aquaria containing 500 ppm sodium perchlorate for 1, 10, or 30 days. 2) Food only: bass were placed in aquaria with clean water, and fed minnows (in the amount of approximately 10% of the body weight of the bass each feeding period) once every other day. The bass were fed by placing the minnows in the aquaria with the bass and allowing the bass to feed on them. Minnows were weighed in tared weighboats filled with water prior to being fed to the bass. All minnows that were used in one day of the study (at least 200) were placed in a single 80L aquarium and exposed to sodium perchlorate for 2 days before being fed to the bass (each tank of minnows constitutes a "batch"). Because this was a 2-day exposure, a new batch of minnows was exposed to perchlorate beginning every other day. I.e. for the first group of minnows (at least 200 individuals), exposure to perchlorate started on day 1 and they were fed to the bass on day 3. The second group of minnows (at least 200 individuals) were exposed to perchlorate in a second aquarium starting on day 3 and were fed to the same bass on day 5. 3) Food and water: Bass were exposed to perchlorate via the water and fed perchlorate-exposed minnows for 2 days following the methods outlined above. At the end of the exposure, all bass were collected for analysis of perchlorate. In addition, composite samples of minnows, each weighing at least 5 g, were taken from 13 separate batches and analyzed for whole body perchlorate concentrations. For the 10 and 30 day exposures, the water was changed every other day. The minnows were not fed during the 2-day exposures. Water samples in the bass tanks and minnows tanks were collected and analyzed for perchlorate. The 1, 10 and 30 day exposures were run concomitantly, rather than consecutively.

Rates/concentrations: Bass were exposed to 0 or 500 ppm sodium perchlorate in water. Some bass were exposed to perchlorate in water only, others were exposed to perchlorate in both water and food. Concentration of perchlorate in minnows fed to the bass is reported in section 17.2, below. Minnows were exposed to perchlorate in water only.

Frequency: Bass were exposed to perchlorate in food, water, or food plus water for 1, 10 or 30 days.

Route/Method of Application: Route was via dermal, dietary, and respiratory exposure.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live, and absorb chemicals from their food.

Exposure Verification: A sample of treated water was tested by chemical analysis during each day of the exposure.

14.4. Test System Observation

Aquaria were observed on a daily basis. The number of individuals that expired each day was recorded for each perchlorate concentration as well as any abnormal behavior. In addition, pH, dissolved oxygen, conductivity, temperature, and ammonia were determined at least 3 times per week.

14.5. Animal Sacrifice and Sample Collections

After exposure for 2 days, all bluegill and composite samples of mosquitofish and minnows were weighed, sacrificed with an overdose with MS-222, and frozen in liquid nitrogen. These procedures were also followed for bass after 1, 10, and 30 days of exposure, as outlined in 14.3.2. An overdose of MS222 consisted of immersing the animal in 1 g/L MS-222 for at least 60 seconds until gill ventilation ceased, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Small Amphibians and Fish". Individuals were wrapped in aluminum foil and frozen by immersion in liquid nitrogen. Perchlorate concentration was determined as described in section 14.6.

Labeling: samples were labeled with a unique ID number according to the following scheme: For bluegill: LPFB (laboratory perchlorate food chain bluegill)-sample number-treatment. Treatments were designated by a 2-letter abbreviation as follows: WO was for exposure through water only; FO for exposure through food only; FW for exposure through food and water. E.g., LPFB-0001-FO was bluegill # 0001 exposed to perchlorate through food (contaminated mosquitofish) only. The mosquitofish composite samples were labeled as LPFM-0001, LPFM-0002, etc.

Information included on the label was project number and unique ID (SOP IN-03-02 Sample Labeling/Logging Procedure), date collected, elimination time period, exposure concentration. All of this information was recorded on the fish dissection/tissue collection sheet. Fish weight as well as the standard length for the catfish, were also recorded. Any information not determined was entered as "ND".

14.6. Endpoint Analysis

Whole body extraction of perchlorate was performed according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Quantification of perchlorate in water and tissue extracts were performed according to SOP AC-2-11 "Analysis of Perchlorate by IC". The endpoint was whole body concentration of perchlorate.

15. STATISTICAL METHODS

Mean values and standard deviations (SD) were calculated for each group test. Differences among groups were tested using one way and two-way analysis of variance (ANOVA) followed by the Tukey multiple comparisons test. Normality and homogeneity of variances of the data were verified prior to analysis.

16. PROTOCOL CHANGES/REVISIONS:

See attached change in study documentation forms.

17. RESULTS

17.1. Bluegill sunfish and mosquitofish

Body burden concentrations of perchlorate in mosquitofish exposed to 100 ppm sodium perchlorate for 2 days and fed to bluegill sunfish are reported in Table 1. As reported in Table 2, bluegill sunfish fed mosquitofish exposed to perchlorate (food only) did not accumulate perchlorate in their tissues. In contrast, perchlorate was detected in bluegill sunfish exposed to sodium perchlorate in the water (100 ppm). Concentrations of perchlorate were also measured in fish tissues after the two days of exposure in for all treatments (Table 3) to ensure correct exposure concentrations.

Table 1: Concentrations of perchlorate (mean ± SD) in mosquitofish exposed to 100 ppm sodium perchlorate for 2 days and offered as food to bluegills sunfish at days 1 and 2.

Feeding time	Concentration (ppm)
Day 1	9.5 ± 1.3
Day 2	6.5 ± 0.1

n=2 (average of 2 composites)

Table 2: Body burden concentrations (mean ± SD) of perchlorate in bluegill sunfish exposed to sodium perchlorate for 2 days via water, food or by a combination of food and water. nd = not detected. Means with different letter are significantly ($P<0.05$) different from each other.

Treatment	Concentration (ppm)
Water only	5.6 ± 1.1[a]
Food only	nd[b]
Water and food	5.4 ± 1.3[a]

n=10 (average of 10 fish)

Table 3: Concentrations of perchlorate (mean ± SD) in fish tissues throughout the experiment.

Treatment[1]	Concentration (ppm)
Mosquitofish[2]	
0 ppm	0.04 ± 0.01
100 ppm	63.37 ± 2.37
Bluegill sunfish[3]	
Water only	0.004 ± 0.007
Food only	70.05 ± 3.28
Water and food	70.60 ± 2.79

[1]Mosquitofish exposed to clean water or perchlorate contaminated water, bluegill exposed to perchlorate through water, food, or both.
[2]n=2 (average of 2 samples collected at the end of exposure)
[3]n=10 (average of 10 samples collected at the end of exposure)

17.2. Largemouth bass and fathead minnows

A preliminary experiment was conducted to determine which concentrations of perchlorate should be used to ensure detectable levels of perchlorate in fish fed exposed prey. In this experiment, largemouth bass and fathead minnows were exposed to sodium perchlorate for 2 days (Table 4). This study revealed that fathead minnows should be exposed to 1000 ppm to ensure deposition of perchlorate in largemouth bass.

Based upon these results, a subsequent exposure using fathead minnows were exposed to 1000 ppm sodium perchlorate for 2 days and then were fed every other day to largemouth bass. Perchlorate body burden concentrations in 13 separate batches of fathead minnows (see 14.3.2) are reported in Table 5. Average concentration was 51.5 ± 12.2 ppm. Average perchlorate concentration in the water was lower than the nominal concentration (1000 ppm) (Table 5). The body burden concentrations of perchlorate in largemouth bass indicated that food chain transfer of perchlorate occurred after 1 day of feeding (Table 6). Moreover, fish exposed to perchlorate via food and water contained higher concentrations of perchlorate than fish exposed to water only. The two-way ANOVA indicated that there was a significant difference between the length of exposure and mode of exposure ($P < 0.05$). However, the interaction term was not significant. Finally, the concentration of perchlorate in the water throughout the experiment was measured (Table 7). The low perchlorate concentrations were reported in the "food only" aquaria are probably due to the elimination of perchlorate from the fathead minnows. The concentrations of perchlorate in the "water only" and "water and food" aquaria were below the nominal concentration (500 ppm), but were not significantly different from each other.

Table 4: Body burden concentrations (mean ± SD) of perchlorate in largemouth bass and

fathead minnows exposed to sodium perchlorate in water for 2 days.

Species and exposure concentration	Concentration (ppm)
Largemouth bass[1]	
100 ppm	1.5 ± 0.6
500 ppm	6.9 ± 2.5
Fathead minnows[2]	
1000 ppm	87.5 ± 19.8
5000 ppm	213.2 ± 15.9

[1]n=5 (average of 5 fish)
[2]n=5 (average of 5 composites)

Table 5: Concentrations of perchlorate (mean ± SD) in water and fathead minnows exposed to 1000 ppm sodium perchlorate for 2 days and offered as food to largemouth bass throughout the experiment.

Batch	Concentration (ppm)	
	Water[1]	Body burden[2]
1	618.9	35.2 ± 7.4
2	640.0	48.2 ± 18.1
3	668.1 ± 6.9	36.9 ± 16.4
4	788.9 ± 144.9	65.4 ± 32.7
5	686.9 ± 0.4	48.6 ± 8.5
6	684.6 ± 0.2	52.2 ± 13.4
7	678.1 ± 2.0	46.5 ± 9.1
8	710.9 ± 4.9	55.1 ± 7.7
9	680.3 ± 10.3	79.7± 11.8
10	675.2 ± 6.6	61.9
11	706.8 ± 0.8	41.8 ± 2.9
12	722.0 ± 5.8	44.8 ± 14.0
13	749.1 ± 19.4	53.7
Average	693.1 ± 44.2	51.5 ± 12.2

[1]n=2 (average of 2 water samples collected at the end of exposure, expect feeding 1 and 2 n=1)
[2]n=2 (average of 2 composites, except feeding 10 and 13 n=1)

Table 6: Body burden concentrations (mean ± SD; ppm) of perchlorate in largemouth bass

exposed to sodium perchlorate for 1, 10, and 30 days via water, food or by a combination of food and water. Means with different letter are significantly ($P<0.05$) different form each other.

Time of exposure (day)	Treatment		
	Water only	Food only	Water and food
1	8.8 ± 3.2^{a}	1.3 ± 0.6^{x}	11.1 ± 5.7^{w}
10	10.6 ± 4.3^{b}	3.1 ± 2.3^{y}	21.0 ± 4.3^{z}
30	15.2 ± 2.4^{b}	2.0 ± 0.8^{y}	21.9 ± 12.1^{z}

n=5 (average of 5 fish)

Table 7: Concentrations of perchlorate (mean ± SD) in water throughout the experiment in regards to the treatment and time of exposure.

Treatment and days of exposure	Concentration (ppm)
Water only	
1	381.5 ± 18.8
10	403.3 ± 19.0
30	401.6 ± 31.2
average	395.4 ± 24.2
Food only	
1	0.08 ± 0.13
10	0.17 ± 0.15
30	0.28 ± 0.36
average	0.18 ± 0.10
Water and food	
1	411.4 ± 26.7
10	442.5 ± 57.7
30	347.1 ± 20.5
average	400.1 ± 45.7

n=10 for day 1 (water collected on days 0 and 1), n=15 for day 10 (water collected on days 0, 6, and 10) and n=30 for day 30 (water collected on days 0, 6, 13, 18, 24, and 30)

18. DISCUSSION

The results of the first experiment (bluegill sunfish-mosquitofish) indicated that food chain transfer did not occur if the concentration of perchlorate is low in the prey fish and/or if the exposure period (feeding period of exposed fish) is reduced. However, when prey fish were exposed to higher dose of perchlorate (1000 ppm), a transfer of perchlorate was observed immediately after the first feeding. Moreover, it is important to notice that the body burden concentration of perchlorate in largemouth bass did not differ significantly between day 10 and day 30. Therefore, we concluded that transfer of perchlorate might occur only if prey

items contain high levels of perchlorate.

The concentrations of perchlorate used in the present study were significantly higher than the ones usually reported in the field samples (water and fish). However, fish collected from the field presented higher levels of perchlorate than the ones measured in the water where they were collected (Smith et al., 2001; Theodorakis et al., unpublished data). Thus, the present results indicate that water is not the only route of perchlorate uptake but food chain transfer may occur and be responsible for the higher concentration of perchlorate found in fish collected from the field.

The present results are also important to ecological risk assessments of perchlorate exposure as well as for input into models used in predicting fate and food chain transport of perchlorate in natural environments.

19. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20. REFERENCES:

Manzon, R.G., and Youson, J.H., 1997. The effects of exogenous thyroxine (T-4) or triiodothyronine (T-3), in the presence and absence of potassium perchlorate, on the incidence of metamorphosis and on serum T-4 and T-3 concentrations in larval sea lamprey (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Paz, D.A., Dezi, R.E., and Pisano, A., 1996. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

Smith, N.S., Theodorakis, C.W., Anderson, T.A., and Kendall, R.J., 2001. Preliminary assessment of pechlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. Ecotoxicology 10: 305-313.

21. APPENDICES:

Study Protocol
Changes to Study Documentation
List of Key Standard Operating Procedures

Project No. T9700

A STUDY PROTOCOL

ENTITLED

Food Chain Transfer of Perchlorate in Fish

STUDY NUMBER: FISH-02-03

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

ADMINISTRATOR: The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

TESTING FACILITY

Name/Address:

The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

PROPOSED EXPERIMENTAL
START DATE: April 15, 2002

1 **DESCRIPTIVE STUDY TITLE**: Food Chain Transfer of Perchlorate in Fish

2 **STUDY NUMBER:** FISH-02-03

3 **SPONSOR**: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4 **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

5 **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: (date of chemical application) April 15, 2002
Termination Date: (date of last data collected) September 31, 2002

6 **KEY PERSONNEL:**
Christopher Theodorakis, Study Director
Ronald Kendall, Testing Facility Management
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager

7 **DATED SIGNATURES:**

	4/23/02	Dr. Christopher Theodorakis Study Director
	4/26/02	Dr. Ronald Kendall Testing Facility Management
	25-Apr-02	Dr. Lou Chiodo Assistant Director for Science
	4/23/02	Mr. Ryan Bounds Quality Assurance Manager
	4-25-02	Dr. Todd Anderson Analytical Chemist

8 REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to TIEHH and to the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:

Dr. Ronald Kendall
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

9 STUDY OBJECTIVES / PURPOSE:

To determine contribution of food vs. water in uptake of perchlorate in fish.

10 TEST MATERIALS:

Test Chemical name: Sodium perchlorate
CAS number: 7601-89-0
Characterization: Determination of concentration in environmental samples.
Source: Aldrich Chemical Company

Reference Chemical name: (list any standards, positive or negative control materials) ultrapure water with added sea salts ("Instant Ocean®" or any other brand of sea salts with identical or nearly identical composition).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water. 60 mg/L salts will be added.

11 JUSTIFICATION OF TEST SYSTEM

Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently,

fish and wildlife population stability as well as human health. However, the transfer of perchlorate through the food chain in this species has not been investigated. Bluegill sunfish will be used as the test species because they are native Texas species. Mosquitofish will be used as a food item for bluegill, because they are a natural prey item of bluegill.

12 **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: *Gambusia spp.,* mosquitofish (any native Texas species weighing less than 1 g may be substituted for mosquitofish); *Lepomis macrochirus,* bluegill sunfish (any other species native to Texas weighing at least 30 g may be substituted for bullhead bluegill).

Strain: Feral organisms or bred in hatcheries

Age: Juveniles.

Number: 30 bluegill, 800 mosquitofish

Source: Captured in the wild, or purchased from hatcheries, Carolina Biological Supply or other commercial suppliers

13 **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**

The test system will consist of laboratory exposures constructed according to the experimental design described below. Wild fish will be identified in the field by the project manager or personel trained in the identification of such fish. Identity of all fish will be confirmed in the laboratory by visual inspection before tests are begun. Aquaria will be labeled with the aquaria number, species name, animal use protocol number, project number, test system, date of exposure and date of collection, concentration, and person responsible.

14 **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**

Fish will be exposed to 100 ppm of sodium perchlorate for 2 days. There will be 3 treatments for the bluegill: exposed to perchlorate via the food, via the water, or by a combination of food and water. The food will consist of mosquitofish exposed to 100 ppm sodium perchlorate in the water. Fish will be placed into precleaned aquaria (1 bluegill per aquaria). Aquaria/beakers will be cleaned by washing each aquarium according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays." For exposures, aquaria will be located on shelves capable of supporting such weight. Each shelf will hold five 20L aquaria. The experimental design will consist of a

randomized block design, with each shelf constituting a block. Determine arrangement of the aquaria or beakers within each block by a random number generator, random number table or by rolling dice. Each block will contain at least 1 beaker or aquarium of each treatment.

METHODS:

15.1 Test System acquisition, quarantine, acclimation

Fish will be obtained from the wild populations according to SOP AQ-3-05, commercial vendors, or fish hatcheries. They will be treated commercially available antibiotics for 5 days, as instructed by the manufacturer. After five days, any debris at the bottom of the tank and 1/3 of the tank water will be cleaned and replaced with our fish water. Every other day, debris at the bottom of the tank will be cleaned by suction. Water will be continuously aerated and filtered using mechanical and biological filtration. Water will consist of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts or other brands of identical composition. Animal husbandry will be according to SOPs AQ-1-08, "General Fish Husbandry" and AQ-01-09 "Mosquitofish Husbandry". Total acclimatization period will be a minimum of one week. Once acclimated, fish will be exposed to sodium perchlorate dissolved in water for 2 days. They will then be placed into clean water for elimination of perchlorate anion.

15.2 Test Condition Establishment

Exposures will begin after fish have become acclimatized.

15.3 Test Material Application

Bluegill will be weighed in a tared beaker filled with water prior to being placed in the aquaria. The weights will be recorded in laboratory notebooks or data sheets. The three treatments will be as follows: 1) Water only: Bluegill will be placed into aquaria containing 100 ppm sodium perchlorate for 2 days. 2) Food only: bluegill will be placed in aquaria with clean water, and fed mosquitofish (in the amount of 5% of the body weight of the bluegill per day) once per day. The bluegill will be fed by placing the mosquitofish in the aquaria with the bluegill and allowing the bluegill to feed on them. The mosquitofish will weigh approximately 0.19-0.20 g, and the bluegill will weigh approximately 25-35 g. Mosquitofish will be weighed in tared weighboats filled with water prior to being fed to the bluegill. All mosquitofish to be used in one day of the study (at least 200) will be placed in a single 80L aquarium and exposed to sodium perchlorate for 2 days before being fed to the bluegill. Because this will be a 2-day exposure, the mosquitofish will be exposed in 2 different tanks on consecutive days. I.e. for the first group of mosquitofish (at least 200 individuals) exposure to perchlorate will start on day 1 and they will be fed to the bluegill on day 3. For the second group of mosquitofish (at least 200 individuals) they will be exposed to perchlorate in a second aquarium starting on day 2 and will be fed to the same bluegill on day 4. 3) Food and

water: Bluegill will be exposed to perchlorate via the water and fed perchlorate-exposed mosquitofish for 2 days following the methods outlined above. At the end of the exposure, all bluegill will be collected for analysis of perchlorate. In addition, a composite sample of mosquitofish weighing at least 5 g. will be taken from each tank after being exposed for 2 days, and these fish will be analyzed for perchlorate. Perchlorate will be analyzed in whole bodies. Because this is only a 2-day exposure, the water will not be changed and the mosquitofish will not be fed for the duration of the exposure. However, water samples in the bluegill tanks and mosquitofish tanks will be collected and analyzed for perchlorate.

Rates/concentrations: Fish will be exposed to 100 ppm sodium perchlorate in water or in contaminated food..

Frequency: Bluegill will be exposed to perchlorate in food, water, or food plus water for 2 days.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker/aquaria water.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live, and absorb chemicals from their food..

Exposure Verification:
A sample of treated water will be tested by chemical analysis during each day of the exposure.

15.4 Test System Observation

Tanks or beakers will be observed on a daily basis. The number of individuals that expire each day will be recorded for each perchlorate concentration. In addition, pH, dissolved oxygen, conductivity, temperature, and any other water chemistry parameters deemed appropriate by the project manager will be determined at least 3 times per week.

15.5 Animal Sacrifice and Sample Collections

After exposure for 2 days, all bluegill and composite samples of mosquitofish will be weighed, sacrificed with an overdose with MS 222, and frozen in liquid nitrogen. An overdose of MS222 will consist of immersing the animal in 1 g/L MS222 for at least 60 seconds after all gill ventilation has ceased, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Small Amphibians and Fish". Individuals will be wrapped in aluminum foil or placed in cryogenic tubes suitable for liquid-phase liquid nitrogen and

will be frozen by immersion in liquid nitrogen. Perchlorate concentration will be determined as described in section 15.6.

Fish will be anesthetized in 1.5 g/L MS222 until the animal loses righting reflex and does not respond to physical stimuli, but before gill ventilation ceases, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Small Amphibians and Fish".

Labeling: samples will be labeled with a unique ID number according to the following scheme:

For bluegill: LPFB (laboratory perchlorate food chain bluegill)– sample number-treatment. Treatments will be designated by a 2-letter abbreviation as follows: WO is exposure through water only; FO is exposure through food only; FW is exposure through food and water. e.g., LPFB-0001-FO is bluegill # 0001.exposed to perchlorate through food (contaminated mosquitofish) only.

The mosquitofish composite samples will be labeled as LPFM-0001, LPFM-0002 .etc.

Minimum information to be included on the label is project number and unique ID (SOP IN-03-02 Sample Labeling/Logging Procedure). Additional information can include treatment, species, date collected, in decreasing order of importance. All of this information should be recorded on the fish dissection/tissue collection sheet and/or bound laboratory notebook. Fish weight should also be recorded, and standard length (from the tip of the nose to the end of the caudal peduncle) may also be included on this form or in the notebook. Any information not determined should be entered as "ND".

15.6 Endpoint Analysis

Tissue or whole body extraction of perchlorate will be performed according to SOP AC-2-15 "Extraction and Cleanup of Tissue samples to be Analyzed for Perchlorate Using Ion Chromatography". Analysis of water and of tissue extracts will be performed according to SOP AC-2-11 "Analysis of Perchlorate by IC". The endpoint will be tissue/whole body concentration of perchlorate.

15 PROPOSED STATISTICAL METHODS

To statistically determine differences between treatments in terms of perchlorate body concentrations, 1-way ANOVA will be used to determine effects of concentration. If warranted by lack of normality and/or homogeneity of variances of the data, nonparametric 2-way (e.g. Friedman test) and correlation/regression will be performed.

16 REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include: Room temperature and water temperature, dissolved oxygen, salinity, and pH will be collected. Date, time, and amount of feedings per tank

will be recorded. Relative tissue distribution in bluegill, mosquitofish, and water, and relationship between perchlorate body burden and exposure concentration will be included in the report.
Report content will include presentation of data, interpretation, and discussion of the following endpoints:

List individual endpoints and analyses.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

17 RECORDS TO BE MAINTAINED / LOCATION:

A draft of the final report will be delivered to the Sponsor on or before September 31, 2002. The final report will be delivered to the Sponsor on or before December 27, 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point, for final archive within six months of study completion (upon request). All data, the protocol, and a copy of the final report shall be maintained by the testing facility.

18 QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

19 PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and Test Facility Manager and maintained with the protocol and the Quality Assurance Unit.

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment ____Deviation __X__ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer
Dated: _____10/3/02_____
Document # (if appropriate): ____FISH-02-03_____
Page #(s): _____4_and 5_____
Section #: _____12__and ___15_____
Text to reference: ___
Section 12: Species: *Gambusia* spp., mosquitofish; *Lepomis macrochirus* bluegill sunfish
All section 15.3

Change in Document: __
Species: Largemouth bass Micropterus salmonides; fathead minnows: *Pimaphales promelas*

Largemouth bass will be weighed in a tared beaker filled with water prior to being placed in the aquaria. The weights will be recorded in laboratory notebooks or data sheets. The three treatments will be as follows: 1) Water only: largemouth bass will be placed into aquaria containing 500 ppm sodium perchlorate. Largemouth bass will be fed minnows at 10% of their body weight every other day throughout the experiment. 2) Food only: largemouth bass will be placed in aquaria with clean water, and fed minnows (in the amount of 10% of the body weight of the largemouth bass) every other day. All minnows to be used will be exposed to sodium perchlorate at 1000 ppm for 2 days before being fed to the largemouth bass. 3) Food and water will be exposed to perchlorate via the water (500 ppm) and fed perchlorate-exposed minnows for 2 days (1000 ppm) following methods outlined above. After 1, 10 and 30 days, largemouth bass will be collected for perchlorate analysis. In addition composite samples of minnows will be taken after each exposure to perchlorate. Perchlorate will be analyzed in whole bodies. Water will be change every other day and water quality checked once a week. Water samples will be collected throughout the experiment and analyzed for perchlorate.

Rates/concentrations: Fish will be exposed to 500 ppm sodium perchlorate in water and/or 1000 ppm in contaminated food.

Frequency: Largemouth bass will be exposed to perchlorate in food, water, or food plus water for 1, 10 and 30 days.

*** Sequentially numbered in order of the date that the change is effective**

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Form No. 014 Rev. 3.06/00
Project No.: _T9700 _____
*Change No: ___1_____
Page: _2____ of ___2__

Change In Study
Documentation Form

Route/Method of application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the aquarium water or food.

Justification and Impact on Study:

We were not able to detect any perchlorate in bluegills fed contaminated mosquitofish (100 ppm) for two days. Therefore, we decided to repeat the experiment and extend the feeding period to 30 days (with samplings on days 1, 10 and 30). We also increased the dose exposure 500 ppm for the largemouth bass and 1000 ppm for the fathead minnows.

Submitted by: Signature: J. RINCHARD Date: 10/3/02

Authorized by: Study Director: Date: 12/12/02

Received by: Quality Assurance Unit: Date: 12-20-02

* Sequentially numbered in order of the date that the change is effective

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Form No. 014 Rev. 3.06/00
Project No.: _____T9700_____
*Change No: ___2_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Food Chain Transfer of Perchlorate in Fish

Dated: 12/08/02
Document # (if appropriate): Fish 02-03
Page #(s): _____8_____
Section #: _____17_____
Text to reference: The final report will be delivered to the Sponsor on or before December 27, 2002.

Change in Document: The final report will be delivered to the Sponsor on 31 March 2003

Justification and Impact on Study: _____

Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/20/03

Authorized by: Chris Theodorakis Study Director: Date: 2/20/03

Received by: Brian Birdwell Quality Assurance Unit: Date: 2-21-03
Brian Birdwell

* Sequentially numbered in order of the date that the change is effective

Project No.: T9700
Study No.: FISH-02-03

SOPs Referenced in the Protocol

1. AQ-1-02 Cleaning Glassware and Aquaria for Perchlorate Assays
2. AQ-3-05 Fish and Amphibian Field Collection Methods
3. AQ-1-08 General Fish Husbandry
4. AQ-1-09 Mosquitofish (Gambusia spp.) Husbandry
5. AQ-1-03 MS-222 Anesthesia and Euthanasia of Amphibians and Fish
6. IN-3-02 Sample Labeling/Logging Procedure
7. AC-2-15 Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate using Ion Chromatography
8. AC-2-11 Analysis of Perchlorate by Ion Chromatography (IC)

A FINAL REPORT

ENTITLED

EFFECTS OF PERCHLORATE ON GROWTH, REPRODUCTION, AND SURVIVAL OF MOSQUITOFISH

STUDY NUMBER:	FISH 02-02
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	03/01/2002
RESEARCH COMPLETION:	12/31/2002

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GOOD LABORATORIES PRACTICES STATEMENT

Project FISH 02-02, entitled "Effects of Perchlorate on Growth, Reproduction, and Survival in Mosquitofish", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

3/27/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase/Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	3-7-02	3-17-02	3-17-02	4-22-02
Test Material Application	4-23-02	4-23-02	5-1-02	5-6-02
Test System Care and Application	4-23-02	4-23-02	5-1-02	5-6-02
Test Material Mixing Dosing	4-23-02	4-23-02	5-1-02	5-6-02
Final Report and Raw Data Review	1-9-03	1-31-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

03/23/03
Date

1. DESCRIPTIVE STUDY TITLE:

Effects of Perchlorate on Growth, Reproduction, and Survival in Mosquitofish

2. STUDY NUMBER:

FISH 02-02

3. SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. TESTING FACILITY NAME AND ADDRESS:

The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start: 03/01/2002
Termination: 12/31/2002

6. KEY PERSONNEL:

Ron Kendall, Principal Investigator
Christopher Theodorakis, Study Director
Renaldo Patino, Senior Scientist
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Brian Birdwell, Quality Assurance Officer
Jacques Rinchard, Postdoctoral research Associate
June-Woo Park, Graduate Student
Sandeep Mukhi, Graduate Student

7. STUDY SUMMARY:

To determine the effect of perchlorate on fish reproduction, adult mosquitofish were exposed to 0, 1, 10, and 100 ppm sodium perchlorate for 8 weeks. Growth and fecundity of mosquitofish females did not significantly ($P>0.05$) differed among treatments. In males, the incidence of macrophage aggregates in kidneys was positively associated with perchlorate exposure concentration and the difference was significant between the control (0 ppm) and 100 ppm groups.

Survival of mosquitofish fry exposed to 0, 100, 300, 500, 800, 1000, and 2000 ppm sodium perchlorate for 5 days were determined and the 5-days lethal concentration (LC_{50})was 404 ppm. Finally, growth of mosquitofish fry exposed to 0, 1, 10, and 100 ppm sodium perchlorate for 4 weeks. Weight gain and specific growth weight of mosquitofish fry were significantly ($P<0.05$) affected by the increasing levels of perchlorate. Fry exposed to 1 ppm

sodium perchlorate grew significantly faster than fry from the other groups.

8. STUDY OBJECTIVES / PURPOSE:

To determine the effects of perchlorate on reproduction of adult mosquitofish and growth and survival of mosquitofish fry.

9. TEST MATERIALS:

Test Chemical: Sodium perchlorate
CAS Number: 7601-89-0
Characterization: Determination of concentration in environmental samples
Source: EM Science

Reference Chemical: Ultra-pure water with added sea salts ("Instant Ocean®")
CAS Number: Not applicable
Characterization: Determination of pH and conductivity
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultra-pure water. 60 and 180 mg/L sea salts ("Instant Ocean®") were added for fry and adult exposure, respectively.

10. JUSTIFICATION OF TEST SYSTEM:

Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, fish and wildlife population stability as well as human health.

11. TEST ANIMALS:

Species: *Gambusia holbrooki*, mosquitofish
Strain: Bred in hatcheries
Age: Adults and fry less than 1 week old
Number: Approximately 120 mosquitofish (80 females and 40 males), and 750 fry
Source: Purchased from hatcheries

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Aquaria were labeled with the aquaria number, species name, animal use protocol number, project number, test system, date of exposure and date of collection, concentration, and responsible personnel.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Mosquitofish fry were exposed to five concentrations of sodium perchlorate plus a zero control for survival study, three concentrations of sodium perchlorate plus a zero control for growth study, and mosquitofish adults were exposed to three concentrations of sodium perchlorate plus a zero control for reproductive study. Fish were placed into precleaned aquaria or beakers. Aquaria and beakers were cleaned according to SOP AQ-1-02 "Cleaning

Glassware and Aquaria for Perchlorate Assays". For exposures, aquaria or beakers were located on shelves capable of supporting such weight. Each shelf held all the 1 L beakers (for the fry survival and growth studies) or four 15 L aquaria (for the adult reproductive study). The experimental design consisted of a randomized block design, with each shelf constituting a block for the adult exposures. The arrangement of the aquaria within each block was randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. A random number generator determined the arrangement of the aquaria within each block. Each block contained at least 1 aquarium of each treatment. Fish were placed in the aquaria in random order, within blocks, using the procedure described below.

For the adult mosquitofish, each block consisted of 4 aquaria, each with 4 females and 2 males. There were 5 blocks, for a total of 120 fish. All female fish were placed in one or more aquaria and all male fish were placed in one or more aquaria. Within each block, each aquarium was assigned a number from 1-4. A random number generator was used to randomly order the numbers 1-4, and this was done 4 times for the female fish and 2 times for the male fish: e.g., 3412, 4123, 4312, 4213 (female fish) 3421, 1342 (male fish). A random number table was used for this purpose. The fish were placed into the 4 aquaria in the 1st block according to this list of numbers. For example, one female fish was placed into aquarium 3, then aquarium 4, aquarium 1, and finally aquarium 2. A second female fish was placed in each aquarium in the order 4123. A third female fish was then placed in each aquarium in the order 4312. Finally a fourth female fish was paced in each aquarium in the order 4213. Male fish were placed in each aquarium in the order 3412, then 1342. This was then done for the other 4 blocks in the experiment.

For the fry mosquitofish, all beakers were on one shelf in random order. Ten fry were randomly assigned to each beaker in a similar manner as described above.

14. METHODS:

14.1. Test System acquisition, quarantine, acclimation

Fish were obtained from fish hatcheries. They were treated with commercially available antibiotics for 5 days, as instructed by the manufacturer. After five days, any debris at the bottom of the tank was removed and 1/3 of the tank water replaced with fresh water. Every other day, debris at the bottom of the tank was removed by suction. Water was continuously aerated and filtered using mechanical and biological filtration. Water consisted of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts. Animal husbandry was conducted according to SOPs AQ-1-08, "General Fish Husbandry" and AQ-01-09 "Mosquitofish Husbandry". Total acclimation period was one week. Once acclimated, fish were exposed to sodium perchlorate dissolved in water. Mosquitofish were fed commercial flake goldfish food at the rate of 5 mg per gram of fish, on a daily basis. Debris and uneaten food were removed from the bottom of the tank 1-3 hours post feeding.

Gravid females were obtained from hatcheries and fry were collected according to SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry". All females were placed in one 80 L aquaria and fry were collected for one week. Fry that were less than 1 week old were used for fry survival and growth experiments.

14.2. Test Condition Establishment

Exposures were begun after fish had become acclimatized.

14.3. Test Material Application

Fish were placed into beakers or aquaria containing various concentrations of sodium perchlorate, with 10 fish per beaker and 5 replicate beaker per treatment (for fry exposures) or 6 fish per aquaria and 5 replicate aquaria per treatment (for adult exposure). Stock solutions of 1 g/L, 10 g/L, 100 g/L and 1,000 g/L perchlorate in reconstituted fresh water were used to dose the fish. The mosquitofish aquaria were filled with 15 L of reconstituted fresh water, and the mosquitofish beakers were filled with 500 mL of reconstituted fresh water, and an appropriate amount of stock solution was added according to the desired concentration of the aquarium or beaker water. Every other day, debris was cleaned out of the aquaria and 1/3 of the water was replaced in each tank with undosed water (as described in 15.1.), and perchlorate stock solution was added to maintain the desired concentration.
For fry survival, ten fry were randomly assigned to each 500 mL beaker and immediately exposed to 0, 100, 500, 1000, 2000, and 3000 ppm sodium perchlorate for five days. Fish were fed according to SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry."
For fry growth, ten fry were randomly assigned to each 500 mL beaker and were weighed prior to placement in beakers. Fry were continuously exposed to 0, 1, 10, and 100 ppm sodium perchlorate in water for 4 weeks. Fish were fed according to SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry."
For adult reproduction, four females and two males were randomly assigned to each aquaria and continuously exposed to 0, 1, 10, and 100 ppm sodium perchlorate in water for six weeks in aquaria conditions of 18 h light, 6 h dark with water temperature at 25°C, 180 mg/L salinity, and 50% tank coverage with artificial plants. A 0.5 cm thick layer of activated charcoal was added to the filtration unit to remove any contaminants released from the artificial plants.

Rates/concentrations: Mosquitofish were exposed to 0, 100,500, 1000, 2000, and 3000 ppm (for fry survival) or 0, 1, 10, and 100 ppm (for fry growth and adult reproduction) sodium perchlorate in water.

Frequency: Five replicate tanks of each concentration were continually exposed for 5 days for determination of fry survival, 4 weeks for determination of fry growth, and 6 weeks for adult reproduction. Adult exposures were extended to 8 weeks (see attached change in study documentation form).

Route/Method of Application: Route was via dermal, oral, and respiratory exposure as the chemical was in the aquaria water.

Stock solutions for study were mixed in precleaned glass containers as indicated in SOP AQ-1-02. Stock solutions were made by dissolving sodium perchlorate in reconstituted fresh water (60 mg/L Instant Ocean® sea salts in ultrapure water, pH adjusted to 7.4 with 1N HCl or 1N NaOH, as appropriate). The appropriate amount of sodium perchlorate compound was weighed on a calibrated balance, and mixed into reconstituted fresh water. The pH was

checked on a calibrated pH meter (calibrate according to SOP IN-4-06) and adjusted as above. After 1/3 of the aquarium water had been removed and replaced (see above instructions), an appropriate amount of stock solution was added to adjust the concentration to the original value.

For the adult mosquitofish, the 10 g/L stock was used for the 1 ppm (1 mg/L) and 10 ppm (10 mg/L) treatments and the 100 g/L stock was used for the 100 ppm (100 mg/L) treatments. For example, if there was 15L water in a mosquitofish tank, 5L was removed and replaced every other day. For the aquaria that had the 1 ppm and 10 ppm, 0.0015 L (1.5 ml) of perchlorate stock solution was added to the 1 ppm aquarium and 0.015 L (15 ml) was added to the 10 ppm aquarium initially, and 0.5 mL and 5 mL of perchlorate stock solution were added following water changes (e.g., (15L x 10 mg/L)/10,000 mg/L = 0.015 L, one-third of this was replaced after each water change). Stock solutions were measured out in an appropriate pipette.

For the fry exposures, the 1 g/L stock was used for the 1 ppm (1mg/L), the 10 g/L stock was used for the 10 ppm (10 mg/L), the 100 g/L was used for the 100 ppm (100 mg/L), and the 1,000 g/L stock was used for the 1000 ppm (1000 mg/L), 2000 ppm (2000 mg/L), and 3000 ppm (3000 mg/L) treatments.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification: A sample of each concentration of treated water was collected on the first day of exposure and whenever animals were removed from the aquarium for analysis. Water samples were also collected periodically throughout the exposure period. The concentration of perchlorate was tested using ion chromatography.

14.4. Test System Observation

Aquaria and beakers were observed on a daily basis. The number of individuals that expired and any abnormal behavior was recorded each day for all treatments. In addition, pH, dissolved oxygen, conductivity, temperature, and ammonia were determined at least 3 times per week.

14.5. Animal Sacrifice and Sample Collections

Fry used to determine survival were euthanized with 1.0 g/L MS-222 and the number of surviving fry following exposure to sodium perchlorate was recorded. Fry used to determine growth rate were euthanized in 1.0 g/L MS-222 and the final weight were recorded. Adult mosquitofish were euthanized in 1.0 g/L MS-222 and the females were dissected. The ovaries were removed and the eggs were put in water. The egg volume was determined by measuring the change in water volume divided by the number of eggs. All mosquitofish were then either disposed or preserved for future analysis.

14.6. Endpoint Analysis

For fry survival, the endpoint was the number of survivors as after 5 day exposures. For fry growth, percent mass gain was used as the endpoint [(final weight-initial weight)/initial

weight] x 100. For adult reproduction, fecundity as expressed by the ratio of number of eggs to body weight was used as the endpoint. Histopathology of male testis and kidneys were performed after 8 weeks of perchlorate exposure. In males, histopathology of the gonads and the kidneys were examined to determine the effect of perchlorate exposure. Macrophages aggregates as well as clusters of necrotic cells in testes were the parameters used. Briefly, for the incidence of macrophage aggregates in the testis, we placed a 1-mm square grid containing 121 crosshairs over the center of a randomly chosen testicular section at a total magnification of 200. The section was chosen only according to the quality of the preparation (absence of damage to the section and adequate staining) starting with the first section of the first row on the slide preparation. We counted the numbers of crosshairs that fell on macrophage aggregates as well as those that, sometimes, fell outside the testis (the latter were subtracted from the total of 121). Percent incidence was determined by dividing the number of crosshairs over macrophage aggregates by 121 (minus those falling outside) and multiplying by 100. The same section and general analytical procedure were used to determine the incidence of necrosis, except that the grid was placed on the lower right portion of the testis since preliminary observations indicated that necrosis normally did not occur in the center of the testis but around the edges. The same general procedures for section selection and observations were used for determining the incidence of macrophage aggregates in the kidney. The right lobe of the kidney was chosen for the observations prior to data collection.

15. STATISTICAL METHODS:

Mean values and standard deviations (SD) were calculated for each group test. Percent data were transformed (arcsine) prior to statistical analysis. Homogeneity of variances was assessed and one-way ANOVA followed by Duncan's multiple range tests were used to determine differences between mean values. Statistical differences were considered significant at overall α of 0.05. The 5-day lethal concentration (LC_{50}) for mosquitofish fry was calculated using a nonlinear regression (logistic model).

16. PROTOCOL CHANGES/REVISIONS:

Adult exposures were extended to 8 weeks to allow the females to reach complete maturity. Histopathology of male testis and kidneys were performed after 8 weeks of perchlorate exposure. See attached changes in study documentation forms.

17. RESULTS:

17.1. Effects of perchlorate on mosquitofish reproduction

Analysis of perchlorate in water during the experiment revealed that the actual concentrations of perchlorate in the different treatments were close to the nominal concentrations (Table 1). Survival of mosquitofish females did not significantly differ among perchlorate exposure doses and reached 85, 95, 75 and 80% for 0, 1, 10, and 100 ppm, respectively. Standard length, weight and fecundity of mosquitofish females did not significantly ($P>0.05$) differ among treatments (Table 2).

Table 1: Nominal and actual concentrations (ppm) of perchlorate in water throughout the perchlorate exposure.

Nominal	Actual (n=5)
0	0.004 ± 0.003
1	1.14 ± 0.04
10	8.30 ± 0.17
100	91.55 ± 2.92

Table 2: Survival, standard length, weight, and fecundity of mosquitofish females after 8 weeks of perchlorate exposure.

Dose (ppm)	n	Length (cm)	Weight (g)	Fecundity (# eggs/g of fish)
0	11	4.3 ± 0.3	0.82 ± 0.23	18.9 ± 8.1
1	14	4.2 ± 0.4	0.74 ± 0.20	28.4 ± 11.0
10	11	4.4 ± 0.3	0.88 ± 0.24	24.7 ± 9.2
100	12	4.3 ± 0.4	0.88 ± 0.29	27.1 ± 12.1

There was a tendency for testicular macrophage aggregates and focal necrosis to increase with increasing pechlorate exposure concentration, but this tendency was not statistically significant (Figure 1). In kidneys, the incidence of macrophage aggregates was positively associated with perchlorate exposure concentration and the difference was significant between the control (0 ppm) and 100 ppm groups (Figure 2).

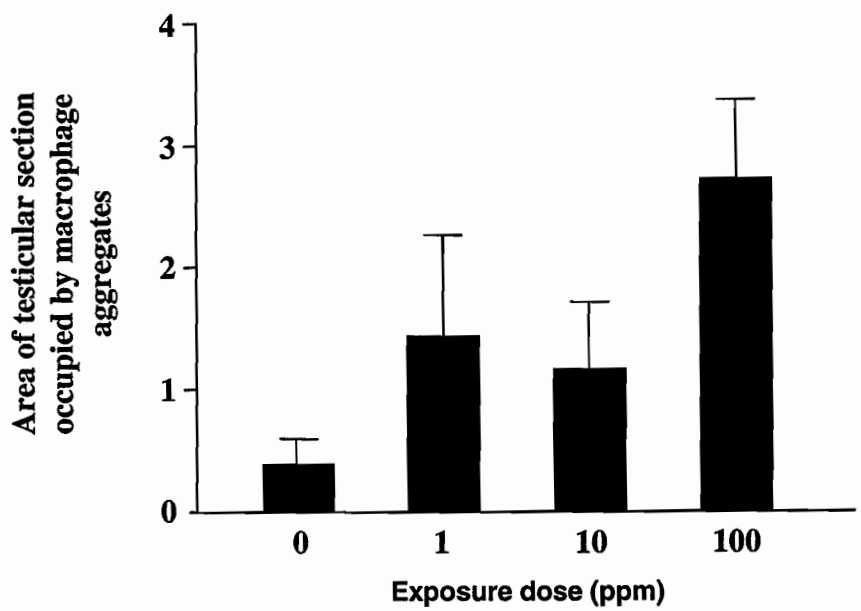

Figure 1: Incidence of macrophage aggregates in testis of mosquitofish exposed to perchlorate for 8 weeks.

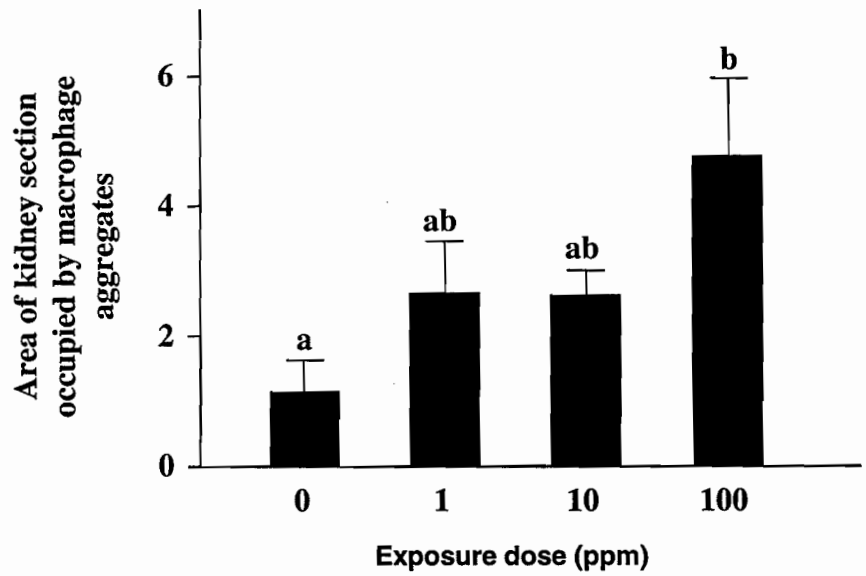

Figure 2: Incidence of macrophage aggregates in kidney of mosquitofish males exposed to perchlorate for 8 weeks. Means with different letter are significantly ($P<0.05$) different form each other.

17.2. Effects of perchlorate on fry survival

Analysis of perchlorate in water during the experiment revealed that the actual concentrations of perchlorate in the different treatments were close to the nominal concentrations, except for 800 ppm (Table 3). Survival of mosquitofish embryos decreased significantly ($P<0.05$) with the increasing doses of perchlorate (Figure 3). The 5-day lethal concentration calculated with actual perchlorate concentrations was estimated to be 404 ppm (LC_{50}).

Table 3: Nominal and actual concentrations (ppm) of perchlorate in water throughout the perchlorate exposure (n = 7 for 0 ppm and n = 5 for all perchlorate exposures).

Nominal	Actual
0	0.003 ± 0.004
100	81.57 ± 0.98
300	270.27 ± 7.84
500	425.73 ± 19.77
800	565.22 ± 13.08
1000	928.19 ± 43.32
2000	1802.85 ± 123.10

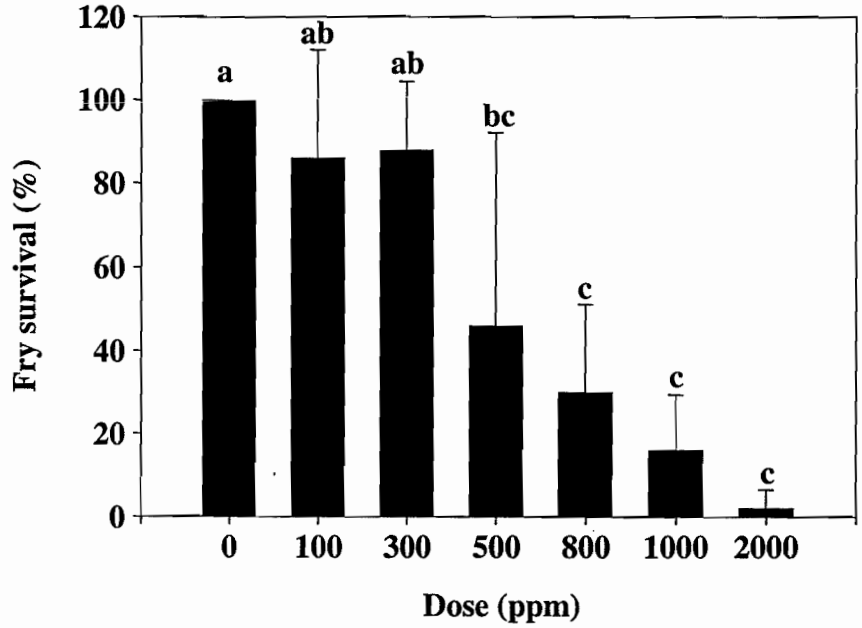

Figure 3: Survival of mosquitofish fry exposed to perchlorate for 5 days. Means with different letter are significantly (P<0.05) different form each other.

17.3. Effects of perchlorate on fry growth

Analysis of perchlorate in water during the experiment revealed that the actual concentrations of perchlorate in the different treatments were close to the nominal concentrations (Table 4). Weight gain and specific growth weight of mosquitofish fry were significantly (P<0.05) affected by the increasing levels of perchlorate in the water (Table 5). Fry exposed to 1 ppm sodium perchlorate grew significantly longer than fry from the other groups. No significant difference was observed between control fry and fry exposed to 100 ppm sodium perchlorate.

Table 4: Nominal and actual concentrations (ppm) of perchlorate in water throughout the perchlorate exposure.

Nominal	n	Actual
0	3	0.009 ± 0.015
1	5	0.80 ± 0.02
10	5	17.32 ± 3.78
100	5	98.73 ± 0.86

Table 5: Mass gain and specific growth rate (SGR) of mosquitofish fry exposed to perchlorate for 4 weeks.

Dose (ppm)	n	Mass gain[1] (%)	SGR[2] (%)
0	3	526. 7 ± 124.9[ab]	6.5 ± 0.8[a]
1	5	928.9 ± 104.8[c]	8.2 ± 0.4[b]
10	5	338.7 ± 23.6[a]	5.2 ± 0.2[c]
100	5	560.9 ± 140.0[b]	6.8 ± 0.8[a]

[1]Mass gain = [(final weight-initial weight)/initial weight] x 100
[2]SGR = [ln(final weight)–ln(initial weight)] x 100/period of exposure

18. DISCUSSION:

18.1. Effects of perchlorate on mosquitofish reproduction

The results of the present study indicate that perchlorate does not appear to affect fecundity of mosquitofish at the dosages associated with effects in the thyroid. Preliminary experiments indicated that 10 and 100 ppm perchlorate exposures induce an increase of the epithelial cell height as well as hyperplasia (Theodorakis et al., unpublished data). Patino et al. (in press) reported that at environmentally relevant concentrations (18 ppm), ammonium perchlorate does not affect the packed egg volume (index of spawning success) and the rate of natural egg fertilization (index of egg quality) of zebrafish. Those results are consistent with the ones reported by York et al. (2001). These authors indicated that although ammonium perchlorate did affect thyroid gland of rats, it has little effect on their reproductive performances. In contrast, ammonium perchlorate at high concentration (677 ppm) suppressed spawning activity of zebrafish (Patino et al., in press). However, these authors suggest that this response may have been due to toxic effects of perchlorate at sites other than the thyroid follicles.

The higher incidence of macrophage aggregates in kidney of mosquitofish males exposed to perchlorate indicates that perchlorate may affect kidney function. This observation is consistent with the results of earlier observations on correlations between the incidence of macrophage aggregates in kidney and exposure to environmental contaminants (Wolke, 1992). It is unknown if the effect of perchlorate is direct or indirect. Direct toxic effects of perchlorate on kidneys have been previously reported (e.g., Yen et al., 1973). However, receptors for thyroid-stimulating hormone (TSH) have been described in kidneys (Dutton et al., 1997; Sellitti et al., 2000), and thus it is possible that higher levels of TSH caused by perchlorate-induced hypothyroid conditions could also affect kidney function. Finally, the present observations of the kidneys of mosquitofish are consistent with those of zebrafish kidneys exposed to perchlorate (T. Capps, Masters Thesis in preparation). Preliminary observations with zebrafish indicate that perchlorate may also increase the incidence of kidney macrophage aggregates as well as other histopathologies. Although there was a tendency for higher incidences of macrophage aggregates and focal necrosis in the testis of

mosquitofish exposed to increasing concentrations of perchlorate, this tendency was not statistically significant. In our opinion, this observation is inconclusive. Most of the replicate tanks used for these analyses contained only one fish and we feel that a higher number of fish per replicate would have added more confidence to our results.

18.2. Effects of perchlorate on fry survival

Increasing levels of perchlorate significantly affected survival of mosquitofish fry. LC_{50} for 5 days perchlorate exposure was 404 ppm. In *Xenopus* larvae, the 5- and 70-d median lethal concentrations (LC50s) were 510 mg /L and 223 mg /L. respectively (Goleman et al., 2002). The LC_{50} concentration determined in the present study is high in comparison to the water concentrations found in the field (e.g., Longhorn Army Ammunition Plant, Karnack, Texas and Naval Weapons Industrial Reserve Plant, McGregor, Texas) (Smith et al., 2001; Theodorakis et al., unpublished data). Therefore, we concluded that perchlorate does not represent a direct threat to mosquitofish larvae survival in natural environments. However, an indirect effect through the alteration of the thyroid may be possible, as it has been shown that thyroid hormones are involved in the development of the larvae.

18.3. Effects of perchlorate on fry growth

Growth of fry mosquitofish was significantly enhanced when they were exposed to sodium perchlorate at 1 ppm. Moreover, there was no significant difference between growth of control fry and growth of fry exposed to 1000 ppm. Those results indicate that perchlorate does not represent a direct threat to mosquitofish larvae in natural environments.

19. STUDY RECORDS AND ARCHIVE:

Study Records were maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20. REFERENCES:

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21. APPENDICES:

Study Protocol
Changes to Study Documentation
List of Key Standard Operating Procedures

Project No. T9700

A STUDY PROTOCOL

ENTITLED

Effects of Perchlorate on Growth, Reproduction, and Survival of Mosquitofish

STUDY NUMBER: FISH-02-02

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

ADMINISTRATOR: The Institute of Environmental and Human Health
Texas Tech University/Texas Tech University Health Sciences Center
PO Box 41163
Lubbock, Texas 79409-1163

TESTING FACILITY

Name/Address:

The Institute of Environmental and Human Health
Texas Tech University/Texas Tech University Health Sciences Center
PO Box 41163
Lubbock, Texas 79409-1163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

PROPOSED EXPERIMENTAL
START DATE: MARCH 1, 2002

1. **DESCRIPTIVE STUDY TITLE:** Effects of Perchlorate on Growth, Reproduction, and Survival in Mosquitofish

2. **STUDY NUMBER:** FISH-02-02

3. **SPONSOR:** United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: (date of chemical application) March 1, 2002

Termination Date: (date of last data collected) September 31, 2002

6. **KEY PERSONNEL:**
Christopher Theodorakis, Study Director
Ronald Kendall, Testing Facility Management
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager

7. **DATED SIGNATURES:**

	3/17/02	Dr. Christopher Theodorakis Study Director
	4/26/02	Dr. Ronald Kendall Testing Facility Management
	22-APR-02	Dr. Lou Chiodo Asst. Director for Science
	4/17/02	Mr. Ryan Bounds Quality Assurance Manager

Todd Anderson 4-18-02 Dr. Todd Anderson
Analytical Chemist

8. **REGULATORY COMPLIANCE STATEMENT:**

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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Dr. Ronald Kendall
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

9. **STUDY OBJECTIVES / PURPOSE:**

To determine the effects of perchlorate on reproduction of adult mosquitofish and growth and survival of mosquitofish fry.

10. **TEST MATERIALS:**

Test Chemical name: Sodium perchlorate
CAS number: 77601-89-0
Characterization: Determination of concentration in environmental samples.
Source: EM Science

Reference Chemical name: ultrapure water with added sea salts ("Instant Ocean®" or any other brand of sea salts with identical or nearly identical composition).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water. 60 mg/L sea salts will be added for fry exposures and 180 mg/L sea salts will be added for breeding exposures.

11. **JUSTIFICATION OF TEST SYSTEM:**
Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, fish and wildlife population stability as well as human health.

12. **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: *Gambusia holbrooki*, mosquitofish

Strain: Feral organisms or bred in hatcheries

Age: Adults and fry less than 1 week old.

Number: Approximately 120 adult mosquitofish (80 female and 40 male) and 750 fry

Source: Captured in the wild, or purchased from hatcheries, Carolina Biological Supply or other commercial suppliers

13. **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**
The test system will consist of laboratory exposures constructed according to the experimental design described below. Wild fish will be identified in the field (upon capture) by the project manager or personel trained in the identification of such fish. Identity of all fish will be confirmed in the laboratory by visual inspection before tests are begun. Aquaria will be labeled with the aquaria number, species name, animal use protocol number, project number, test system, date of exposure and date of collection, concentration, and person responsible.

14. **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**
Mosquitofish fry will be exposed to five concentrations of sodium perchlorate plus a zero control for survival study, three concentrations of sodium perchlorate plus a zero control for growth study, and mosquitofish adults will be exposed to three concentrations of sodium perchlorate plus a zero control for reproductive study. Fish will be placed into precleaned aquaria or beakers. Aquaria and beakers will be cleaned by washing according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays". For exposures, aquaria or beakers will be located on shelves capable of supporting such weight. Each shelf will hold all the 1 L beakers (for the fry survival and growth studies) or four 20L aquaria (for the adult reproductive study). The experimental design will consist of a randomized block design, with each shelf constituting a block for the adult

exposures. The arrangement of the aquaria within each block will be randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. Determination of the arrangement of the aquaria or beakers within each block by a random number generator, random number table or by rolling dice. Each block will contain at least 1 aquarium or beaker of each treatment. Fish will be placed in the aquaria in random order, within blocks, using the procedure described below.

For the adult mosquitofish, each block will consist of 4 aquaria, each with 4 female and 2 male fish. There will be 5 blocks, for a total of 120 fish. All female fish will be placed in one or more aquaria and all male fish will be paced in one or more aquaria. Within each block, each aquarium will be assigned a number from 1-4. A random number generator will be used to randomly order the numbers 1-4, and this will be done 4 times for the female fish and 2 times for the male fish: e.g., 3412, 4123, 4312, 4213 (female fish) 3421, 1342 (male fish). A pair of dice, a random number table, or a computerized random number generator can be used for this purpose. The fish will be placed into the 4 aquaria in the 1st block according to this list of numbers. For example, one female fish will be placed into aquarium 3, then aquarium 4, aquarium 1, and finally aquarium 2. A second female fish will be placed in each aquarium in the order 4123. A third female fish will then be placed in each aquarium in the order 4312. Finally a fourth female fish will be paced in each aquarium in the order 4213. Male fish will be placed in each aquarium in the order 3412, then 1342. This will then be done for the other 4 blocks in the experiment.

For the fry mosquitofish, all beakers will be on one shelf in random order. Ten fry will be randomly assigned to each beaker in a similar manner as described above.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation

Fish will be obtained from the wild populations, commercial vendors, or fish hatcheries. If fish are captured in the wild, they will be transported back from the field in plastic buckets or other containers with constant aeration. Upon arrival to the lab, they will be treated commercially available antibiotics for 5 days, as instructed by the manufacturer. After five days, any debris at the bottom of the tank and 1/3 of the tank water will be removed using a siphon hose or electric pump and replaced with fresh water. Fresh water will consist of reverse osmosis (RO) water supplemented with 60 mg/L (for fry exposures) or 180 mg/L (for adult exposures) Instant Ocean sea salts or other brands of identical composition. Fresh water will be replaced in each tank by siphon or electric pump from a reservoir (e.g., 100 gallon aquarium). Each day, debris at the bottom of the tank will be cleaned by suction. Water will be continuously aerated and filtered using mechanical and biological filtration. Animal husbandry will be according to SOP AQ-1-08, "General Fish Husbandry" and SOP AQ-1-09 "Mosquitofish *Gambusia spp.*

Husbandry". Total acclimatization period will be a minimum of one week. Once acclimated, fish will be exposed to sodium perchlorate dissolved in water. Mosquitofish will be fed commercial flake goldfish food at the rate of 5 mg per gram of fish, on a daily basis. Debris and uneaten food will be removed from the bottom of the tank 1-3 hours post feeding.

Gravid females will be obtained from hatcheries and fry will be collected according to SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry". All females will be placed in one 80 L aquaria and fry will be collected for one week. Fry that are less than 1 week old will be used for fry survival and growth experiments.

15.2 **Test Condition Establishment**

Exposures will begin after fish have become acclimatized.

15.3 **Test Material Application**

Fish will be placed into beakers or aquaria containing various concentrations of sodium perchlorate, with 10 fish per beaker and 5 replicate beaker per treatment (for fry exposures) or 6 fish per aquaria and 5 replicate aquaria per treatment (for adult exposure). Stock solutions of 1 g/L, 10 g/L, 100 g/L and 1,000 g/L perchlorate in reconstituted fresh water will be used to dose the fish. The mosquitofish aquaria will be filled with 15 L of reconstituted fresh water, and the mosquitofish beakers will be filled with 1 L of reconstituted fresh water, and an appropriate amount of stock solution will be added according to the desired concentration of the aquarium or beaker water. Every other day, debris will be cleaned out of the aquaria and 1/3 of the water will be replaced in each tank with undosed water (as described in 15.1), and perchlorate stock solution will be added to maintain the desired concentration.

For fry survival, ten fry will be randomly assigned to each 1 L beaker and immediately exposed to 0, 100, 500, 1000, 2000, and 3000 ppm sodium perchlorate for five days. Fish will be feed according to SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry."

For fry growth, ten fry will be randomly assigned to each 1 L beaker and will be weighed prior to placement in beakers. Fry will be continuously exposed to 0, 1, 10, and 100 ppm sodium perchlorate in water for 4 weeks. Fish will be fed according to SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry."

For adult reproduction, four females and two males will be randomly assigned to each aquaria and continuously exposed to 0, 1, 10, and 100 ppm sodium perchlorate in water for six weeks in aquaria conditions of 18 h light, 6 h dark with water temperature at 25°C,

180 mg/L salinity, and 50% tank coverage with artificial plants. A 0.5 cm thick layer of activated charcoal will be added to the filtration unit to remove any contaminants released from the artificial plants.

Rates/concentrations: Mosquitofish will be exposed to 0, 100,500, 1000, 2000, and 3000 ppm (for fry survival) or 0, 1, 10, and 100 ppm (for fry growth) or 0, 1, 10, and 100 ppm (for adult reproduction) sodium perchlorate in water.

Frequency: Five replicate tanks of each concentration will be continually exposed for 5 days for determination of fry survival, 4 weeks for determination of fry growth, and 6 weeks for adult reproduction. Exposure periods may be extended if initial results warrant.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker/aquaria water.

Stock solutions for study will be mixed in precleaned glass containers as indicated in SOP AQ-1-02. Stock solutions will be made by dissolving sodium perchlorate in reconstituted fresh water (60 mg/L Instant Ocean® sea salts or equivalent, in ultrapure water, pH adjusted to 7.4 with 1N HCl or 1N NaOH, as appropriate). The appropriate amount of sodium perchlorate compound will be weighed on a calibrated balance, and mixed into reconstituted fresh water. The pH will be checked on a calibrated pH meter (calibrate according to SOP IN-4-06) and adjusted, if necessary, as above. After 1/3 of the aquarium water has been removed and replaced (see above instructions), an appropriate amount of stock solution will be added to adjust the concentration to the original value.

For the adult mosquitofish, the 10 g/L stock will be used for the 1 ppm (1 mg/L) and 10 ppm (10 mg/L), treatments and the 100 g/L stock will be used for the 100 (100 mg/L) treatments. For example, if there is 15L water in a mosquitofish tank, 5L will be removed and replaced every other day. For the aquaria that have the 1 ppm and 10 ppm, 0.0015 L (1.5 ml) of perchlorate stock solution will be added to the 1 ppm aquarium and 0.015 L (15 ml) will be added to the 10 ppm aquarium initially, and 0.5 mL and 5 mL of perchlorate stock solution will be added following water changes (e.g., (15L x 10 mg/L)/10,000 mg/L = 0.015 L, one-third of this will be replaced after each water change). Stock solutions will be measured out in an appropriate pipette.

For the fry exposures, the 1 g/L stock will be used for the 1 ppm (1mg/L), the 10 g/L stock will be used for the 10 ppm (10 mg/L), the 100 g/L will be used for the 100 ppm (100 mg/L), and the 1,000 g/L stock will be used for the 1000 ppm (1000 mg/L), 2000 ppm (2000 mg/L), and 3000 ppm (3000 mg/L) treatments.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification: A sample of each concentration of treated water will be collected on the first day of exposure and whenever animals are removed from the aquarium for analysis. Water samples will also be collected periodically throughout the exposure period (at least once per week). The concentration of perchlorate in the water will be tested using ion chromatography.

15.4 **Test System Observation**

Tanks or beakers will be observed on a daily basis. The number of individuals that expire each day will be recorded for each perchlorate concentration as well as any abnormal behavior. In addition, pH, dissolved oxygen, conductivity, temperature, ammonia, and any other water chemistry parameters deemed appropriate by the project manager will be determined at least 3 times per week.

15.5 **Animal Sacrifice and Sample Collections**

Fry used to determine survival will be euthanized with 1.0 g/L MS-222 and the number of surviving fry following exposure to sodium perchlorate will be recorded. Fry used to determine growth rate will be euthanized in 1.0 g/L MS-222 and the final weight will be recorded. Adult mosquitofish will be euthanized in 1.0 g/L MS-222 and the females will be dissected. The ovaries will be removed and the eggs will be put in water. The egg volume will be determined by measuring the change in water volume divided by the number of eggs. All mosquitofish will then either be disposed or preserved for future analysis.

15.6 **Endpoint Analysis**

For fry survival, the number of survivors will be counted as the endpoint. For fry growth, percent mass gain will be used as the endpoint [(final weight-initial weight)/initial weight] x 100. For adult reproduction, fecundity as expressed by the ratio of number of eggs to body weight will be used as the endpoint.

16. **PROPOSED STATISTICAL METHODS:**

ANOVA or nonparametric analysis such as Kruskal-Wallis will be used to determine effects of perchlorate on fry growth and reproduction. Probit analysis to test for LC_{50} will be used to determine effects of perchlorate on fry survival.

17. **REPORT CONTENT/RECORDS TO BE MAINTAINED:**

Records to be maintained include: Room temperature and water temperature, dissolved

oxygen, salinity, ammonia, and pH will be collected. Date, time, and amount of feedings per tank will be recorded. Perchlorate effects on fry survival and growth and on adult reproduction will be included in the final report.
Report content will include presentation of data, interpretation, and discussion of the following endpoints:

List individual endpoints and analyses.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. **RECORDS TO BE MAINTAINED / LOCATION:**
The final report will be delivered to the Sponsor on or before December 31, 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point, upon request. All data, the protocol and a copy of the final report shall be archived at the testing facility.

19. **QUALITY ASSURANCE:**
The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. **PROTOCOL CHANGES / REVISIONS:**
All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and Test Facility Manager and maintained with the protocol and the Quality Assurance Unit.

21. **REFERENCES:**
Manzon RG and Youson JH. 1997. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

Miranda, LA, Paz, DA, Dezi, RE and Pisano, A. 1996. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

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Form No. 014 Rev. 3.06/00
Project No.: _T9700_____
*Change No: ___~~2~~ 1 (WE) 1-28-03 MBB
Page: _1____ of ___1__

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment ____Deviation __X__ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Effects of Perchlorate on Growth, Reproduction, and Survival in Mosquitofish
Dated: _____4/3/02_____
Document # (if appropriate): ____FISH-02-02_____
Page #(s): _____10_____
Section #: _____14.6_____
Text to reference: Endpoint analysis: Histopathology of male testis and kidneys were performed after 8 weeks of perchlorate exposure.

Change in Document: In males, histopathology of the gonads and the kidneys were examined to determine the effect of perchlorate exposure. Macrophages aggregates as well as clusters of necrotic cells in testes were the parameters used. Briefly, for the incidence of macrophage aggregates in the testis, we placed a 1-mm square grid containing 121 crosshairs over the center of a randomly chosen testicular section at a total magnification of 200. The section was chosen only according to the quality of the preparation (absence of damage to the section and adequate staining) starting with the first section of the first row on the slide preparation. We counted the numbers of crosshairs that fell on macrophage aggregates as well as those that, sometimes, fell outside the testis (the latter were subtracted from the total of 121). Percent incidence was determined by dividing the number of crosshairs over macrophage aggregates by 121 (minus those falling outside) and multiplying by 100. The same section and general analytical procedure were used to determine the incidence of necrosis, except that the grid was placed on the lower right portion of the testis since preliminary observations indicated that necrosis normally did not occur in the center of the testis but around the edges. The same general procedures for section selection and observations were used for determining the incidence of macrophage aggregates in the kidney. The right lobe of the kidney was chosen for the observations prior to data collection.

Justification and Impact on Study: Earlier observations indicated correlations between the incidence of macrophage aggregates in kidney and testis and exposure to environmental contaminants

* Sequentially numbered in order of the date that the change is effective

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Form No. 014 Rev. 3.06/00
Project No.: _T9700
*Change No: ___~~2~~ WE 1-28-03 mBB
Page: _1____ of ___1__

Change In Study Documentation Form

Submitted by: June Woo Park Signature: June Woo Park Date: 1/26/03

Authorized by: Chris Theodorakis Study Director: Chris Theodorakis Date: 1/27/03

Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 1-28-03

* Sequentially numbered in order of the date that the change is effective

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Form No. 014 Rev. 3.06/00
Project No.: _T9700 ___
*Change No: Z ~~2~~ ~~1~~ (WE) 1-30-03 MBB
Page: _1____ of ___1__

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: _____ Amendment _____ Deviation __X__ Addendums

Document Reference Information
Check One: __X__ Protocol _____ SOP _____ Other _____
Title: Effects of Perchlorate on Growth, Reproduction, and Survival in Mosquitofish
Dated: _____4/3/02_____
Document # (if appropriate): ____FISH-02-02_____
Page #(s): _____10_____
Section #: _____14.6_____
Text to reference: Endpoint analysis: Histopathology of male testis and kidneys were performed after 8 weeks of perchlorate exposure.

Change in Document: In males, histopathology of the gonads and the kidneys were examined to determine the effect of perchlorate exposure. Macrophages aggregates as well as clusters of necrotic cells in testes were the parameters used. Briefly, for the incidence of macrophage aggregates in the testis, we placed a 1-mm square grid containing 121 crosshairs over the center of a randomly chosen testicular section at a total magnification of 200. The section was chosen only according to the quality of the preparation (absence of damage to the section and adequate staining) starting with the first section of the first row on the slide preparation. We counted the numbers of crosshairs that fell on macrophage aggregates as well as those that, sometimes, fell outside the testis (the latter were subtracted from the total of 121). Percent incidence was determined by dividing the number of crosshairs over macrophage aggregates by 121 (minus those falling outside) and multiplying by 100. The same section and general analytical procedure were used to determine the incidence of necrosis, except that the grid was placed on the lower right portion of the testis since preliminary observations indicated that necrosis normally did not occur in the center of the testis but around the edges. The same general procedures for section selection and observations were used for determining the incidence of macrophage aggregates in the kidney. The right lobe of the kidney was chosen for the observations prior to data collection.

Justification and Impact on Study: Earlier observations indicated correlations between the incidence of macrophage aggregates in kidney and testis and exposure to environmental contaminants

Submitted by: Jacques Rinchard Signature: _____ Date: 4/3/02
Authorized by: Chris Theodorakis Study Director: _____ Date: 1-30-03
Received by: Brian Birdwell Quality Assurance Unit: _____ Date: 1-27-03

* Sequentially numbered in order of the date that the change is effective

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Form No. 014 Rev. 3.06/00
Project No.: _T 9700_____
*Change No: __3_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment ____Deviation __X__ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Effects of Perchlorate on Growth, Reproduction, and Survival of Mosquitofish
Dated: _____12/1/02_____
Document # (if appropriate): ___Fish-02-02_____
Page #(s): _____6_____
Section #: _____15.3_____
Text to reference: For fry survival, ten fry will be randomly assigned to each 1 L beaker and immediately exposed to 0, 100, 500, 1000, 2000, and 3000 ppm sodium perchlorate for five days.

Change in Document: For fry survival, ten fry will be randomly assigned to each 1 L beaker and immediately exposed to 0, 100, 300, 500, 800, 1000, and 2000 ppm sodium perchlorate for five days.

Justification and Impact on Study: _3000 ppm was not tested because almost all mosquitofish larvae died when they were exposed to 2000 ppm. Moreover, to calculate more accurately the LC50, the concentrations 300 and 800 ppm were added.

Submitted by: Jacques Rinchard Signature: _____ Date: 12/1/02

Authorized by: Chris Theodorakis Study Director: _____ Date: 1-30-03

Received by: Brian Birdwell Quality Assurance Unit: _____ Date: 1-27-03

* Sequentially numbered in order of the date that the change is effective

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Form No. 014 Rev. 3.06/00
Project No.: _____T9700_____
*Change No: ___3_____
Page: __1___ of __1___

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Effects of Perchlorate on Growth, Reproduction, and Survival of Mosquitofish
Dated: 12/08/02
Document # (if appropriate): Fish 02-02
Page #(s): _____9_____
Section #: _____18_____
Text to reference: The final report will be delivered to the Sponsor on or before December 31, 2002.

Change in Document: The final report will be delivered to the Sponsor on 31 March 2003

Justification and Impact on Study: _____

Submitted by: Chris Theodorakis Signature: Chris Theodorakis Date: 2/20/03

Authorized by: Chris Theodorakis Study Director: Chris Theodorakis Date: 2/20/03

Received by: Brian Birdwell Quality Assurance Unit: Brian Birdwell Date: 2-20-03

* Sequentially numbered in order of the date that the change is effective

Project No.: T9700
Study No.: FISH-02-01

SOPs Referenced in the Protocol

1. AQ-1-02 Cleaning Glassware and Aquaria for Perchlorate Assays
2. AQ-3-05 Fish and Amphibian Field Collection Methods
3. AQ-1-08 General Fish Husbandry
4. AQ-1-09 Mosquitofish (Gambusia spp.) Husbandry
5. AQ-1-03 MS-222 Anesthesia and Euthanasia of Amphibians and Fish
6. IN-3-02 Sample Labeling/Logging Procedure
7. AC-2-15 Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate using Ion Chromatography
8. AC-2-11 Analysis of Perchlorate by Ion Chromatography (IC)

A FINAL REPORT

ENTITLED

EFFECTS OF SUBLETHAL CONCENTRATIONS OF AMMONIUM PERCHLORATE ON *RANA* SPECIES EMBRYOS AND DEVELOPING JUVENILES THROUGH METAMORPHOSIS

STUDY NUMBER:	AMPH-02-02
SPONSOR:	United States Air Force AFIERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	Department of Biological Sciences –**AP** Texas Tech University Box 4-3131 Lubbock, Texas 79409-3131
TEST SITE:	Department of Biological Sciences –**AP** Texas Tech University Box 4-3131 Lubbock, Texas 79409-3131
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	July 18, 2002
RESEARCH COMPLETION:	March 12, 2003

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GOOD LABORATORIES PRACTICES STATEMENT

Project AMPH-02-02, entitled " Effects of sublethal concentrations of ammonium perchlorate on *Rana* species embryos and developing juveniles through metamorphosis", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

James A. Carr, Ph.D

3/27/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Protocol Review	7-22-02	7-29-02	7-29-02	7-29-02
Final Report and Raw Data Review	3-6-03	3-14-03		

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/03
Date

1.0 DESCRIPTIVE STUDY TITLE:

Effects of sublethal concentrations of ammonium perchlorate on *Rana* species embryos and developing juveniles through metamorphosis.

2.0 STUDY NUMBER:

AMPH-02-02

3.0 SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4.0 TESTING FACILITY NAME & ADDRESS:

Department of Biological Sciences –**AP**
Texas Tech University
Box 4-3131
Lubbock, Texas 79409-3131

5.0 EXPERIMENTAL START & TERMINATION DATES:

Start Date: July 18, 2002
Termination Date: March 12, 2003

6.0 KEY PERSONNEL:

James A. Carr, Co-Principle Investigator, Testing Facility Management
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist/ Assistant Director of Science
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principle Investigator

7.0 STUDY OBJECTIVES / PURPOSE:

To determine the sublethal effects of ammonium perchlorate (AP) on development and metamorphosis on *Rana* species embryos and developing juveniles through metamorphosis.

8.0 STUDY SUMMARY

Embryonic and larval *R. utriculata* were exposed to one of two concentrations of ammonium perchlorate or 0.5x magnesium Holtfreter's solution. Hatching in test concentrations was not significantly different from controls. Exposures remained constant for 168 days. Mortality was extremely high with losses of 50% or greater. Additionally, many larvae developed an "ulcer" in the thorax region, therefore this study was terminated prior to 60% metamorphosis in controls.

9.0 TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number : 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.

Source: Aldrich Chemical Company

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: 0.5x Magnesium Holtfreter's Solution, a mixture of reagent grade salts, prepared in 100% ultrapure water.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations: NaCl, 1.75 g/L; $NaHCO_3$, 0.1 g/L; KCl, 0.025 g/L; $CaCl_2 \cdot 2\ H_2O$, 0.65 g/L; $MgSO_4 \cdot 7H_2O$, 0.1 g/L (http://www.indiana.edu/~axolotl).

10.0 JUSTIFICATION OF TEST SYSTEM

Perchlorate occurs in ground and surface waters in 44 states in the USA, primarily as a result of AP discharge from rocket fuel manufacturing facilities or from the demilitarization of missiles (Urbansky, 1998). AP is highly water-soluble and, as a result of the very slow reduction of the central chlorine atom, can persist in the environment for decades (Urbansky, 1998).

Perchlorate is known to prevent intake of iodine from water or food and thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Ionic perchlorate also alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Calcium is a ubiquitous chemical messenger that is involved in the regulation of cellular function. Endocrine glands require calcium for the normal secretion of hormones and therefore contaminant-induced disruption of calcium balance can lead to systemic endocrine disruption. Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health.

We have previously examined aspects of growth and development and thyroid function in anuran larvae collected from AP-contaminated sites at the Longhorn Army Ammunition Plant (LHAAP) located in Karnack, Texas, and identified two possible cases of thyroid disruption. Bullfrog larvae (*Rana catesbeiana*) collected from an AP-contaminated pond exhibited decreased hindlimb growth than larvae from a reference pond, even though the animals from both sites were of identical body length, and presumably, identical age class. Additionally, chorus frog larvae (*Pseudacris triseriata*) collected from another AP-contaminated site at LHAAP presented evidence of thyroid follicle hypertrophy and colloid depletion, both indicators of thyroid disruption.

In previous studies with *Xenopus laevis* we found 5-d and 70-d LC_{50}s for AP to be 510 $\pm$ 36 mg/L and 223 $\pm$ 13 mg/L, respectively (Goleman et al., 2002a). While AP did not cause any concentration-related developmental abnormalities at concentrations below the 70-d LC_{50}, it did inhibit, in a concentration-dependent manner, several thyroid-hormone-dependent aspects of growth and metamorphosis in *X. laevis* larvae including hindlimb growth, forelimb emergence, and tail resorption. Although *Xenopus* is a widely used animal model in basic toxicological, developmental, and reproductive research, it is not native to North America. The effects of perchlorate on thyroid hormone-sensitive indices in native amphibian species throughout metamorphosis have yet to be determined.

11.0 TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: *Rana utriculata* (Southern leopard frog)
Strain: wild type
Age: embryos and larvae
Number: Approximately 1131
Source: Carolina Biological Supply and Charles Sullivan Company

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:
Each tank was labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:
Approximately fifty embryos/ larvae were exposed to three conditions, two concentrations of AP in 0.5x magnesium Holtfreter's solution (38 ppb and 14040 ppb) and 0.5x magnesium Holtfreter's solution alone. At present the larval period of *R. utriculata* under the standard rearing conditions at TCFWRU (20 $\pm$ 2° C, 12: 12 h photoperiod) remains unknown. Each treatment consisted of 5 replicate exposures. Additionally, after hatching was complete, each tank was supplemented with additional larvae from remaining, non-exposed larvae being held in 0.5x Mg Holtfreter's solution to bring the total number of larvae per tank back to 50. This gave approximately 250 embryos plus an additional 110-137 Taylor-Kollros pre-stage I larvae per treatment, for a study total of 1131 animals.

14.0 METHODS:

14.1 Test System Acquisition, Quarantine, Acclimation
Rana embryos were obtained from Carolina Biological Supply or Charles Sullivan Company. Embryos and larvae were maintained as stated in SOP AQ-1-14.

14.2 Test Condition Establishment
Naturally fertilized *Rana* embryos were used. They were obtained from Carolina Biological Supply or Charles Sullivan Company. Embryos from 3 clutches were counted into 15 groups of approximately 50. Each group of 50 embryos was added to 10 L glass tanks containing 2 L of 0.5x magnesium Holtfreter's solution. Pre-mixed 2x test concentrations of AP or 1x 0.5x magnesium Holtfreter's solution were then be added to each tank to the final volume to 4 L of the appropriate concentration. On days 7 and 98 animals were transferred to 21 L glass tanks containing 8 L of like solutions and 40 L glass tanks containing 18 L of like solution, respectively, to accommodate tadpole growth. Just prior to the beginning of forelimb emergence (FLE) each tank was equipped with a silicone-coated ramp to provide a dry area (to prevent drowning). Each tank was labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure,

treatment, and the name of the person responsible for animal care.

14.3 Test Material Application

Embryos were added to tanks containing 2 L of 0.5x magnesium Holtfreter's solution. Test material was pre-mixed to appropriate 2x concentrations and added to the appropriate tank. Tanks were labeled appropriately (see section 15.2). Fifty percent of the medium was changed 3 times per week (Mon., Wed., Fri.).

Rates/concentrations: 0, 38, 14040 ppb.

Frequency: Constant exposure for 167+ days.

Route/Method of Application: Embryos and larvae were exposed to AP in the tank medium. Embryos/ larvae were maintained in 4 L of the test solution in 10 L tanks. Room temperature will be maintained at an average of 21 $\pm$ 1° C with a photoperiod of 12 h light: 12 h dark. On day 7 larvae were moved to 20 L tanks containing 8 L of like solutions to accommodate growth. Larvae were again moved on day 98 to 40 L tanks containing 18 L of like solutions to accommodate growth. Fifty percent of the test and reference solutions were changed 3 x weekly. Method of application was immersion. Route of exposure was via dermal, oral, and respiratory as the chemical was in the tank medium.

Justifications for Exposure Route: *R. utriculata* are fully aquatic as larvae and semi-aquatic as adults.

Exposure Verification Samples of freshly prepared test and reference solutions were analyzed for perchlorate content (Table 1).

14.4 Test System Observation

Beginning on the day of hatch, hatching success (# unhatched eggs/total # eggs), % deformities (# showing bent tails, asymmetric tails/total hatched), edema (% showing distention of body with fluid/total hatched), and abnormal swimming (% showing abnormal swimming/total) were noted daily for each test and reference solution. For free-swimming larvae, % mortality (#dead larvae/#hatched), percent showing deformities, percent displaying abnormal swimming behavior and percent metamorphosed animals (complete tail resorption) were noted every day. Time to metamorphosis for each animal was recorded. Dead animals were removed daily and preserved in 10% neutral buffered formalin (NBF).

Water quality parameters (temperature, pH, specific conductivity, salinity, dissolved oxygen, and ammonia) from each replicate tank were analyzed on day 2 (Table 2) and immediately following each 50% solution change (Table 3).

14.5 Animal Sacrifice and Sample Collections

At the termination of exposure all remaining were euthanized by immersion in MS-222 (1g/L) according to SOP AQ-1-03 and weighed. Stage, SVL, and HLL were measurements

were collected from approximately 5 haphazardly chosen larvae per tank. These animals were then frozen, with remaining larvae placed into Bouin's fixative (SOP IN-1-05). To reduce cannibalism animals completing metamorphosis were removed, euthanized in MS-222, weighed, measured for SVL and HLL and. placed into Bouin's fixative for 48 hrs, washed in running tap water for 24 hrs, then transferred to 70% ethanol (SOP IN-1-05).

14.6 Endpoint Analysis

Hatching success, deformities (bent axial skeletons, asymmetric tails), edema (distention of body with fluid), and abnormal swimming were noted for *Rana* hatchlings. Percent mortality (#dead/#hatched), deformities, abnormal swimming behavior and percent metamorphosed animals (complete tail resorption) were recorded for larvae. Time to metamorphosis for each animal was recorded.

15.0 STATISTICAL METHODS:

Differences in hatching success and metamorphic (% FLE, % tail resorption) and growth parameters (SVL, HLL) were analyzed by one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

16.0 PROTOCOL CHANGES / REVISIONS:

See attached change in study documentation forms.

17.0 RESULTS:

A total of two trials were conducted from July 2002 to March 2003. In the initial trial no larvae survived to hatching (Data not shown). Because of total mortality in the initial trial, only data from the second trial will be presented here. Exposures in the second trial were continued for 168 days prior to termination. As shown in Table 4, no significant differences were seen in % hatching (one-way ANOVA, $F = 0.8232$, $p = 0.46$) compared to controls. Post-hatch mortality was, on the other hand, significantly different between the treatments (one-way ANOVA, $F = 9.779$, p 0.003). The highest percentage of post-hatch mortality was, however, in the reference tanks (Table 4).

While incidences of bent axial skeletons and abnormal swimming behaviors in treatment tanks were not different from reference tanks, exposure to 38 ppb AP resulted in a significantly higher incidence of edema (one-way ANOVA, $F = 6.92$, $p < 0.01$) compared to controls (Table 5). No significant differences were found between reference and treatments for either forelimb emergence or complete tail resorption (Table 6). Likewise, no difference was found in mean developmental stage attained or hindlimb length (Table 7). Mean snout-vent length (Table 7) of animals exposed to 14040 ppb AP were significantly greater than controls (one-way ANOVA, $F = 8.03$, $p < 0.01$).

Table 1. Mean Perchlorate Concentrations from Freshly Prepared Test and Reference Solutions.

Nominal AP (ppb)	Measured AP (ppb)
14 040	13728
38	45
Holtfreter's*	0

*0.5x Magnesium Holtfreter's Solution

Table 2. Mean Water Quality Parameters Analyzed from Five Replicate Test Solutions on 09/27/02.

Nominal AP	Temperature (° C)	pH	Specific Conductivity (µS/cm)	Salinity (ppt)	Dissolved Oxygen (mg/L)	Non-Ionized Ammonia[a] (mg/L)
14 ppm	21.2	7.9	3770	2.0	7.0	0.0704
38 ppb	21.2	7.8	3803	2.0	6.9	0.0001
Holtfreter's[b]	21.2	7.8	3859	2.1	7.0	0.0000

All parameters were measured on exposure day 2.
[a]Measured as ammonia nitrogen on 9/27/02; corrected to un-ionized ammonia.
[b]0.5x Magnesium Holtfreter's Solution

Table 3. Mean Water Quality Parameters Analyzed from Five Replicate Test Solutions over the Length of the Study.

Nominal AP	Temperature (° C)	pH	Specific Conductivity (µS/cm)	Salinity (ppt)	Dissolved Oxygen (mg/L)	Non-Ionized Ammonia[a] (mg/L)
14 ppm	20.7	7.4	3951	2.1	5.5	0.0736
38 ppb	20.7	7.4	3957	2.1	5.5	0.0480
Holtfreter's[b]	20.8	7.4	3980	2.1	5.7	0.0363

Average of water quality parameters for the entire study.

[a]Measured as ammonia nitrogen; corrected to un-ionized ammonia.
[b]0.5x Magnesium Holtfreter's Solution

Table 4. Percent Hatching and Mortality in Larval *R. utriculata* Exposed to Ammonium Perchlorate for Trial 2.

Nominal AP (ppb)	Perchlorate[a] (ppm)	N	%Hatch[b]	%Mortality[c]
14040	13365.8	277	85.92	49.49*
38	39.8	285	90.53	74.43*
Holtfreter's[d]	0.0	278	88.85	90.20

[a]Average measured from tank water samples 9/25/02
[b]Calculated as a percent of total embryos exposed.
[c]Calculated as a percent of total hatchlings exposed.
[d]0.5x Magnesium Holtfreter's Solution.
*Significantly different from controls; one-way ANOVA followed by Tukey-Kramer multiple comparisons test.

Table 5. Developmental Abnormalities in Larval *R. utriculata* Exposed to Ammonium Perchlorate during Trial 2.

Nominal AP (ppb)	N	Bent Axial Skeletons (%)	Edema (%)	Abnormal Swimming (%)
14040	396	35.35	1.26	34.34
38	395	29.11	5.32*	32.91
Holtfreter's[a]	357	52.94	0.00	57.42

Calculated as a percent of total hatchlings exposed.
[a]0.5x Magnesium Holtfreter's Solution
*Significantly different from controls; one-way ANOVA followed by Tukey-Kramer multiple comparisons test.

Table 6. Metamorphic Parameters for *R. utriculata* Exposed to Ammonium Perchlorate during Trial 2.

Nominal AP (ppb)	N	FLE (%)[a]	Tail Resorption (%)
14040	396	3.03	1.01
38	395	0.76	0.00
Holtfreter's[b]	357	0.00	0.00

Calculated as a percent of total hatchlings exposed.

[a]FLE – Forelimb emergence.
[b]0.5x Magnesium Holtfreter's Solution

Table 7. Growth Parameters for Larval *R. utriculata* Exposed to Ammonium Perchlorate during Trial 2.

Nominal AP (ppb)	TK Stage	Snout-Vent Length (mm)	Hindlimb Length (mm)
14040	XII	26.6 ± 0.7*	14.2 ± 3.0
38	X	23.2 ± 0.7	3.8 ± 0.5
Holtfreter's[a]	X	23.0 ± 0.8	7.5 ± 2.8

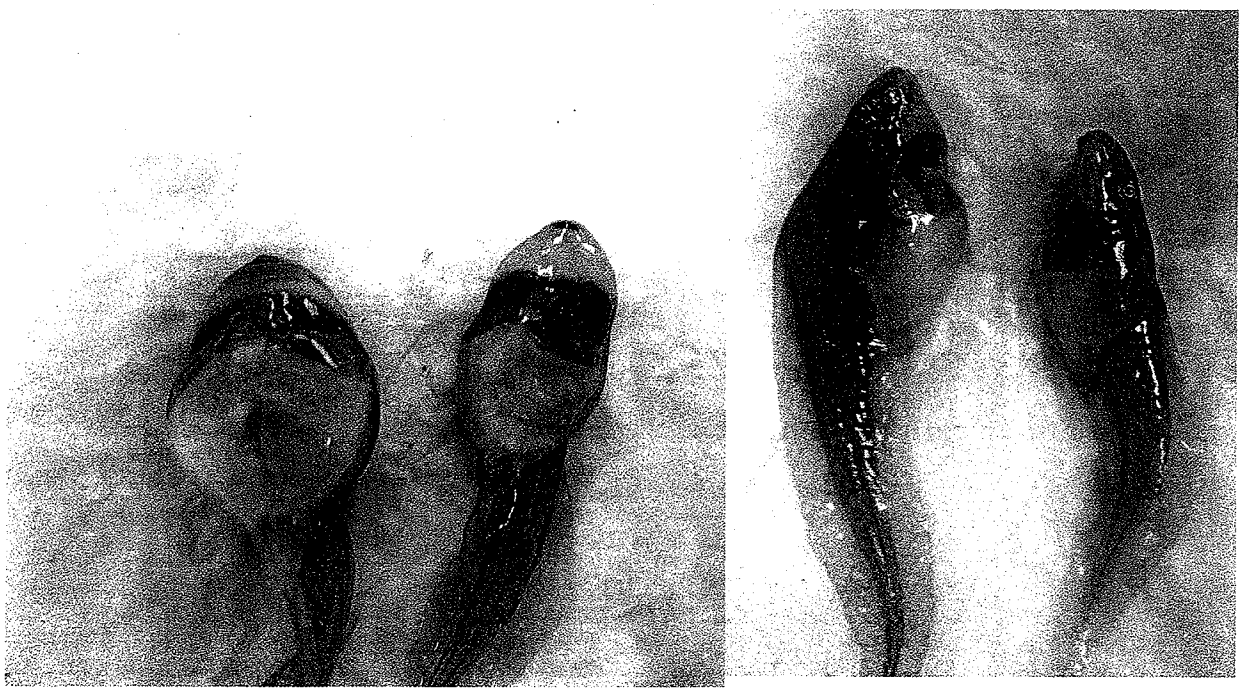
Calculated as mean ± SEM from animals surviving to day 168.
TK – Taylor and Kollros (1946) staging system
[a]0.5x Magnesium Holtfreter's Solution
*Significantly different from controls; one-way ANOVA followed by Tukey-Kramer multiple comparisons test.

18.0 DISCUSSION

As previously noted in *Xenopus laevis* (Goleman et al., 2002b), there were no significant differences in hatching success for *Rana utriculata* at the AP concentrations used in this study. Our current findings to date suggest a high incidence of bent tails and abnormal swimming, however, these findings may be, at least in part, due to the lack of experience with *R. utriculata* larvae. As this study continued further observations revealed that the apparent deformity in the axial skeleton resulting in bent tails may actually be a normal phase of larval development in *R. utriculata*. Likewise, the observer may have also over estimated abnormal swimming behavior, which generally follows the same trend as bent tails.

In mid October 2002 some larvae (in all tanks) developed an "ulcer" across their thorax; their vital organs appear to be exposed (Figure 1). While most have died, these animals do not account for all the mortality, as there were still many present at study termination.

Rana utriculata normally completes metamorphosis in approximately 90 d (Taylor and Kollros, 1946). Metamorphosis was not observed until day 133, with a single animal completing tail resorption. Two additional animals completed tail resorption on day 140. All 3 animals surviving to complete tail resorption had been exposed to 14040 ppb AP. A single control animal entered the metamorphic climax phase, nearing complete tail resorption before expiring, but never exhibited forelimb emergence. Two larvae exposed to 38 ppb AP exhibited forelimb emergence, but never completed tail resorption. At termination of the study (day 168) only 7 additional animals were exhibiting forelimb emergence, all were exposed to 14040 ppb AP.



19.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20.0 REFERENCES:

Dawson, D.A. and Bantle, J. A. (1987). Development of a reconstituted water medium and preliminary validation of the frog embryo teratogenesis assay – *Xenopus* (FETAX). J. Appl. Toxicol.7: 237-244.

Goleman, W.L., Urquidi, L.J, Anderson, T.A., Smith, E.E., Kendall, R.J., Carr, J.A. (2002a). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environ. Toxicol. Chem. 21: 424-430.

Goleman, W.L., Carr, J.A., and Anderson, T.A. (2002b). Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. Environ. Toxicol. Chem. 21: 590-597.

Luttgau, H.C., Gottschalk, G., Kovacs, L., Fuxreiter, M. (1983). How perchlorate improves excitation-contraction coupling in skeletal muscle fibers Biophys. J. 43: 247-249.

Jong, D.S., Stroffekova, K., Heiny, J.A. (1997). A surface potential change in the membranes of frog skeletal muscle is associated with excitation-contraction coupling. J. Physiol.499: 787-808.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. Biocell 20: 147-153.

Taylor AC and Kollros JJ. (1946). Stages in the normal development of *Rana pipens* larvae. Anat Rec 94: 7-23.

Thevenod, F. and Jones, S.W. (1992). Cadmium block of calcium current in frog sympathetic neurons. Biophys. J. 63: 162-168.

Urbansky, ET. (1998). Perchlorate chemistry: implications for analysis and remediation. Bioremediation J. 2: 81-95.

21.0 APPENDICES:

Study Protocol
Changes in Study Documentation
List of Key Standard Operating Procedures

A STUDY PROTOCOL

ENTITLED

EFFECTS OF SUBLETHAL CONCENTRATIONS OF AMMONIUM PERCHLORATE ON *RANA* SPECIES EMBRYOS AND DEVELOPING JUVENILES THROUGH METAMORPHOSIS

STUDY NIMBER: AMPH-02-02

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY

Name/Address: The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. Ronald J. Kendall

Study Director: Wanda L. Goleman

PROPOSED EXPERIMENTAL
START DATE JULY 18, 2002

1. **DESCRIPTIVE STUDY TITLE:** Effects of sublethal concentrations of ammonium perchlorate on *Rana* species embryos and developing juveniles through metamorphosis.

2. **STUDY NUMBER:** AMPH-02-02

3. **SPONSOR:** United States Air Force United States Air Force
   AFIERA/RSE
   2513 Kennedy Circle
   Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
   The Institute of Environmental and Human Health
   Texas Tech University
   Texas Tech University Health Sciences Center
   Box 41163
   Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
   Start Date: July 18, 2002
   Termination Date: December 31, 2002

6. **KEY PERSONNEL:**
   James A. Carr, Principle Investigator
   Wanda L. Goleman, Study Director
   Todd Anderson, Analytical Chemist/ Assistant Director of Science
   Ryan Bounds, Quality Assurance Manager
   Ron Kendall, Testing Facility Management

7. DATED SIGNATURES:

Wanda L. Goleman	7/18/02	Ms. Wanda L. Goleman Study Director
James Carr	7/26/02	Dr. James Carr Principle Investigator
[illegible]	7/29/02	Mr. Ryan Bounds Quality Assurance Manager
Todd Anderson	7-29-02	Dr. Todd Anderson Analytical Chemist/ Assistant Director of Science
Ronald F. Kendall	7/29/02	Dr. Ron Kendall Testing Facility Management

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to and the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:

Dr. James A. Carr
Department of Biological Sciences
Texas Tech University
Box 4-3131
Lubbock, Texas 79409

9. STUDY OBJECTIVES / PURPOSE:

To determine the sublethal effects of ammonium perchlorate (AP) on development and metamorphosis on *Rana* species embryos and developing juveniles through metamorphosis.

10. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number : 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.
Source: Aldrich Chemical Company

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Dawson and Bantle, 1987): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

11. JUSTIFICATION OF TEST SYSTEM

Perchlorate occurs in ground and surface waters in 44 states in the USA, primarily as a result of AP discharge from rocket fuel manufacturing facilities or from the demilitarization of missiles (Urbansky, 1998). AP is highly water-soluble and, as a result of the very slow reduction of the central chlorine atom, can persist in the environment for decades (Urbansky, 1998).

Perchlorate is known to prevent intake of iodine from water or food and thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Ionic perchlorate also alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Calcium is a ubiquitous chemical messenger that is involved in the regulation of cellular function. Endocrine glands require calcium for the normal secretion of hormones and therefore contaminant-induced disruption of calcium balance can lead to systemic endocrine disruption. Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health.

We have previously examined aspects of growth and development and thyroid function in anuran larvae collected from AP-contaminated sites at the Longhorn Army Ammunition Plant (LHAAP) located in Karnack, Texas, and identified two possible cases of thyroid disruption. Bullfrog larvae (*Rana catesbeiana*) collected from an AP-contaminated pond exhibited decreased hindlimb growth than larvae from a reference pond, even though the animals from both sites were of identical body length, and presumably, identical age class. Additionally, chorus frog larvae (*Pseudacris triseriata*)

collected from another AP-contaminated site at LHAAP presented evidence of thyroid follicle hypertrophy and colloid depletion, both indicators of thyroid disruption.

In previous studies with *Xenopus laevis* we found 5-d and 70-d LC_{50}s for AP to be 510 ± 36 mg/L and 223 ± 13 mg/L, respectively (Goleman et al., 2002). While AP did not cause any concentration-related developmental abnormalities at concentrations below the 70-d LC_{50}, it did inhibit, in a concentration-dependent manner, several thyroid-hormone-dependent aspects of growth and metamorphosis in *X. laevis* larvae including hindlimb growth, forelimb emergence, and tail resorption. Although *Xenopus* is a widely used animal model in basic toxicological, developmental, and reproductive research, it is not native to North America. The effects of perchlorate on thyroid hormone-sensitive indices in native amphibian species throughout metamorphosis have yet to be determined.

12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: *Rana pipiens* (Northern leopard frog) or *R. utriculata* (Southern leopard frog)
Strain: wild type
Age: embryos and larvae
Number: Approximately 750
Source: Carolina Biological Supply or Charles Sullivan Company

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:
Each tank will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:
Approximately fifty embryos/ larvae will be exposed to three conditions, two concentrations of AP in FETAX medium (38 ppb and 14040 ppb) and FETAX medium alone for 90 d or until at least 60% of control animals complete metamorphosis. At present the larval period of *R. pipiens* under the standard rearing conditions at TIEHH (20 ± 2° C, 12: 12 h photoperiod) are unknown. Each treatment will consist of 5 replicate exposures. This will give approximately 250 eggs/larvae per treatment, for a study total of approximately 450 animals.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation
Rana embryos will be obtained from Carolina Biological Supply or Charles Sullivan Company. Embryos and larvae will be maintained as stated in SOP AQ-1-14.

15.2 Test condition establishment

Naturally fertilized *Rana* embryos will be used. They will be obtained from Carolina Biological Supply or Charles Sullivan Company. Eggs will be counted into 9 groups of approximately 50 (if more than one egg mass is obtained, eggs will be divided equally for a total of approximately 50 per tank). Each group of 50 embryos will be added to 10 L glass tanks containing 2 L of FETAX solution. Pre-mixed 2x test concentrations of AP or 1x FETAX solution will then be added to each tank to the final volume to 4 L of the appropriate concentration. Animals will be transferred to 21 L glass tanks containing 8 L of like solutions at approximately day 45 to accommodate tadpole growth. Each 21 L tank will be equipped with a silicone-coated ramp to provide a dry area for animals with emerging forelimbs. Each tank will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

15.3 Test Material Application

Embryos will be added to tanks containing 2 L of FETAX medium. Test material will be pre-mixed to appropriate 2x concentrations and added to the appropriate tank. Tanks will be labeled appropriately (see section 15.2). Fifty percent of the medium will be changed every 3 d.

Rates/concentrations: 0, 38, 14040 ppb

Frequency: Constant exposure for 90 days or until at least 60% of control animals have completed metamorphosis.

Route/Method of Application: Embryos and larvae will be exposed to AP in the tank medium. . Embryos/ larvae will be maintained in 4 L of the test solution in 10 L tanks. Room temperature will be maintained at 22 $\pm$ 2° C with a photoperiod of 12 h light: 12 h dark. On approximately day 45 larvae will be moved to 20 L tanks containing 8 L of like solutions, as appropriate, to accommodate growth. Fifty percent of the test and reference solutions will be changed every 3 d. Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory as the chemical will be in the tank medium.

Justification for Exposure Route: *R. pipiens* and *R. utriculata* are fully aquatic as larvae and semi-aquatic as adults.

Exposure Verification: Samples of test and reference solutions will be analyzed for perchlorate content. At the end of the study, 5 animals from each test and reference solution will be euthanized and frozen for contaminant analysis.

15.4 Test System Observation

Beginning on the day of hatch, hatching success (# unhatched eggs/total # eggs), % deformities (# showing bent tails, asymmetric tails/total hatched), edema (% showing distention of body with fluid/total hatched), and abnormal swimming (% showing abnormal swimming/total) will be noted daily for each test and reference solution. For free-swimming larvae, % mortality (#dead larvae/#hatched), percent showing deformities, percent displaying abnormal swimming behavior and percent metamorphosed animals (complete tail resorption) will be noted every day. Time to metamorphosis for each animal will be recorded. Dead animals will be removed and preserved in 10% neutral buffered formalin (NBF).

15.5 Animal Sacrifice and Sample Collections

At the end of exposure unmetamorphosed larvae will be weighed, staged (Taylor and Kollros, 1946), measured for snout-vent (SVL), tail (TL), and hindlimb (HLL) lengths and euthanized by immersion in MS-222 (1g/L) according to SOP AQ-1-03. To reduce cannibalism animals completing metamorphosis will be removed, euthanized in MS-222, weighed, measured for SVL and HLL and. placed into Bouin's fixative for 48 hrs, then transferred to 70% ethanol. At the end of the exposure approximately 5 animals per tank will be frozen for analysis of perchlorate with the remainder placed into Bouin's fixative for 48 hrs, then transferred to 70% ethanol.

15.6 Endpoint Analysis

Hatching success, deformities (bent axial skeletons, asymmetric tails), edema (distention of body with fluid), and abnormal swimming will be noted for *Rana* hatchlings. Percent mortality (#dead/#hatched), deformities, abnormal swimming behavior and percent metamorphosed animals (complete tail resorption) will be recorded for larvae. Time to metamorphosis for each animal will be recorded. SVL, HLL, TL and developmental stage will be recorded at the end of the exposure.

16. PROPOSED STATISTICAL METHODS

Differences in metamorphic (% FLE, % tail resorption) and growth parameters (SVL, HLL,) will be analyzed by one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include:

- Room and water temperature, salinity, pH, and ammonia will be collected.
- Date, time, and amount of feedings per tank will be recorded. Number of expired larvae removed prior to termination of exposure will be recorded, including date and tank/dish.
- Deformities, abnormal swimming behavior and percent metamorphosed animals will be recorded daily prior to termination of the experiment.

Report content will also include presentation of data, interpretation, and discussion of the following end-points:

- Discussion of the relevance of the findings
- List of all SOPs used.
- List of all personnel.

18. RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before December 31, 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be archived by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

Dawson, D.A. and Bantle, J. A. 1987. Development of a reconstituted water medium and preliminary validation of the frog embryo teratogenesis assay – *Xenopus* (FETAX). J. Appl. Toxicol.7: 237-244.

Goleman, W.L., Urquidi, L.J, Anderson, T.A., Smith, E.E., Kendall, R.J., Carr, J.A. (2002). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environ. Toxicol. Chem. 21: 424-430.

Luttgau, H.C., Gottschalk, G., Kovacs, L., Fuxreiter, M. (1983). How perchlorate improves excitation-contraction coupling in skeletal muscle fibers Biophys. J. 43: 247-249

Jong, D.S., Stroffekova, K., Heiny, J.A. (1997). A surface potential change in the membranes of frog skeletal muscle is associated with excitation-contraction coupling. J. Physiol.499: 787-808.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. Biocell 20: 147-153.

Taylor AC and Kollros JJ. (1946). Stages in the normal development of *Rana pipens* larvae. Anat Rec 94: 7-23.

Thevenod, F. and Jones, S.W. (1992). Cadmium block of calcium current in frog sympathetic neurons. Biophys. J. 63: 162-168.

Urbansky, ET. (1998). Perchlorate chemistry: implications for analysis and remediation. Bioremediation J. 2: 81-95.

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Lubbock, TX 79409-3131
(806) 742-2715

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information

Check One: X Protocol ____ SOP ____ Other _____

Title: Effects of sublethal concentrations of ammonium perchlorate on *Rana* species embryos and developing juveniles through metamorphosis
Dated: July 18, 2002
Document # (if appropriate): AMPH-02-02
Page #(s): 2, 4, 5, 6
Section #: 4, 6, 10, 12, 14, 15.2, 15.3

Text to reference:

Section 4. TESTING FACILITY NAME & ADDRESS:

The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Section 6. KEY PERSONNEL:

James A. Carr, Principle Investigator
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist/ Assistant Director of Science
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Testing Facility Management

Section 10. TEST MATERIALS:

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Dawson and Bantle, 1987): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

Section 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: *Rana pipiens* (Northern leopard frog) or *R. utriculata* (Southern leopard frog)

* Sequentially numbered in order of the date that the change is effective

Change In Study Documentation Form

Strain: wild type
Age: embryos and larvae
Number: Approximately 750
Source: Carolina Biological Supply or Charles Sullivan Company

Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Approximately fifty embryos/ larvae will be exposed to three conditions, two concentrations of AP in FETAX medium (38 ppb and 14040 ppb) and FETAX medium alone for 90 d or until at least 60% of control animals complete metamorphosis. At present the larval period of *R. pipiens* under the standard rearing conditions at TIEHH (20 $\pm$ 2° C, 12: 12 h photoperiod) are unknown. Each treatment will consist of 5 replicate exposures. This will give approximately 250 eggs/larvae per treatment, for a study total of approximately 450 animals.

Section 15.2. Test condition establishment

Naturally fertilized *Rana* embryos will be used. They will be obtained from Carolina Biological Supply or Charles Sullivan Company. Eggs will be counted into 15 groups of approximately 50 (if more than one egg mass is obtained, eggs will be divided equally for a total of approximately 50 per tank). Each group of 50 embryos will be added to 10 L glass tanks containing 2 L of FETAX solution. Pre-mixed 2x test concentrations of AP or 1x FETAX solution will then be added to each tank to the final volume to 4 L of the appropriate concentration. Animals will be transferred to 21 L glass tanks containing 8 L of like solutions at approximately day 45 to accommodate tadpole growth. Each 21 L tank will be equipped with a silicone-coated ramp to provide a dry area for animals with emerging forelimbs. Each tank will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

Section 15.3. Test Material Application

Embryos will be added to tanks containing 2 L of FETAX medium. Test material will be pre-mixed to appropriate 2x concentrations and added to the appropriate tank. Tanks will be labeled appropriately (see section 15.2). Fifty percent of the medium will be changed every 3 d.

Change in Document:

Section 4. TESTING FACILITY NAME & ADDRESS:

Department of Biological Sciences –**AP**
Texas Tech University
Box 4-3131
Lubbock, Texas 79409-3131

*** Sequentially numbered in order of the date that the change is effective**

Dept. of Biological Sciences (DBS)
Box 43131
Lubbock, TX 79409-3131
(806) 742-2715

Change In Study Documentation Form

Section 6. KEY PERSONNEL:

Ronald Kendall, Principle Investigator
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist/ Assistant Director of Science
Ryan Bounds, Quality Assurance Manager
James Carr, Testing Facility Management, Co-Principle Investigator

Section 10. TEST MATERIALS:

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: 0.5x Magnesium Holtfreter's Solution, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations: NaCl, 1.75 g/L; $NaHCO_3$, 0.1 g/L; KCl, 0.025 g/L; $CaCl_2 \cdot 2\ H_2O$, 0.65 g/L; $MgSO_4 \cdot 7H_2O$, 0.1 g/L (http://www.indiana.edu/~axolotl).

Section 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: *Rana pipiens* (Northern leopard frog) or *R. utriculata* (Southern leopard frog)
Strain: wild type
Age: embryos and larvae
Number: Approximately 450-750
Source: Carolina Biological Supply or Charles Sullivan Company

Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Approximately fifty embryos/ larvae will be exposed to three conditions, two concentrations of AP in 0.5x magnesium Holtfreter's solution (38 ppb and 14040 ppb) and 0.5x magnesium Holtfreter's solution alone for 90 d or until at least 60% of control animals complete metamorphosis. At present the larval period of *R. pipiens* under the standard rearing conditions at TCFWRU (20 $\pm$ 2° C, 12: 12 h photoperiod) are unknown. Each treatment will consist of 3-5 replicate exposures. This will give approximately 150-250 eggs/larvae per treatment, for a study total of approximately 450-750 animals.

Section 15.2. Test condition establishment

Naturally fertilized *Rana* embryos will be used. They will be obtained from Carolina Biological Supply or Charles Sullivan Company. Eggs will be counted into 15 groups of approximately 50 (if more than one egg mass is obtained, eggs will be divided equally for a total of approximately 50 per tank). Each group of 50 embryos will be added to 10 L glass tanks containing 2 L of 0.5x magnesium Holtfreter's solution. Pre-mixed 2x test concentrations of AP or 1x 0.5x magnesium Holtfreter's solution will then be added to each tank to the final

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

volume to 4 L of the appropriate concentration. Animals will be transferred to 21 L glass tanks containing 8 L of like solutions at approximately day 45 to accommodate tadpole growth. Each 21 L tank will be equipped with a silicone-coated ramp to provide a dry area for animals with emerging forelimbs. Each tank will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

Section 15.3. Test Material Application

Embryos will be added to tanks containing 2 L of 0.5x magnesium Holtfreter's solution. Test material will be pre-mixed to appropriate 2x concentrations and added to the appropriate tank. Tanks will be labeled appropriately (see section 15.2). Fifty percent of the medium will be changed every Mon, Wed, and Fri.

Justification and Impact on Study:

Section 4. Study will be conducted in the Fisheries and Wildlife Research Building (Room 108) on the main Texas Tech University campus.

Section 6. Due to the location change Dr. James Carr will be the Testing Facility Manager/ Co-Principle Investigator with Dr. Ronald Kendall as Principle Investigator.

Section 10., 14., 15.2., 15.3. 0.5x magnesium Holtfreter's solution will be the reference solution as this solution is may be more suited for raising *Rana* larvae.

Submitted by: Signature: Sranda L. Goleman Date: 9/9/02

Authorized by: Study Director: Sranda L. Goleman Date: 9/9/02

Received by: Quality Assurance Unit: Brian Bidwell Date: 9/13/02

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The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information

Check One: X Protocol ____ SOP ____ Other _____

Title: Effects of sublethal concentrations of ammonium perchlorate on *Rana* species embryos and developing juveniles through metamorphosis
Dated: July 18, 2002
Document # (if appropriate): AMPH-02-02, Amendment 1
Page #(s): Protocol: 2, 4, 5, 6; Amendment 1: 3
Section #: 4, 6, 10, 12, 14, 15.2, 15.3

Text to reference:

Section 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: *Rana pipiens* (Northern leopard frog) or *R. utriculata* (Southern leopard frog)
Strain: wild type
Age: embryos and larvae
Number: Approximately 450-750
Source: Carolina Biological Supply or Charles Sullivan Company

Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Approximately fifty embryos/ larvae will be exposed to three conditions, two concentrations of AP in 0.5x magnesium Holtfreter's solution (38 ppb and 14040 ppb) and 0.5x magnesium Holtfreter's solution alone for 90 d or until at least 60% of control animals complete metamorphosis. At present the larval period of *R. pipiens* under the standard rearing conditions at TCFWRU (20 ± 2° C, 12: 12 h photoperiod) are unknown. Each treatment will consist of 3-5 replicate exposures. This will give approximately 150-250 eggs/larvae per treatment, for a study total of approximately 450-750 animals.

Section 15.2. Test condition establishment

Naturally fertilized *Rana* embryos will be used. They will be obtained from Carolina Biological Supply or Charles Sullivan Company. Eggs will be counted into 15 groups of approximately 50 (if more than one egg mass is obtained, eggs will be divided equally for a total of approximately 50 per tank). Each group of 50 embryos will be added to 10 L glass tanks containing 2 L of 0.5x magnesium Holtfreter's solution. Pre-mixed 2x test concentrations of AP or 1x 0.5x magnesium Holtfreter's solution will then be added to each tank to the finalvolume to 4 L of the appropriate concentration. Animals will be transferred

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to 21 L glass tanks containing 8 L of like solutions at approximately day 45 to accommodate tadpole growth. Each 21 L tank will be equipped with a silicone-coated ramp to provide a dry area for animals with emerging forelimbs. Each tank will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

Change in Document:

Section 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: *Rana pipiens* (Northern leopard frog) or *R. utriculata* (Southern leopard frog)
Strain: wild type
Age: embryos and larvae
Number: 1131
Source: Carolina Biological Supply or Charles Sullivan Company

Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Approximately fifty embryos/ larvae will be exposed to three conditions, two concentrations of AP in 0.5x magnesium Holtfreter's solution (38 ppb and 14040 ppb) and 0.5x magnesium Holtfreter's solution alone for 90 d or until at least 60% of control animals complete metamorphosis. At present the larval period of *R. pipiens/ R. utriculata* under the standard rearing conditions at TCFWRU (20 $\pm$ 2° C, 12: 12 h photoperiod) are unknown. Each treatment will consist of 3-5 replicate exposures. Additionally, after hatching is complete, each tank will be supplemented with additional larvae from remaining, non-exposed larvae being held in 0.5x Mg Holtfreter's solution to bring the total number of larvae per tank to 50. This will give approximately 250 embryos plus an additional 110-137 Taylor-Kollros pre-stage I larvae per treatment, for a study total of 1131 animals.

Section 15.2. Test condition establishment

Naturally fertilized *Rana* embryos will be used. They will be obtained from Carolina Biological Supply or Charles Sullivan Company. Eggs will be counted into 15 groups of approximately 50 (if more than one egg mass is obtained, eggs will be divided equally for a total of approximately 50 per tank). Each group of 50 embryos will be added to 10 L glass tanks containing 2 L of 0.5x magnesium Holtfreter's solution. Pre-mixed 2x test concentrations of AP or 1x 0.5x magnesium Holtfreter's solution will then be added to each tank to the final volume to 4 L of the appropriate concentration. Animals will be transferred to 21 L glass tanks containing 8 L of like solutions on day 7 to accommodate tadpole growth. As animals exhibit forelimb emergence (FLE) they will be transferred to

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a 21 L tank containing 8 L of like solution and equipped with a silicone-coated ramp to provide a dry area (prevent drowning). Each tank will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

Justification and Impact on Study:
Section 12., 14., 15.2. The addition of non-exposed larvae at this point should have no effect on study results as hatching in test solutions is complete, larvae are at approximately Taylor-Kollros pre-stage I, and thyroid function has not yet begun.

Submitted by: Signature: Sandra L. Goleman Date: 10/1/02

Authorized by: Study Director: Sandra L. Goleman 10/9/02 Date: ~~10/9/02~~ 10/9/02

Authorized by: Co-Principle Investigator: Date: 10/9/02

Received by: Quality Assurance Unit: Date: 10-22-02

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Document Reference Information

Check One: X Protocol ____ SOP ____ Other _____

Title: Effects of sublethal concentrations of ammonium perchlorate on *Rana* species embryos and developing juveniles through metamorphosis
Dated: July 18, 2002
Document # (if appropriate): AMPH-02-02, Amendment 1, Amendment 2
Page #(s): Protocol: 6; Amendment 1: 3; Amendment 2: 2
Section #: 15.2

Text to reference: Section 15.2. TEST CONDITION ESTABLISHMENT

Naturally fertilized *Rana* embryos will be used. They will be obtained from Carolina Biological Supply or Charles Sullivan Company. Eggs will be counted into 15 groups of approximately 50 (if more than one egg mass is obtained, eggs will be divided equally for a total of approximately 50 per tank). Each group of 50 embryos will be added to 10 L glass tanks containing 2 L of 0.5x magnesium Holtfreter's solution. Pre-mixed 2x test concentrations of AP or 1x 0.5x magnesium Holtfreter's solution will then be added to each tank to the final volume to 4 L of the appropriate concentration. Animals will be transferred to 21 L glass tanks containing 8 L of like solutions on day 7 to accommodate tadpole growth. As animals exhibit forelimb emergence (FLE) they will be transferred to a 21 L tank containing 8 L of like solution and equipped with a silicone-coated ramp to provide a dry area (prevent drowning). Each tank will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

Change in Document:

Section 15.2. TEST CONDITION ESTABLISHMENT

Naturally fertilized *Rana* embryos will be used. They will be obtained from Carolina Biological Supply or Charles Sullivan Company. Eggs will be counted into 15 groups of approximately 50 (if more than one egg mass is obtained, eggs will be divided equally for a total of approximately 50 per tank). Each group of 50 embryos will be added to 10 L glass tanks containing 2 L of 0.5x magnesium Holtfreter's solution. Pre-mixed 2x test concentrations of AP or 1x 0.5x

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magnesium Holtfreter's solution will then be added to each tank to the final volume to 4 L of the appropriate concentration. Animals will be transferred to 21 L glass tanks containing 8 L of like solutions on day 7 to accommodate tadpole growth. On day 98 animals will be transferred to 40 L glass tanks containing 18 L of like solution to accommodate tadpole growth. As animals exhibit forelimb emergence (FLE) they will be transferred to a 21 L tank containing 8 L of like solution and equipped with a silicone-coated ramp to provide a dry area (prevent drowning). Each tank will be labeled as indicated in section 5.5 of SOP AQ-1-17, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, treatment, and the name of the person responsible for animal care.

Justification and Impact on Study:
Section 15.2. As stated in Amendment 2, section 14, the larval period of *R. utriculata* under the standard rearing conditions at TCFWRU are unknown. As of day 97 no control animals have exhibited forelimb emergence. Therefore, all animals are being transferred to larger tanks containing a greater volume of solution in order to decrease the density of larvae in an effort to encourage continued development.

Submitted by: Signature: Wanda L. Goleman Date: 12/31/02

Authorized by: Study Director: Wanda L. Goleman Date: 12/31/02

Received by: Quality Assurance Unit: Brian Bridwell Date: 1-2-03

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Form No. 014 Rev 3. 06/00
Project No.: T9700.2
*Change No: 4
Page: 1 of 1

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Document Reference Information

Check One: X Protocol ____ SOP ____ Other _____

Title: Effects of sublethal concentrations of ammonium perchlorate on *Rana* species embryos and developing juveniles through metamorphosis
Dated: July 18, 2002
Document # (if appropriate): AMPH-02-02
Page #(s): 8
Section #: 18

Text to reference: Section 18. RECORDS TO BE MAINTAINED / LOCATION:
The final report will be delivered to the Sponsor on or before December 31, 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be archived by the testing facility.

Change in Document: Section 18. RECORDS TO BE MAINTAINED / LOCATION:
The final report will be delivered to the Sponsor on or before February 28, 2003. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be archived by the testing facility.

Justification and Impact on Study: Section 18. This change will allow more time for completion of the final report and will have no impact on the study itself.

Submitted by: Signature: Wanda L. Goleman Date: 1/29/03
Authorized by: Study Director: Wanda L. Goleman Date: 1/29/03
Received by: Quality Assurance Unit: Brian Bridwell Date: 1-30-03

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