FY2004 Final Report

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

SERDP Project ER-1235

March 2006

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FINAL REPORT FY2004
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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

George P. Cobb
Co-Principal Investigator

Date

1.0 **DESCRIPTIVE STUDY TITLE**:
Phase VII Analytical Support

2.0 **STUDY NUMBER**:
MRT-05-01

3.0 **SPONSOR:**
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4.0 **TESTING FACILITY**:
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5.0 **PROPOSED EXPERIMENTAL START & TERMINATION DATES**:
Start Date: July 2004
Termination Date: December 2005

6.0 **KEY PERSONNEL**:
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Dr Kang Tian, Instrument Manager
Ms. Xiaoping Pan, Research assistant
Mr. Jun Liu, Research Assistant
Dr. Ronald Kendall, Principal Investigator

7.0 **STUDY OBJECTIVES / PURPOSE:**
Our group was charged with quantification of energetic compound residues (Figure 1) in environmentally relevant samples.

This required method validation for several sample types and method implementation within ongoing research projects.

8.0 **TEST MATERIALS**:

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazacyclooctane	HMX	2691-41-0
2,4,6-trinitrotoluene	TNT	118-96-7
Hexahydro-1,3,5-trinitro-13,5-triazacyclohexane	RDX	21-82-4
Hexahydro-1,3,5-trinitroso-1,3,5-triazine	TNX	13980-04-6
Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine	MNX	5755-27-1
Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine	DNX	80251-29-2

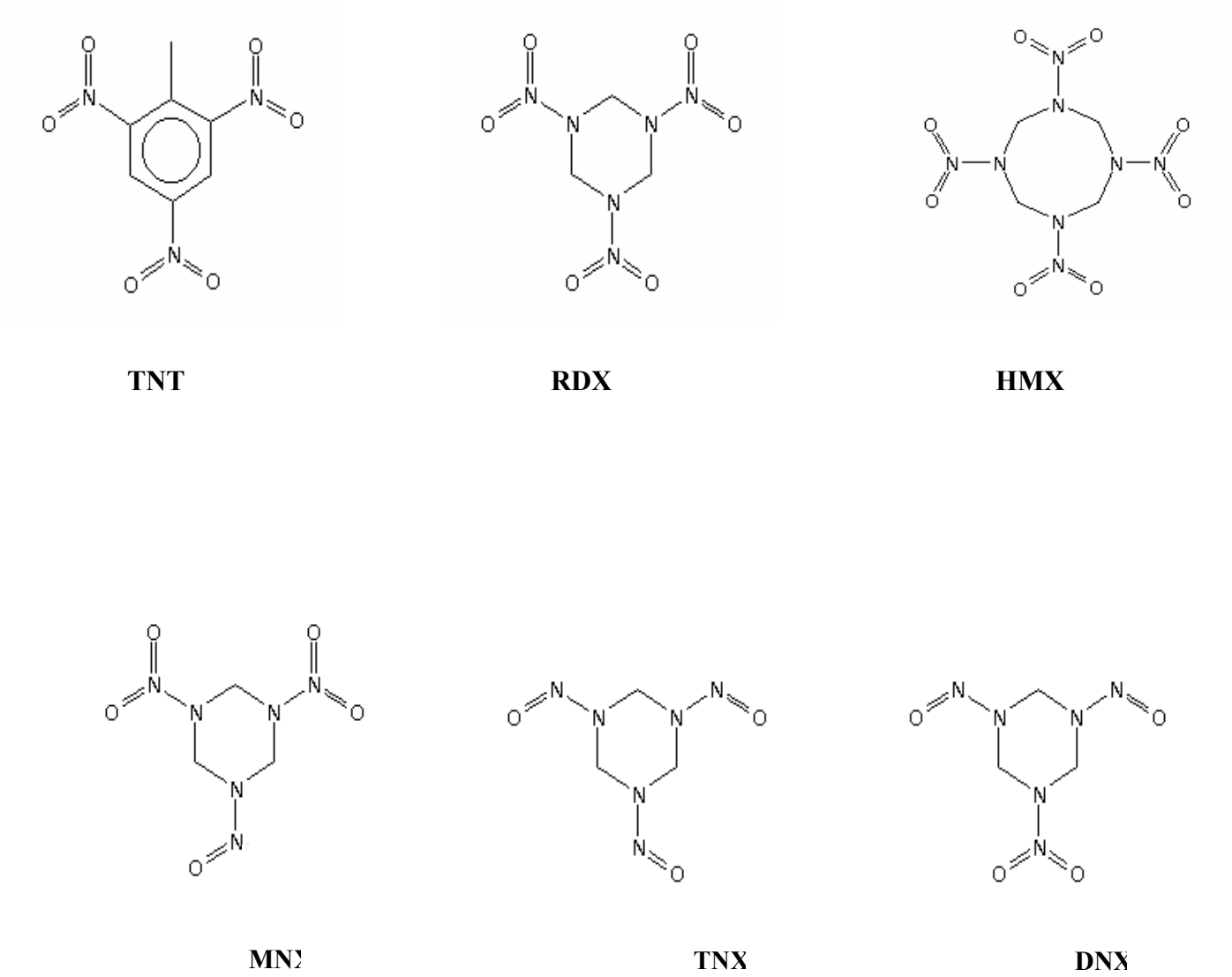


Figure 1. Structures of targeted energetic compounds and their transformation products

9.0 **EXPERIMENTAL DESIGN:**

All methods were validated on three separate days by two individuals. All limits of detection (LODs) were determined using analyses of at least 7 samples that contained analyte concentrations within 8 times the suspected LOD.

All analyses for toxicants in fate and effect studies used techniques previously verified within the Analytical Support section of this research program.

10.0 **METHODS**:

General: In previous years, we developed methods to determine explosives in water, soil, organ tissue, blood, plants, eggs, and animal food With the exception of water analysis each of these methods required Extraction with acetonitrile, and clean-up with florisil solid phase extraction (SPE) columns. In the case of liver and egg samples styrene-divinyl benzene SPE columns were also needed.

GC-ECD: Analyses were performed with a HP 6890 Series gas chromatograph (GC) equipped with an HP 6890 autosampler and an electron capture detector, all controlled by HP 6890 Series Chemstation from Hewlett-Packard (Agilent, Palo Alto, California, USA). Separation was performed on a capillary HP-5 column from Hewlett-Packard (Wilmington, DE, USA). Helium served as carrier gas at a constant flow-rate during the run at 80cm/sec. All gases were supplied by Texas Tech University (Lubbock, TX).

The oven temperature program began at 90°C, held for 2 min, increased to 130°C at a rate of 25°C /min, then made a 10°C /min ramp to 200°C, finally increased to 250°C at a rate of 25°C/min. The temperature of the injection port was 170°C, while that of the detector was 200°C. A 2 µL standard or sample was injected in splitless mode, and the ECD was operated in the constant current mode.

HPLC-UV: The HPLC apparatus was a Hewlett-Packard HP 1100 chromatographic system interfaced with the HP ChemStation software and equipped with a binary pump G1312A, an ultraviolet detector and an autoinjector with a 20 µL loop. The detector was operated at excitation and emission wavelengths of 254 nm. Separations were performed with a reverse-phase C18 column (Supelco, Bellefonate, PA).

For energetic compounds RDX, TNT, MNX, TNX and DNX, the mobile phase consisted of 50% acetonitrile and 50% ultra-pure water. For HMX, gradient mobile phase was employed from 50% acetonitrile and 50% ultra-pure water to 30:70 acetonitrile / water for 5 mins, followed by a gradient to 50:50 acetonitrile / water. The flow rate for all solvents was 1 mL/min, and the injection volume was 15 µL. Chromatography was performed at room temperature (about 25°C). Fresh mobile phase was prepared daily.

LC-MS: A Finnigan LCQ advantage HPLC system consisting of a Surveyor vacuum membrane degasser, a Surveyor gradient pump and a Surveyor autosampler, was coupled to the ion trap mass spectrometer. Chromatographic separation was achieved at room temperature using a Supelco RP C18 column (4.6 * 25 mm, 5-um packing). The isocratic effluent composed of a 50/10/40 (v/v/v), methanol/isopropanol /1.0mM acetic acid running at the flow rate of 0.5 mL/min. The injection volume for all samples was 25 µL and the temperature of the autosampler was kept at room temperature. MS analyses were conducted on a Thermo-Finnigan LCQ advantage ion trap mass spectrometer (San Jose, CA, USA) using ESI interface, in negative-ion mode. Helium was used as damping and collision gas for ion trap, while nitrogen served as sheath and auxiliary gas for ion source. The MS acquisition parameters were: heated capillary temperature 140 ^{o}C; ionization voltage 3.5 kV; sheath gas nitrogen flow rate 28 L/h; auxiliary gas nitrogen flow rate 7 L/h.

11.0 RESULTS

To aid in the evaluation of the potential toxicity nitramine explosives, simple and sensitive quantitative liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) methods were optimized for analysis of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Under negative ionization mode, nitramine explosives can form adduct ions with various organic acids and salts, including acetic acid, formic acid, propionic acid, ammonium nitrate, ammonium chloride, sodium nitrite, and sodium nitrate. Table 1 shows the relative HMX adduct ion abundance when using different additives. Acetic acid was chosen as additive and the ion $[M+CH_3COO]^-$ was used for selective ion monitoring (SIM) in this study (Table 2). Table 3 shows some important LC-ESI-MS operation parameters for HMX analysis, and Table 4 shows some ESI-MS operation parameters for RDX and its N-Nitroso derivatives analysis. Good sensitivity was achieved with low acetic acid concentration in mobile phase and relatively low heated capillary temperature. Figure 2 shows the relationships between target ion abundances with the acetic acid concentrations in the mobile phase across a wide range of concentrations. And Figure 3 shows the relationships between target ion abundances with the heated capillary temperatures. For RDX and its N-nitroso derivatives, the method detection limits (MDLs) were 1.46, 1.46, 1.69, and 1.93 µg/kg for RDX, MNX, DNX, and TNX in soil, respectively. And linearity spanned the range of 5 – 500 µg/L, with correlation coefficients > 0.998. For HMX, the MDL was 0.78 pg in standard solutions and linearity (R^2>0.9998) was obtained at low concentrations (0.5-50 µg/L). Figure 4 shows representative LC-ESI-MS chromatograms for analysis of RDX and its N-nitroso derivatives. And Figure 5 shows LC-ESI-MS chromatograms for HMX analysis.

A liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the analysis of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) was also developed. The electrospray ionization collision-induced dissociation (CID) pattern of HMX was observed and shown in Table 5. And the fragmentation pattern m/z: 355 → m/z: 147 and 174 was chosen for determination of HMX in samples. Important MS/MS operation conditions were also optimized and are shown in Table 6. Using this quantification technique, the method

detection limit was 1.57 µg/L and good linearity was achieved in the range of 5 – 500 µg/L. Figure 6 shows LC-ESI-MS/MS chromatograms for HMX analysis.

We also added to the capabilities for quantification of high explosives in biological tissues by validating an efficient analytical method for HMX in eggs. The method included solvent extraction with ultrasonication followed by cleanup using florisil and styrene-divinyl benzene (SDB) cartridges. Egg extracts were analyzed by high performance liquid chromatography- mass spectrometry (HPLC-MS). Matrix effects to LC-MS were studied and our cleanup procedure proved efficient. Good recoveries and accuracy were achieved. Overall recoveries from eggs containing 10, 50, 250 and 1000 ng/g of HMX were 84.0%, 88.0%, 90.6% and 87.4%. And a method detection limit (MDL) of 0.15 ng/g was achieved by the method.

Applications

These analyses have been applied to several research projects involving different high explosives in biotic (earthworm, lizards, lizard eggs, quail, quail eggs, mice) and abiotic (water, soil, sediment) media. This involved analysis of a significant number of samples (Table 7). Results of these analyses are reported and interpreted in the respective fate and/or effects research projects.

Table 1. Relative HMX adduct ions with different additives (1mM in mobile phase B).

Candidate Additives	Adduct Ion	m/z	Ion Relative Abundance %
Formic acid	$[M + HCOO]^-$	341	30
Acetic acid	$[M + CH_3COO]^-$	355	100
Propionic acid	$[M + CH_3CH_2OO]^-$	369	45
Ammonium nitrate	$[M + NO_3]^-$	358	80
Ammonium chloride	$[M + Cl]^-$	331,333	15
Sodium nitrite	$[M + NO_2]^-$	342	70
Sodium nitrate	$[M + NO_3]^-$	358	80

Table 2. Ions for Selective Ion Monitoring (SIM)

Analyte	CAS #	Molecular Weight	Ion monitored $[M+59]^-$
HMX	2691-41-0	296	355
RDX	121-82-4	222	281
MNX	5755-27-1	206	265
DNX	80251-29-2	190	249
TNX	13980-04-6	174	233

Table 3. Some important optimized LC-ESI-MS operation parameters for HMX analysis

LC conditions	MS conditions
Mobile phase A: Methanol	Mode: Negative
Mobile phase B: 0.5 mM	Ion spray voltage (KV): 3.5
aqueous acetic acid	Sheath gas flow rate (L/hr): 44.0
A: B = 60:40 (v/v)	Aux/Sweep gas flow rate (L/hr): 53.1
Flow rate: 0.5 ml/min	Capillary voltage (V): - 6.3
Injection: 25 µL	Capillary temp (°C): 140.0
	Multipole 1 offset (V): 1.7
	Lens Voltage (V): 25.6
	Multipole 2 offset (V): 7.0
	Multiple RF Amp (Vp-p, sp): 500.0

Table 4. Some important optimized ESI-MS operation parameters for analysis of RDX and its derivatives

ESI Source		**Ion Optics**	
Mode:	Negative	Multiple 1 offset (V):	2.0
Ion spray voltage (KV):	3.52	Lens Voltage (V):	23.6
Sheath gas flow rate(L/hr):	31.9	Multiple 2 offset (V) :	5.4
Aux/Sweep gas flow rate(L/hr):	56.3	Multiple RF Amp (Vp-p, sp) :	500
Capillary voltage (V):	-19.5	Coarse trap DC offset (V) :	10.0
Capillary temp (°C):	140.0	Fine trap DC offset (V):	10.0

Table 5. Collision induced daughter ions of HMX

Parent ion		**Daughter ions**		
m/z	**Structure**	**m/z**	**Relative abundance %**	**Proposed Structure**
355	$[M+CH_3COO]^-$	295	2	$[M-H]^-$
		221	11	$[M - H - CH_2NNO_2]^-$
		192	40	$[M - CH_2NNO_2 - NO]^-$
		174	36	$[M - 3O-CH_2NNO_2]^-$
		147	100	$[M – 2CH_2NNO_2]^-$
		117	12	$[M – 2CH_2NNO_2 - NO]^-$

Table 6. Some important optimized ESI-MS/MS operation conditions for HMX analysis

ESI Source		**Ion optics and ion trap**	
Mode:	Negative	Lens Voltage (V):	14.0
Ion spray voltage (KV):	4.0	Multiple 1 offset (V) :	1.25
Sheath gas flow rate(L/hr):	47.0	Multiple 2 offset (V) :	7.0
Aux/Sweep gas flow rate(L/hr):	27.0	Multiple RF Amp (Vp-p) :	600
Capillary voltage (V):	-12.0	Trap DC offset (V) :	10.0
Capillary temp (°C):	140.0	Normalized collision energy (%):	32

Table 7. Chemical analyses performed within the SERDP Analytical Core during Calendar 2005.

Analytical Activity	**Sample analyses**
Method development	500
Method validation	500
Fish dosing	676
Fish tissue	178
Amphibian dosing	136
Plant dosing	108
Plant	100
Rodent RDX and TNTdosing	12
Rodent RDX and TNTtissue	285
Quail food	58
Quail food stability	60
Quail Eggs	192
Lizard dosing	284
Lizard egg	150
Mesocosm Dosing	230
Mesocosm Output	890
Total Samples Analyzed	**4359**

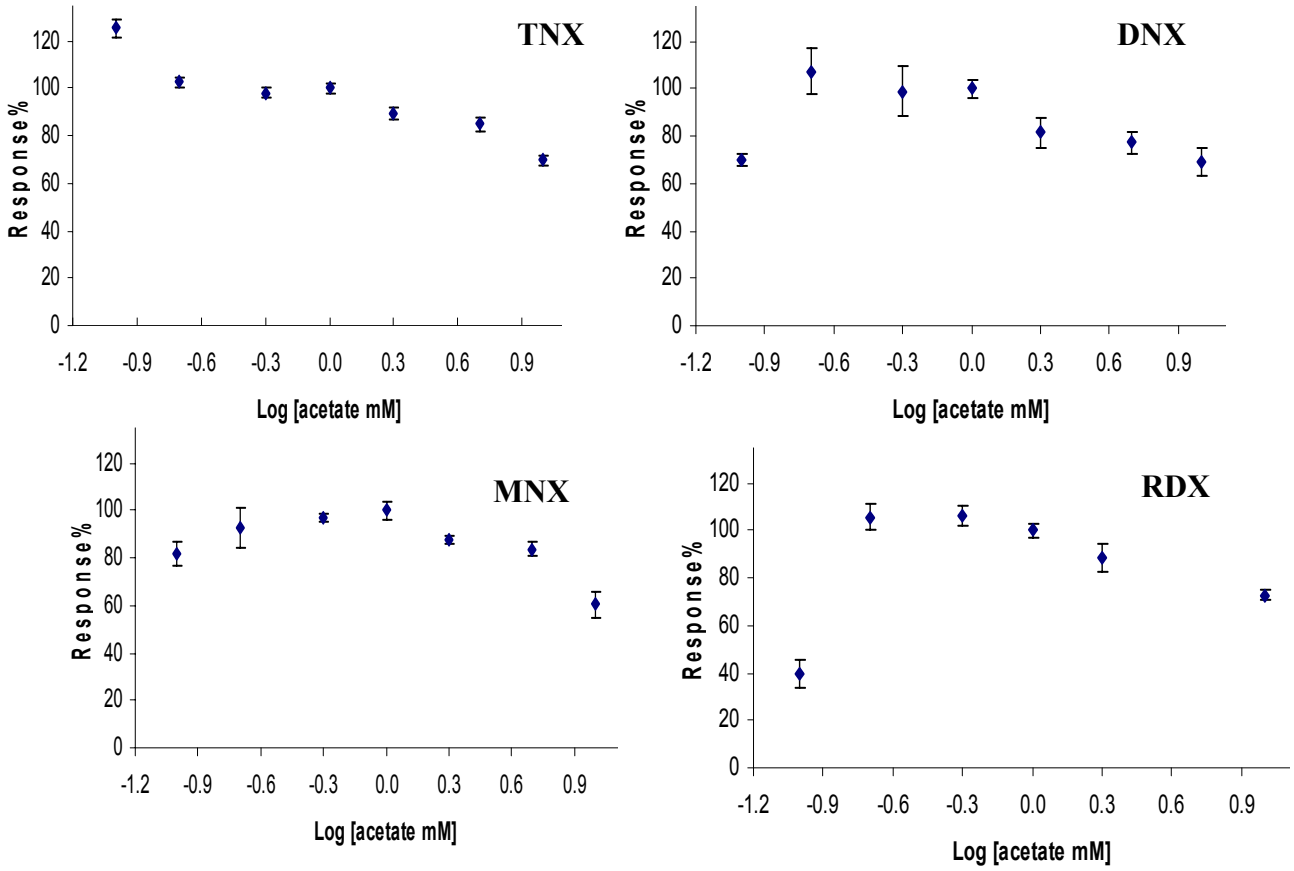


Figure 2. Percent response vs Log [acetate concentration (mM)] in mobile phase B when injecting 250 µg/L of analyte. Y values were calculated as the ratio of signal values at certain acetate concentrations to the signal value obtained at 1 mM acetate at mobile phase B for the same compound. Error bars indicate standard deviation for triplicate injections.

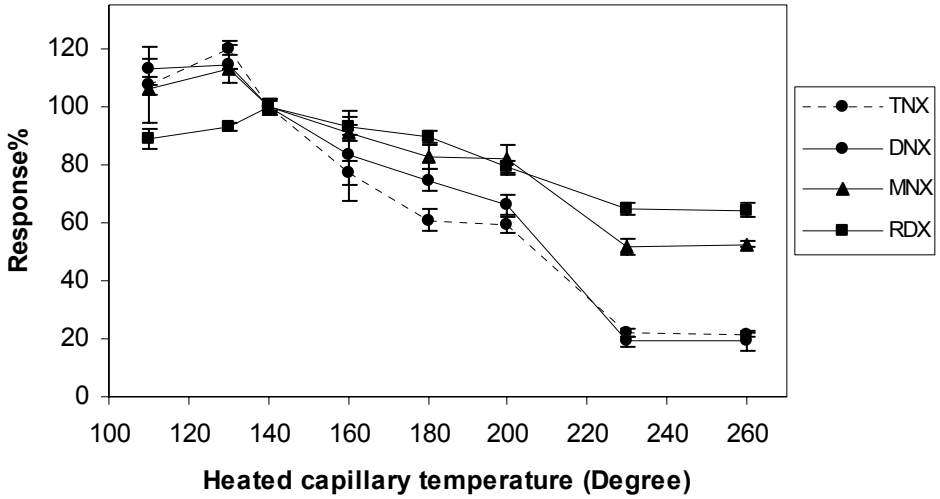


Figure 3. Percent response vs Heated capillary temperature when injecting 20 µg/L analytes. Y values were calculated as the ratio of signal values at certain temperature to the signal value obtained at 140 °C for the same compound. Error bars indicate standard deviation for triplicate injections.

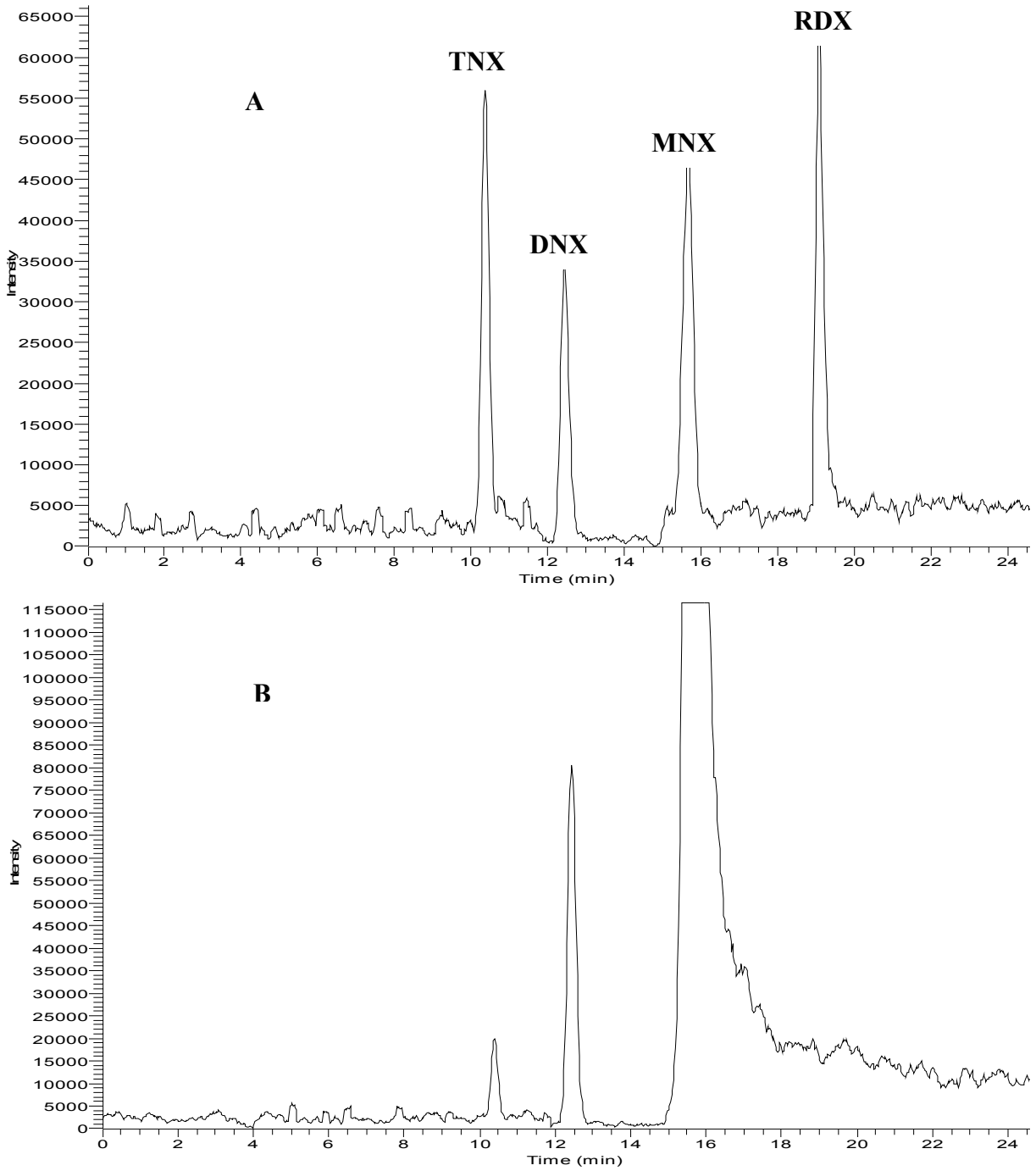
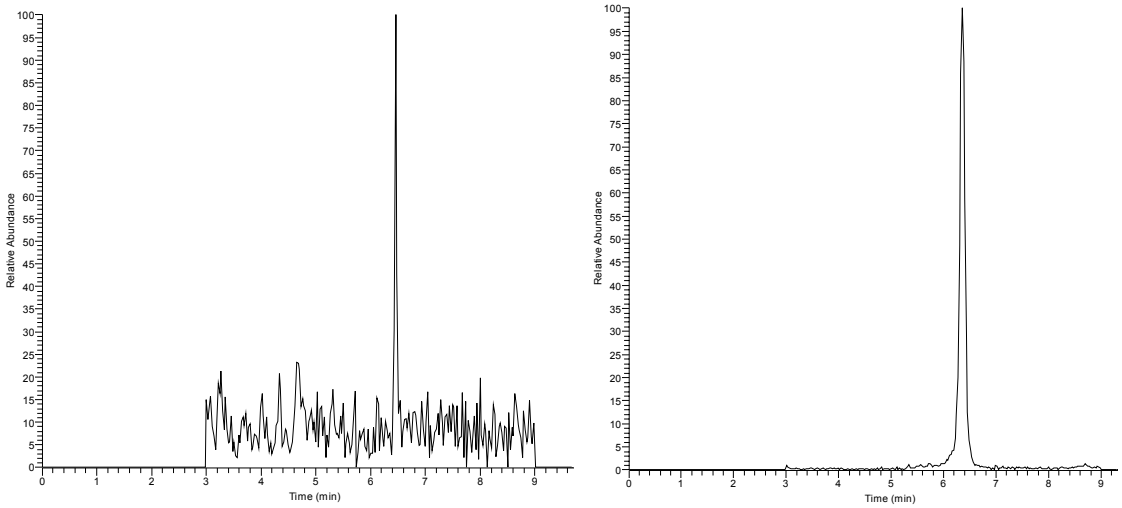
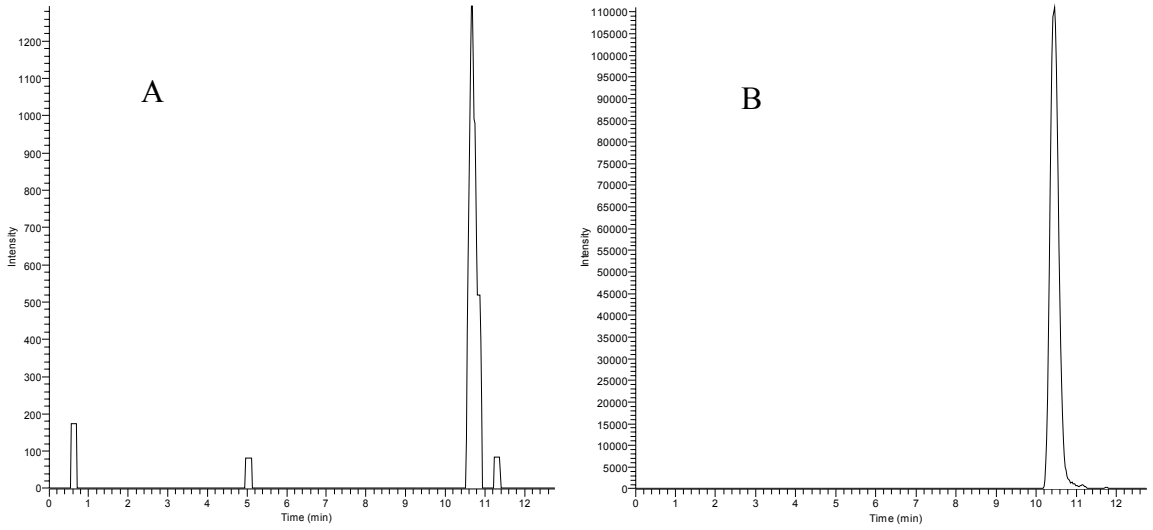


Figure 4. Representative chromatograms of A) a blank soil sample spiked with 20 µg /kg RDX, MNX, DNX, and TNX, and B) a real soil sample containing TNX (7.1 µg /kg), DNX (46.4 µg /kg), and MNX (1543.0) µg /kg (dilution factor = 5).



(A) (B)

Figure 5. LC-ESI-MS (SIM) chromatograms of (A) Mass chromatogram of 0.1 µg/L HMX standard; and (B) Occurred lizard egg sample with HMX 30.6 µg/L.



(A) (B)

Figure 6. Representative LC-ESI-MS/MS chromatograms of A) HMX 5 µg/L in solvent; and B) a real soil sample that contained HMX (210.6 µg /kg) (Diluted sample). SRM: 355>147, 174.

12.0 References:

Pan X, Zhang B, Cobb GP. 2005. Extraction and analysis of cyclonite (RDX) and its nitroso-metabolites in animal tissue using gas chromatography with electron capture detection (GC-ECD). *Talanta*. In Press.

Zhang B, Pan X, Smith J, Anderson TA, Cobb GP. 2006. Determination of Trace Amounts of Energetic Compounds in Blood by Gas Chromatography with Electron Capture Detector (GC/ECD) and by High Performance Liquid Chromatography with Ultraviolet Detector (HPLC/UV). *Anal Chim Acta* (In Press)

Pan X, Tian K, Cobb GP. Analysis of Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by Liquid Chromatography / Electron Spray Ionization / Mass Spectrometry(LC-ESI-MS). Submitted to *Anal. Chim. Acta.*

TITLE: Invertebrate Developmental Toxicity of Explosive Metabolites in Soil

STUDY NUMBER: INVDEV-05-01

SPONSOR: Strategic Environmental and Research Development Program
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TESTING FACILITY: The Institute of Environmental and Human Health
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TEST SITE: The Institute of Environmental and Human Health
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RESEARCH INITIATION: January 1, 2005

RESEARCH COMPLETION: December 31, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Todd A. Anderson Date
Co-Principal Investigator

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 17, 1989.

Submitted By:

_____ _____

Ryan Bounds
Quality Assurance Manager

Date:

1.0 DESCRIPTIVE STUDY TITLE:
Invertebrate Developmental Toxicity of Explosive Metabolites in Soil

2.0 STUDY NUMBER:
INVDEV-05-01

3.0 SPONSOR:
Strategic Environmental Research and Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
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4.0 TESTING FACILITY NAME AND ADDRESS:
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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start: January 1, 2005
Termination: December 31, 2005

6.0 KEY PERSONNEL:
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Mr. Baohong Zhang, co-investigator
Ms. Christina Freitag, co-investigator
Mr. Ryan Bounds, Quality Assurance Manager
Dr. Ronald Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:
The proposed focus of this subproject was to address data gaps related to the potential environmental impact of two degradation metabolites of the explosive RDX (TNX and MNX). These experiments were follow-up studies to those conducted earlier on the biological availability and invertebrate toxicity of TNX and MNX in soil. The developmental toxicity of TNX and MNX to cricket (*Acheta domesticus*) eggs was evaluated. We assessed reproductive/developmental success (egg hatching) upon exposure to TNX and/or MNX.

8.0 STUDY SUMMARY:
The effect of two major hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) metabolites, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), on cricket (*Acheta domesticus*) survival and reproduction was studied. RDX metabolites did not have adverse effects on cricket survival, growth, and egg production. However, MNX and TNX did affect egg hatching. MNX and TNX were more toxic in spiked sand than in topical tests. TNX was more toxic to eggs than MNX. Developmental stage and exposure time affected hatching. After 30 days exposure to

MNX or TNX, the EC20, EC50, and EC95 were 47, 128, and 247 µg/g for TNX, and 65, 140, and 253 µg/g for MNX in topical tests. In sand, the EC20, EC50, and EC95 were 21, 52, and 99 µg/g for MNX, and 12, 48, and 97 µg/g for TNX. No gross abnormalities in cricket nymphs were observed in all experiments indicating that neither TNX or MNX is teratogenic in this assay.

As a side project, the effect of MNX and TNX on earthworm (*Eisenia fetida*) cocoon hatching was also studied. The results indicate that MNX and TNX inhibit cocoon hatching in both topical tests and soil tests. However, there was no significant affect of either chemical on the hatchling survival and growth.

9.0 TEST MATERIALS:

Test Material: laboratory sand
Source: Fisher Scientific

Test Material: silt loam soil
Source: Harlan County, NE

Test Chemical: MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine)
CAS Number: 5755-27-1
Characterization: Purity confirmed by source.
Source: SRI International

Test Chemical: TNX (hexahydro-1,3,5-trinitroso-1,3,5-triazine)
CAS Number: 13980-04-6
Characterization: Purity confirmed by source.
Source: SRI International

Reference Chemical: acetonitrile
CAS Number: 75-05-8
Characterization: ACS-Certified.
Source: Fisher Scientific

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:

Recently, much research effort has been focused on filling data gaps related to the fate/toxicity of explosive materials (EMs) in soil. With the exception of CL-20, the results of this effort have been a better characterization of the bioaccumulation/bioavailability, invertebrate toxicity, and plant toxicity and uptake of a variety of parent EMs and the development of Ecological Soil Screening Level (Eco-SSL) benchmarks for use in ecological risk assessments at explosives-contaminated sites.

While much invertebrate and plant toxicity information has been recently obtained through SERDP-sponsored research on the parent EMs, to our knowledge very little data exist on the potential environmental impact of the degradation metabolites of compounds such as HMX and RDX. The products of the biotic and abiotic degradation of these compounds may also pose toxicological risk to terrestrial and aquatic organisms.

11.0 TEST ANIMALS:

Invertebrate Eggs or Cocoons

Cricket (*Acheta domesticus*)

Earthworm (*Eisenia fetida*)

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

All test systems (crickets, cricket eggs, earthworm cocoons) were placed in bottles with labels containing the appropriate identification information for the test system. Collected samples were placed in individually labeled bags/containers and stored appropriately according to TIEHH SOP IN-3-02.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

A variety of controls were used throughout the course of the experiments to ensure the quality of the data generated. Solvent controls (sand or soil amended with acetonitrile only) and negative controls (sand or soil without test compound or solvent) were included in all trials. The solvent controls were prepared in the same manner as toxicant-spiked systems without the toxicant. All data analyses were conducted on measured metabolite concentrations rather than nominal concentrations. Data were processed using standard statistical software (SigmaPlot, Version 8.0, and SigmaStat, Version 2.03, SPSS, Chicago, Illinois, USA).

14.0 METHODS:

14.1. Test organisms

Crickets (*Acheta domesticus*) were purchased from Carolina Biological Supply Company (Burlington, NC). They were fed a diet consisting of dog food. Water was supplied daily. Crickets were maintained in aquaria on a 12 h light:12 h dark photoperiod at room temperature (~20°C). Each aquaria hosted about 15-20 pairs of adult crickets. Cricket eggs were collected as needed for use in experiments.

Earthworm (*Lumbricus rubellus*) cocoons were obtained from Advanced Biotechnology, Inc. (Elliott, IL).

14.2. Chemicals

MNX (purity > 99%) and TNX (purity > 99%) were obtained as solids from SRI International (Menlo Park, CA). Ultra-pure water (> 18 MΩ) was prepared by a Barnstead NANOpure infinity ultrapure water system (Dubuque, IA). Stock solutions (1000 µg/mL) of MNX and TNX were individually made in acetonitrile and stored at -20 °C until use. All concentrations of MNX and TNX in water, sand, and soil were confirmed using GC-ECD as described previously (Pan et al., 2005; Zhang et al., 2005).

14.3. Cricket egg production
A total of 150 g of fine colored sand (Activa Products Inc., Marshall, TX) was weighed and placed into a 200-mL beaker. Then, 20 mL ultra-pure water was used to wet the sand. Sand was evenly divided into 10 groups and placed into 10 individual 50-mm Petri dishes (VWR International, West Chester, PA). Finally, the sand-filled Petri dishes were put into a 500-mL Redi-Pak straight-sided jar (VWR International, West Chester, PA). Then, two adult female (presence of an ovipositor) crickets were put into the jar and placed in an incubator, overnight, at 28 °C in the dark. After about 12 h of incubation, eggs were harvested for topical tests or other experiments.

14.4. Effect of RDX metabolites on adult cricket survival and egg production
Acclimated adult crickets were fed MNX- or TNX-contaminated dog food for two weeks. Contaminated food was prepared according to the following procedure. Dog food was spiked with 1000 µg/mL MNX or TNX in acetonitrile to obtain final food concentrations of 10 and 100 µg/g. An identical volume of acetonitrile was also added to dog food for the control group (0 µg/g MNX or TNX). MNX and TNX were spiked individually. Spiked food samples were thoroughly mixed in order to distribute the contaminant evenly and allow the solvent to evaporate. Spiked food samples were further stored for 24 hours in the dark under a chemical hood to permit the complete evaporation of acetonitrile. Each treated group (40 adult crickets) was housed in one individual aquarium. Food and water were supplied daily. The behavior and survival of crickets was observed daily. After two weeks of treatment, the number of surviving crickets was recorded. Then, the crickets were allowed to lay eggs in clean colored sand following the same procedure described above.

All laid eggs were maintained (in clean sand) in an incubator at 28 °C in the dark. After 45 days, the number of hatched eggs and nymphs was determined.

14.5. Topical test: Effect of MNX and TNX on cricket eggs and earthworm cocoons
Two layers of filter paper were placed in 10-cm Petri dishes. Then, 30 cricket eggs harvested from the egg production experiments described earlier were placed on the filter paper. Milli-Q water with different concentrations (0, 10, or 100 µg/mL) of TNX or MNX was added to the exterior of each egg. Three replicates were constructed for each of the exposure concentrations. All treatments were incubated in the dark at 28 °C. Petri dishes were opened each day for observation. The earthworm cocoon experiments were performed in a similar manner.

14.6. Sand test: Effect of MNX and TNX on cricket egg hatching
Two healthy adult female crickets were put into a 500-mL glass jar. Each jar contained a 50-mm Petri dish in which 15 g of MNX- or TNX-contaminated sand was contained. Contaminated sand was prepared according to the following procedure. First, 75 g sand was weighed for each treatment. Then, the sand was spiked with 1000 µg/mL MNX or TNX in acetonitrile to final concentrations of 10 or 100 µg/g, individually. Spiked sand was thoroughly mixed in order to distribute the contaminant evenly and allow the solvent to evaporate. Spiked sand was further stored for 24 hours in the dark under a chemical

hood to permit the complete evaporation of acetonitrile. After 24 hours, the sand was wetted with 10 mL of ultra-pure water (> 18 MΩ). Finally, the spiked sand was evenly divided into five 50-mm Petri dishes (5 replicates). Another 75 g of sand, as control, was treated using the same procedure except that it was spiked only with acetonitrile.

Crickets were kept in the jars overnight (~12 hours), then removed to allow the laid eggs to incubate in the sand in the presence of MNX or TNX. After 45 days, the number of eggs and nymphs was recorded.

14.7. Soil test: Effect of MNX and TNX on cricket eggs and earthworm cocoons
Thirty-five grams of silt loam soil (Harlan County, NE) was placed into a 50-mL glass jar (VWR International, West Chester, PA). Silt loam soil contained 2.5% organic matter, 34% sand, 54% silt, and 12% clay (pH = 7.0). Soil was spiked with MNX or TNX in acetonitrile to final concentrations of 0, 10, 100, and 1000 mg/kg. The spiked soils were mixed thoroughly and placed in a chemical hood overnight to allow the acetonitrile to evaporate completely. Then, 5 mL of Milli-Q water was added to each jar to moisten the soil. Finally, 20 cricket eggs were put into each jar and covered by a thin layer of spiked soil. Each treatment group contained three replicates. After 40 days of exposure, the number of nymphs was recorded. The earthworm cocoon experiments were performed in a similar manner, except that the soil used was a sandy loam (Terry County, TX).

14.8. Statistical analysis
All obtained data were statistically analyzed using statistical software (SigmaPlot, Version 8.0, and SigmaStat, Version 2.03, SPSS, Chicago, Illinois, USA). Analysis of variance (ANOVA) was used to compare the data among different treatments. All percent data were arcsine transformed. If there was a significant difference among groups or times, LSD multiple comparisons were conducted to compare the mean of each treatment group or time for determining the bounded No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) values. Each measured parameter was fitted and analyzed using a linear regression model ($y = a + bx$). The 95% confidence intervals (C.I.) were calculated using R project (http://www.r-project.org/).

15.0 RESULTS:

15.1 Effect of MNX and TNX on adult cricket survival and egg production
After 14 days of feeding with MNX- or TNX-contaminated food, there was no difference in survival of adult crickets between treatment groups (10 or 100 µg/g MNX or TNX) and the control group.

Feeding MNX- or TNX-contaminated food did not affect egg production or the hatching success of produced eggs (**Figure 15.1** and **15.2**). About 40% of the eggs hatched (regardless of treatment), and no gross abnormalities in the nymphs were observed for any of the test groups.

15.2. Topical test: Effect of MNX and TNX on egg production and hatching
In the topical test, cricket egg hatching decreased as concentrations of MNX or TNX increased. A dose-response relationship was evident for the hatching of cricket eggs

topically exposed to MNX and TNX (**Figure 15.3**). Without MNX or TNX exposure, 50 ± 7.2% of eggs hatched. After 30 days of exposure to 10 µg/mL MNX or TNX, hatching was 51 ± 7.6% and 48 ± 6.0% for MNX and TNX exposure, respectively. Low concentration (10 µg/mL) exposure to MNX or TNX did not significantly affect cricket egg hatching (**Figure 15.3**). However, the high exposure concentration (100 µg/mL) did affect egg hatching ($p = 0.03149$). After 30 days of exposure to 100 µg/mL MNX or TNX, only 33 ± 12.9% and 30 ± 7.2% of eggs hatched, respectively. Compared with the control, the hatching rates decreased by 34% and 40% after 30 days of exposure to 100 µg/mL MNX or TNX. Based on 30 days of exposure to MNX or TNX, the EC20, EC50, and EC95 were for 47, 128, and 247 µg/mL for TNX, and 65, 140, and 253 µg/mL for MNX (**Table 15.1**).

Developmental stage and exposure time affected cricket hatching (**Figure 15.4**). Short-term exposure to MNX or TNX at a late embryonic developmental stage did not cause a significant decrease in cricket egg hatching. **Figure 15.4** clearly indicates that there was no significant difference among hatching rates for the control group and treatment groups after only 10 days of exposure to MNX or TNX for a total incubation time of 45 days. However, if newly laid eggs were immediately treated with MNX or TNX for at least 30 days, 100 µg/mL MNX or TNX did cause a significant decrease in egg hatching ($p = 0.0315$) (**Figure 15.4**).

Nymph crickets from these experiments (observed under a microscope) showed no morphological abnormalities.

15.3. Sand test: Effect of MNX and TNX on egg production and hatching
Crickets laid eggs in both TNX- and MNX-contaminated sand and uncontaminated (control) sand. Each cricket laid approximately 120 eggs during an overnight period; there was no significant difference in the numbers of eggs laid in contaminated sand or control sand (**Figure 15.5**).

Although TNX or MNX in sand did not affect egg production, both contaminants did affect the hatching of eggs, and this effect was concentration-dependent (**Figure 15.6**). Cricket eggs began hatching after 30-35 days of incubation in sand. For the control group, 43 ± 5.1% of eggs hatched after 45 days of incubation in sand without MNX or TNX. Adding 10 µg/g TNX significantly affected egg hatching ($p = 0.00134$); only 31 ± 2.7% of eggs hatched, a decrease of 29% compared with control. However, the same amount of MNX in sand did not statistically reduce cricket egg hatching. It is possible that this lack of an effect was obfuscated by the large variation among the MNX replicates at this concentration (41 ± 28.1% of eggs hatched in sand with 10 µg/g MNX). At the high concentration (100 µg/g), both MNX and TNX significantly inhibited cricket egg hatching ($p < 0.001$). Only 3.4 ± 3.4% and 1.6 ± 3.6% of eggs hatched in TNX- or MNX-contaminated sand (100 µg/g) after 45 days of incubation, respectively. This suggested that TNX inhibited cricket egg hatching more than MNX at the low concentration (10 µg/g); both MNX and TNX inhibited cricket egg hatching at the higher concentration (100 µg/g). The EC20, EC50, and EC95 were 21, 52, and 99 µg/g for MNX and 12, 48, and 97 µg/g for TNX (**Table 15.1**).

15.4. Soil test: Effect of MNX and TNX on egg production and hatching
In the non-contaminated silt loam soil, 40 ± 5.4% of incubated eggs hatched. Adding 10 or 100 mg/kg MNX or TNX in silt loam soil did not significantly affect cricket egg hatching. However, 1000 mg/kg MNX or TNX did significantly affect egg hatching. TNX at 1000 mg/kg in soil completely inhibited hatching, whereas only 3.3 ± 4.7% of eggs hatched in 1000 mg/kg MNX-spiked silt loam soil (**Figure 15.7**).

15.5. Topical test: Effect of MNX and TNX on earthworm cocoon hatching
MNX and TNX significantly affected cocoon hatching in the topical test (**Figure 15.8** and **15.9**). After 9 weeks of incubation, about 50% of the cocoons hatched in the control group (without MNX or TNX). One µg/mL MNX or TNX did not significantly affect the cocoon hatching. However, 10 µg/mL MNX and TNX significantly decreased the hatching of cocoons. Adding 100 µg/mL MNX or TNX almost completely inhibited earthworm cocoon hatching.

15.6. Soil test: Effect of MNX and TNX on earthworm cocoon hatching
The effect of MNX and TNX on earthworm cocoon hatching was also investigated in sandy loam soil. The results indicated that TNX and MNX affect cocoon hatching in a concentration-dependent manner (**Figure 15.10**). However, it appears that there was no effect on hatchling survival and growth (**Figure 15.11** and **15.12**).

16.0 DISCUSSION

In these experiments, three different approaches were evaluated to determine the effect of two major RDX metabolites (MNX and TNX) on cricket reproductive success. All of the tests indicated that MNX and TNX have somewhat adverse effects on cricket reproductive success, as evidenced by egg hatching. In all experiments, TNX inhibited egg hatching more than MNX. This suggests that TNX was more toxic to cricket eggs than MNX, although the difference is not large. These toxicity results are also similar to our results on earthworms. In our previous RDX metabolite toxicity study with earthworms, it was found that the EC50s were 526 mg/kg for MNX, and 364 mg/kg for TNX in the same silt loam soil (Zhang et al., 2006a). Both the topical test and sand test were good approaches as screening tests to determine the toxicity of RDX metabolites to cricket eggs. These methods are simple, easy to control, and are relevant as examples for maximum contaminant bioavailability. Both are suitable for testing other compounds using crickets or other organisms such as earthworms.

MNX and TNX appeared to display greater toxicity in sand than in the topical test or natural soil test, as evidenced by MNX and TNX having much lower Lowest Observed Effect Concentrations (LOEC) or ECx in sand. The reason for this is possibly due to the cricket eggs being exposed to MNX or TNX isotropically in sand as opposed to the topical tests. Exposure from multiple directions may cause the eggs to absorb more contaminant. Some caution is warranted in this interpretation given the difficulty in comparing the exposure for these two somewhat different toxicity tests. For the sand and soil tests, it is important to note that the silt loam soil contained 2.5% organic matter. Organic matter sorbs MNX or TNX and may make them less biologically available

(Zhang et al., 2006b). Thus, MNX and TNX were less toxic in silt loam soil than in laboratory sand.

Embryos of the cricket (*Acheta domesticus*) were highly sensitive to chemicals (benz[g]isoquinoline-5,10-dione, benzo[h]quinoline-5,6-dione) and developed gross morphological abnormalities after exposure to a number of complex organic mixtures (Walton, 1981; Walton et al., 1983). In addition, crickets display a critical period of teratogen sensitivity and an ability to metabolize xenobiotics during development. In this experiment, although MNX and TNX inhibited cricket egg hatching under certain conditions, no gross abnormalities in cricket nymphs were observed. This indicates that MNX and TNX are not mutagens or teratogens in this assay.

Developmental stage and exposure time affected cricket hatching. In this experiment, we found that only eggs exposed to MNX or TNX for at least 30 days produced low hatching rates. There was no significant effect between the control group and the treatment groups that were exposed to MNX or TNX only for 0-10 days. This indicates that early development of cricket embryos may be more sensitive to MNX or TNX, or MNX and TNX only produce a biological affect after they accumulate to high concentrations in eggs with time.

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:

Pan, X., B. Zhang, and G.P. Cobb. 2005. Extraction and analysis of trace amounts of cyclonite (RDX) and its nitroso-metabolites in animal liver tissue using gas chromatography with electron capture detection (GC–ECD). Talanta 67:816-823.

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Zhang, B., X. Pan, G.P. Cobb, and T.A. Anderson. 2005. Use of pressurized solvent extraction (PSE) /gas chromatography-electron capture detection (GC-ECD) for the determination of biodegradation intermediates of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in soils. Journal of Chromatography B. 824:277-282.

APPENDIX

Table 15.1 Toxicity comparison of RDX metabolites (MNX and TNX) to cricket eggs based on sand test and topical test (30 days of exposure).

RDX Metabolite	EC	Sand test * µg/g	Topical test * µg/mL
MNX	20	29 (3-55)	65 (44-86)
	50	52 (33-71)	140 (110-170)
	95	99 (89-109)	253 (210-296)
TNX	20	12 (4-20)	47 (40-54)
	50	48 (41-55)	128 (114-142)
	95	97 (91-103)	247 (133-261)

Data were processed using standard SPSS software (SigmaPlot, Version 8.0.) Each measured parameter was fitted and analyzed using a linear regression model ($y = a + bx$).
* Values in parentheses represent lower and upper 95% confidence limits.

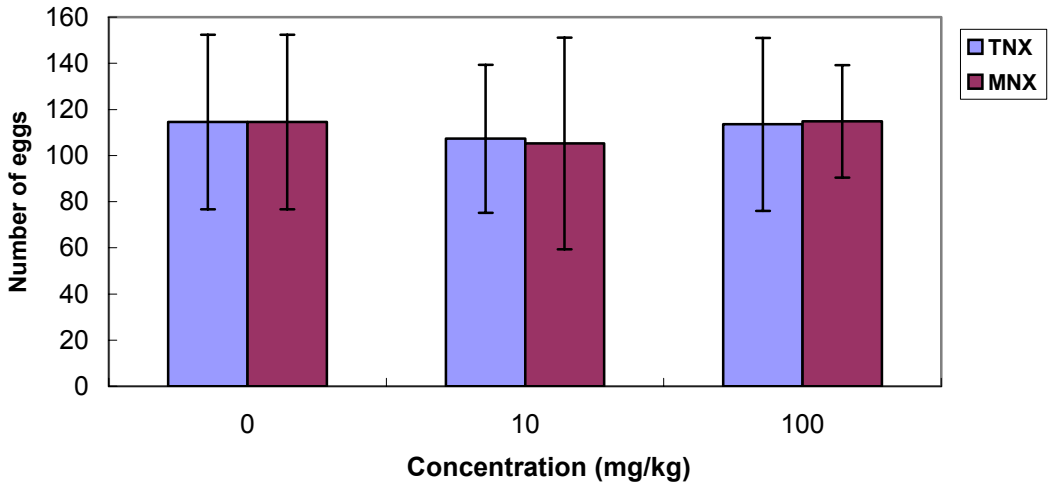

Figure 15.1 Effect of TNX- or MNX-contaminated feed on cricket egg production. Error bars indicate one standard deviation (n = 3).

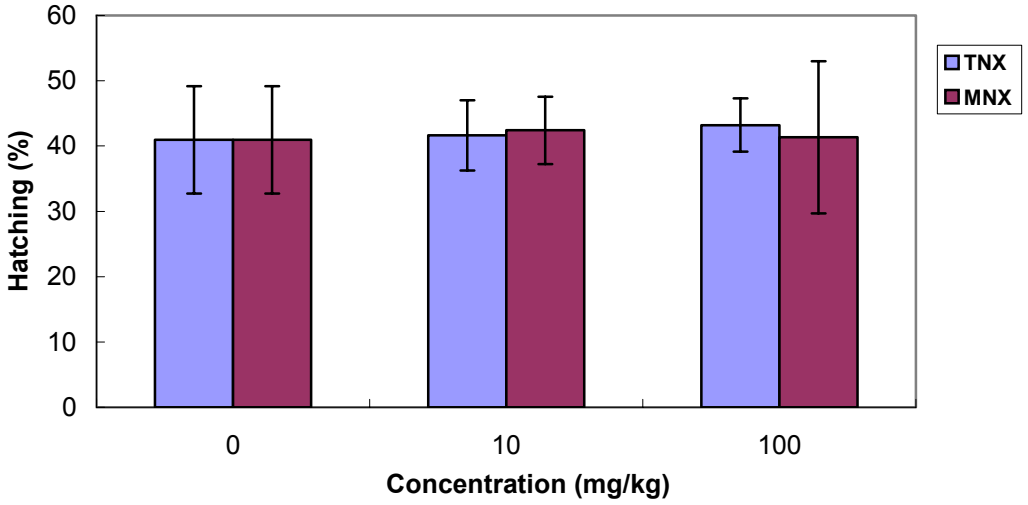

Figure 15.2 Effect of TNX- or MNX-fed crickets on egg hatching. Error bars indicate one standard deviation (n = 3).

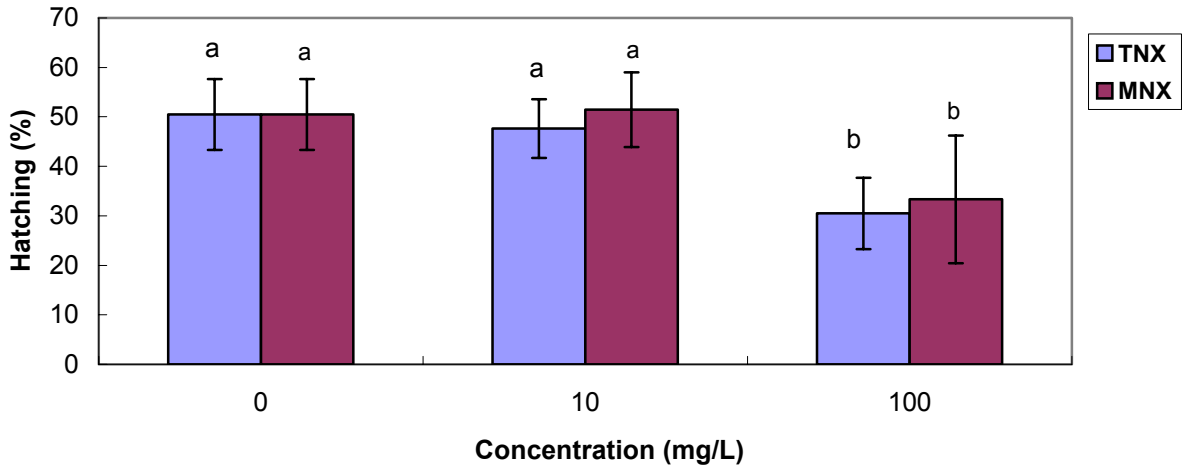

Figure 15.3 Effect of TNX and MNX on egg hatching after 30-d exposure in a topical test. Error bars indicate one standard deviation (n = 3).

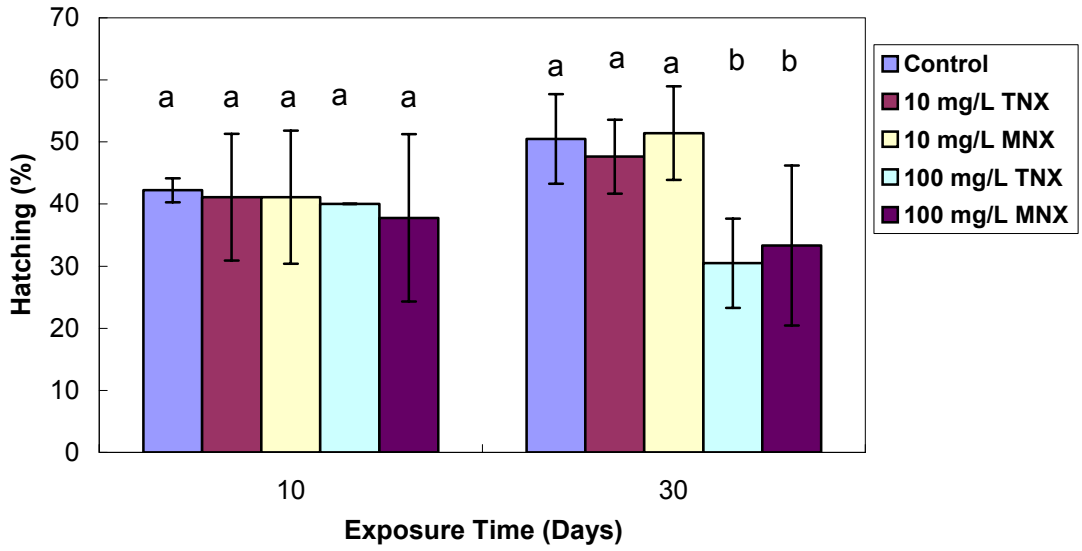

Figure 15.4 Effect of exposure time on cricket egg hatching in a topical test. Error bars indicate one standard deviation (n = 3).

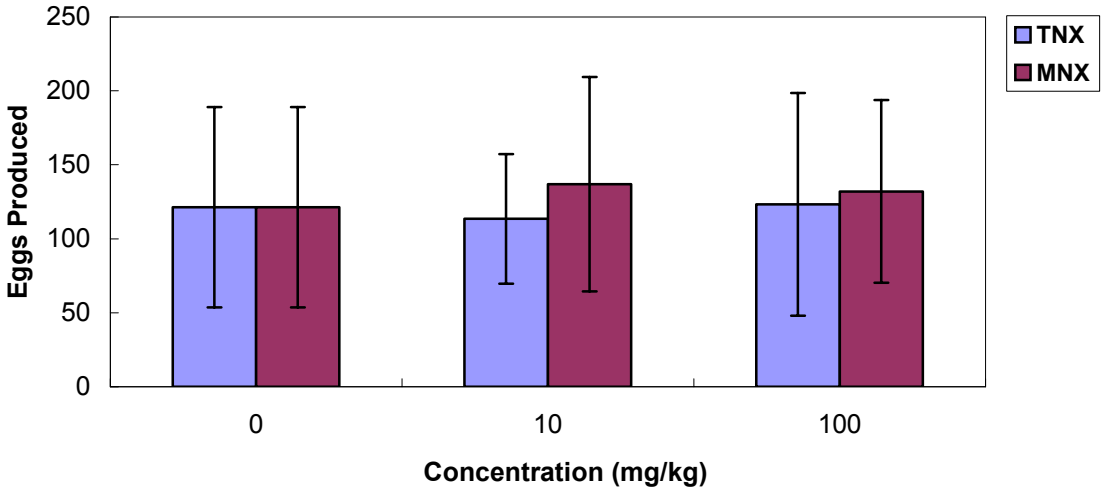

Figure 15.5 Eggs laid on TNX- or MNX-contaminated sand. Error bars indicate one standard deviation (n = 3).

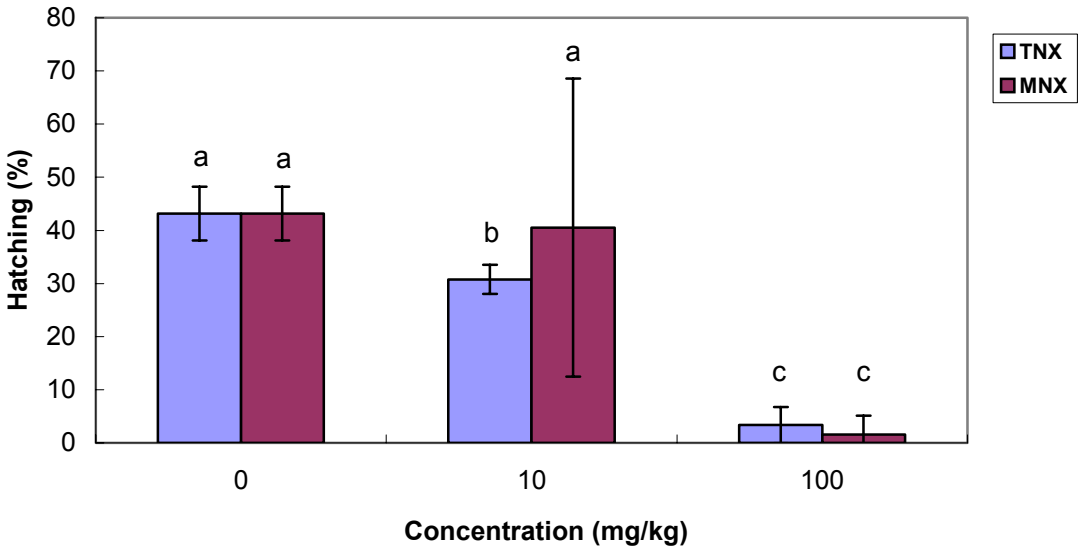

Figure 15.6 Effect of MNX or TNX on cricket egg hatching in contaminated sand. Error bars indicate one standard deviation (n = 5).

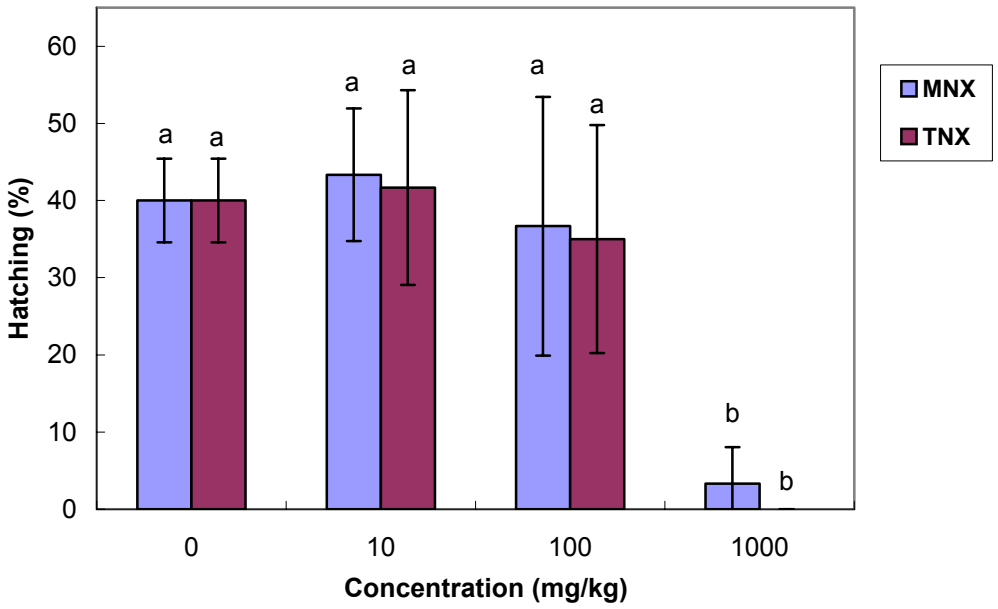

Figure 15.7 Effect of MNX and TNX on cricket egg hatching in silt loam soil. Error bars indicate one standard deviation (n = 3). 1000 mg/kg of TNX completely inhibited egg hatching.

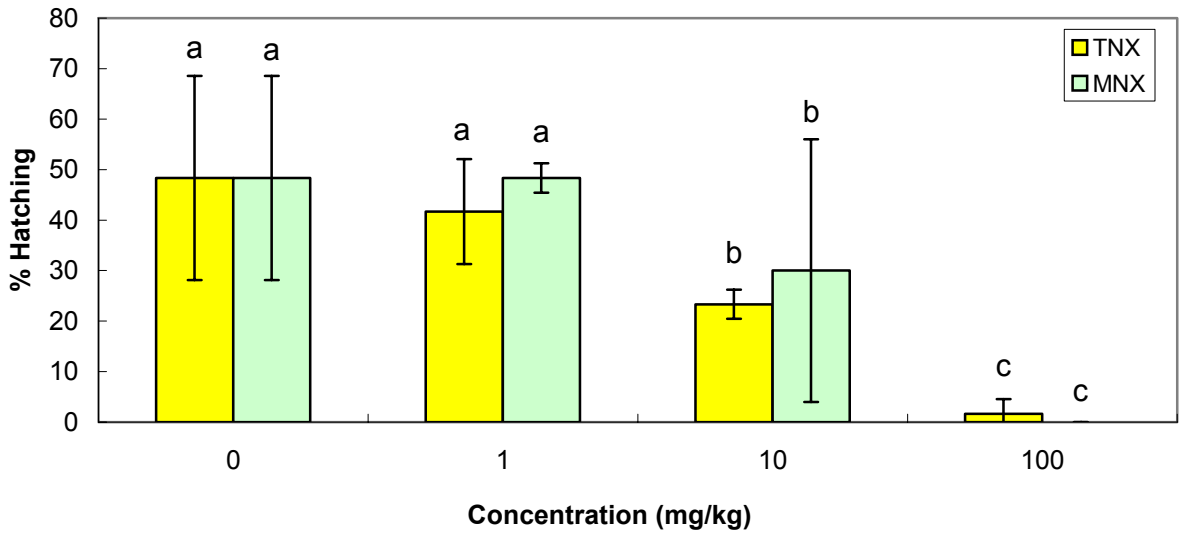

Figure 15.8 Effect of MNX and TNX on cocoon hatching in a topical test (63 day exposure).

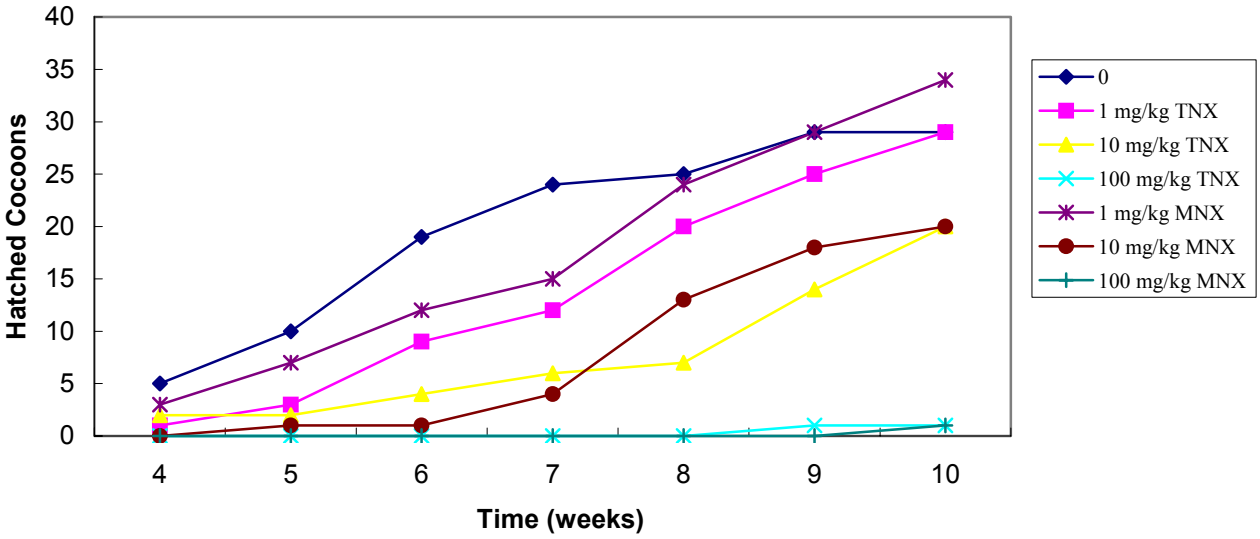

Figure 15.9 Time course of the effect of MNX and TNX on cocoon hatching in a topical test.

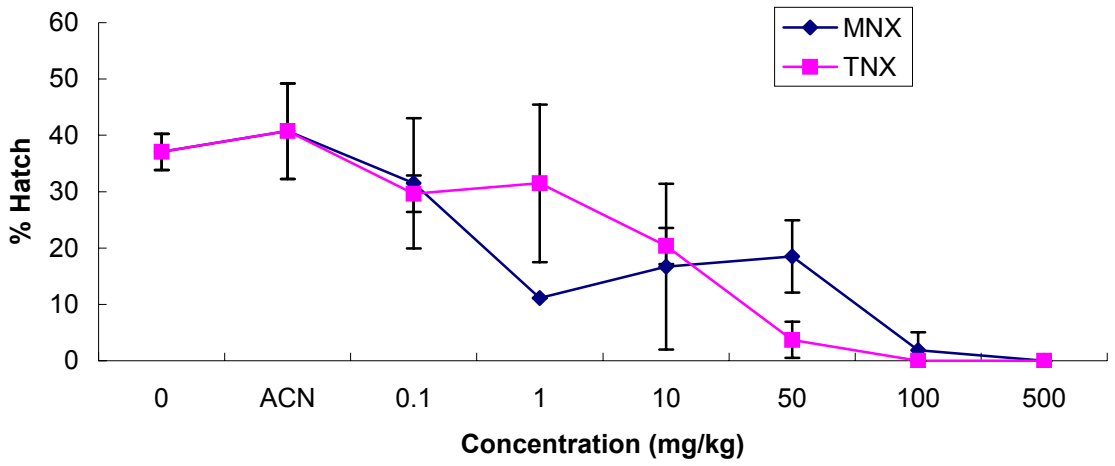

Figure 15.10 Effect of TNX and MNX on cocoon hatching in sandy loam soil (90-d exposure).

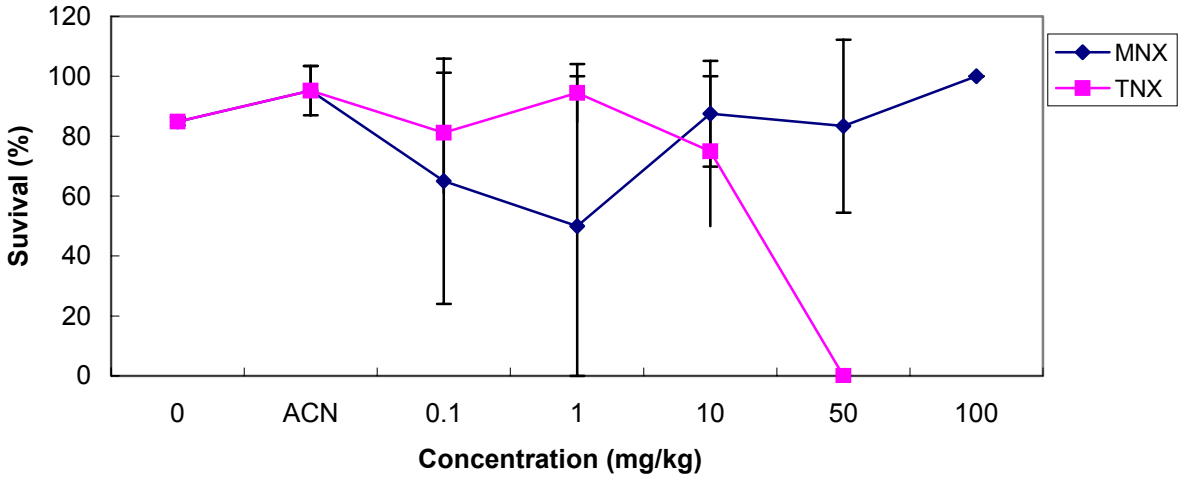

Figure 15.11 Effect of TNX and MNX on earthworm hatchling survival in sandy loam soil (90-d exposure).

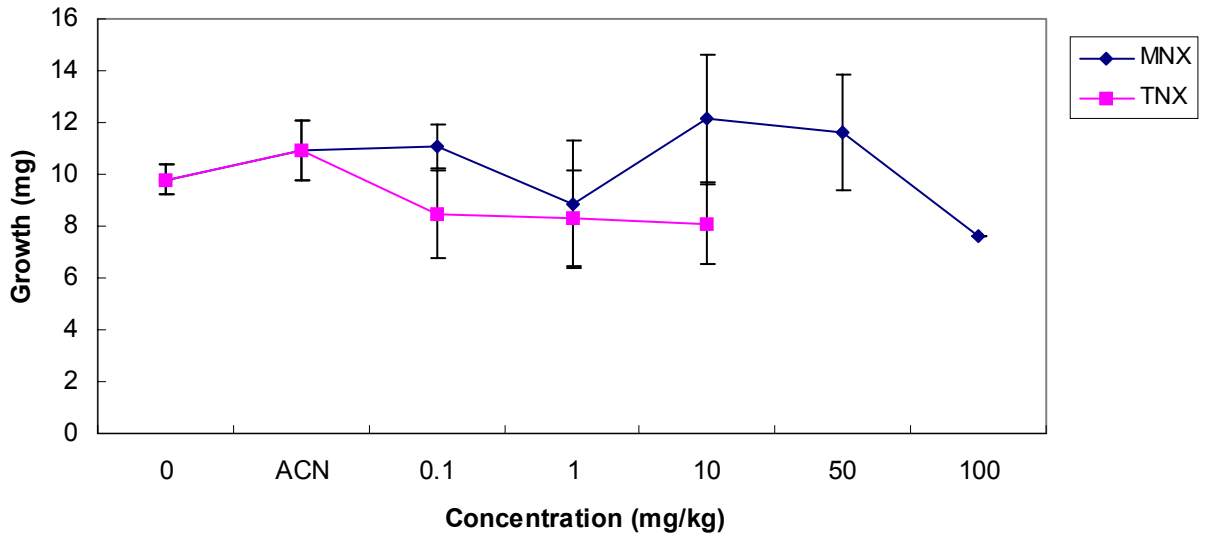

Figure 15.12 Effect of TNX and MNX on earthworm hatchling growth in sandy loam soil (90-d exposure).

Development of Polyclonal Antibody for Biomarkers of Effects following Exposure to RDX Metabolites

STUDY NUMBER:	PABE-05-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
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:	November, 2004
RESEARCH COMPLETION:	December, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in compliance with Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Ernest Smith, PhD 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 17, 1989.
Submitted By:

_____ _____
Quality Assurance Manager Date

1.0 DESCRIPTIVE STUDY TITLE:
Development of Polyclonal Antibody for Biomarkers of Effects following Exposure to RDX Metabolites

2.0 STUDY NUMBER: PABE-05-01

3.0 SPONSOR:

Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME & ADDRESS:
Animal Facility
Human Sciences Building
Texas Tech University

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: November 2004
Termination Date: December 2005

6.0 KEY PERSONNEL:
Ernest Smith, Project Manager / Co-Principal Investigator
Angella Gentles, Co-Principal Investigator
Bharath Ramachandran, Study Director
Ryan Bounds, Quality Assurance
Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:
The purpose of this sub-project is to develop polyclonal antibodies specifically for the deer mice.

8.0 STUDY SUMMARY:
In this study, deer mice were euthanized and liver quickly removed and placed in liquid nitrogen. They were then processed to isolate total RNA, which was used in reverse transcription polymerase chain reaction (PCR) to generate the cDNA for aryl hyrdrocarbon receptor (AhR)gene. A parial deer mouse specific cDNA sequence was obtained. The cDNA was used to generate a deer mouse specific probe and a set of reverse and forward primers that were utilized in real time PCR to quantitate the relative AhR gene expression in various deer mice tissue. The results show the AhR is differentially expressed in deer mouse tissue. The partial sequence was subsequently transcribed to the representative protein sequence. The protien sequence was used to generate polyclonal antibodies antisera against the deer mouse AhR protein.

9.0 TEST MATERIALS:

Peptide sequence (C)SKRHRDRLNTELDR-cooh

10.0 JUSTIFICATION OF TEST SYSTEM

Deer mice were used in this project because they are ubiquitous, opportunistic and are sentinel for wildlife. They are also easily handled, permit large sample sizes, and are more economically feasible in a study of this nature. Live animals are necessary because culture and computer models cannot simulate changes in general homeostasis. In addition, culture and computer models would not provide pertinent scientific data for future use in risk assessment.

11.0 TEST ANIMALS:

Species:	Deer Mice
Strain:	Wild type
Age:	adults
Sex:	Males
Number:	Deer mice = 10 of adults
Source:	In house breeding colony

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each cage was labeled as indicated in TIEHH SOP **IN-3-06;** label included 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 of pups, 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:

Adult animals were selected for tissue collection and isolation of mRNA. Male were selected for this procedure as we do not expect that the AhR would be different between males and females.

14.0 METHODS

14.1 TEST SYSTEM ACQUISITION, QUARANTINE, AND ACCLIMATION

Adult deer mice were obtained from our breeding colony at Texas Tech University. They were maintained in standard cages lined with sani-chip bedding and kept on a 16L: 8D light regimen.

14.2 ANIMAL EUTHANASIA AND SAMPLE COLLECTIONS

Adult animals were euthanized using carbon dioxide asphyxiation and selected tissues were quickly extirpated, wrapped in pre-labeled foil, and placed in liquid nitrogen.

RNA Isolation

Total RNA was extracted, according to manufacturer's procedure, from kidneys using Trizol Reagent (BRL, Gaithersburg, MD). The tissues were homogenized in Trizol Reagent (0.5 mg tissue/ml Trizol) and incubated at room temperature (RT) for 5 min. Chloroform (0.2 ml/ml Trizol) was then added to each tube. After this, the tubes were then shaken and incubated at room temperature (rt) for 3 min. They were then centrifuged at 12,000-x g for 15 min at 4 C. The aqueous phase was transferred to a new tube and 0.5 ml isopropyl alcohol (per ml supernatant) and incubated for 10 min at RT. These were then centrifuged at 12,000 x g for 10 min at 4 C. The supernatant was removed and the pellet washed with 75% ethanol. The pellets were air dried for 5-10 min and dissolved in 50µl nuclease-free water. The RNA concentration was then determined spectrophotometrically at 260nm.

cDNA Cloning, Sequencing and RT-PCR analysis of the deer mice mRNA

An aliquot of total mRNA (2µg) of Deer mouse mRNA was reverse transcribed, using an oligodT-primed first-strand kit (Ambion, TX) to generate deer mice cDNA for amplification and gene isolation.

For PCR amplification, reverse and forward oligonucleotide primers were designed according to the sequence for AhR in *Mus musculus*. PCR was carried out using Failsafe kit (Epicentre, WI). PCR was conducted for 40 cycles of denaturation (92 C, 30 sec), annealing (53 C, 30 sec.) and extension (72 C, 45 sec.), with a 5 min final extension. The deer mouse-AhR PCR product was sequenced for identification and verification using ABI (Perkin Elmer) DNA sequencer by Texas Tech Biotechnolgy Center.

Subsequently, the deer mice specific cDNA sequence was submitted to ABI primer design software for the development of Taqman specific probes and primers for Real Time PCR quantification of mRNA equivalents (Smith et al., 2002).

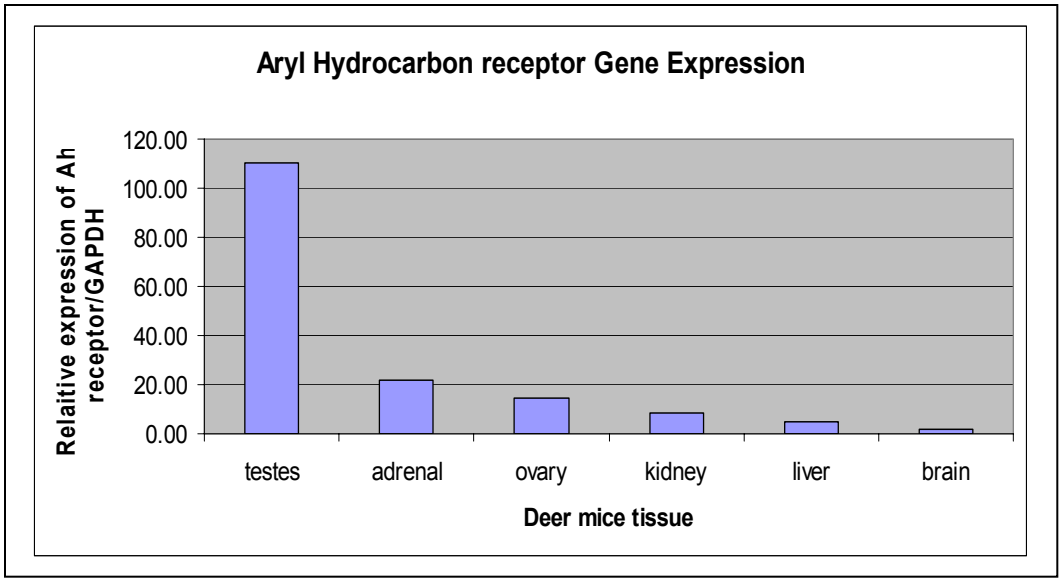
Sense primer sequence – CGC ACA TGG TAC CCA CCT GTA
anti-sense primer – GCC GGT CCC CCT CAA G
Taqman probe – 6fam-CAT GGC CGT CTC TCA CAT GAA GT - tamara

Antibody Development
Expressed deer mouse cDNA gene sequence was used to synthesize deer mouse specific protein at Zymed laboratories. The protein product was using HPLC. The purified peptide was combined with an immunogen for the development of AhR polyclonal antisera over a 90 day period of time. Polyclonal antibody titer was monitored using an ELISA protocol reading at wavelength of 405/490nm.

15.0 RESULTS

Deer mouse mRNA was isolated and successfully transcribed to cDNA. A partial cDNA sequence for AhR was generated by PCR and using deer mouse specific primers. The PCR product was sequence and compared to sequence provided by Dickerson and Frame (2004, personal communication), as outline below. Deer mouse specific real time primer set and probe were subsequently used to determine the relative expression of AhR in different tissues of the deer mouse.

Figure 1. Relative expression of AhR in deer mouse tissues.



Anti-peptide Sequence

A standard 15 residue amino acid peptide sequence (C)SKRHRDRLNTELDR-cooh *(NH-serine-lysine-arginine-histidine-arginine-asparic acid-arginine-leucine-asparagine-threonine-glutamic acid-leucine-aspartic acid-arginine-cooh*) was used for the generation of this polyclonal antibody. This peptide is equivalent to the deer mouse AhR amino acid number 35 to 48.

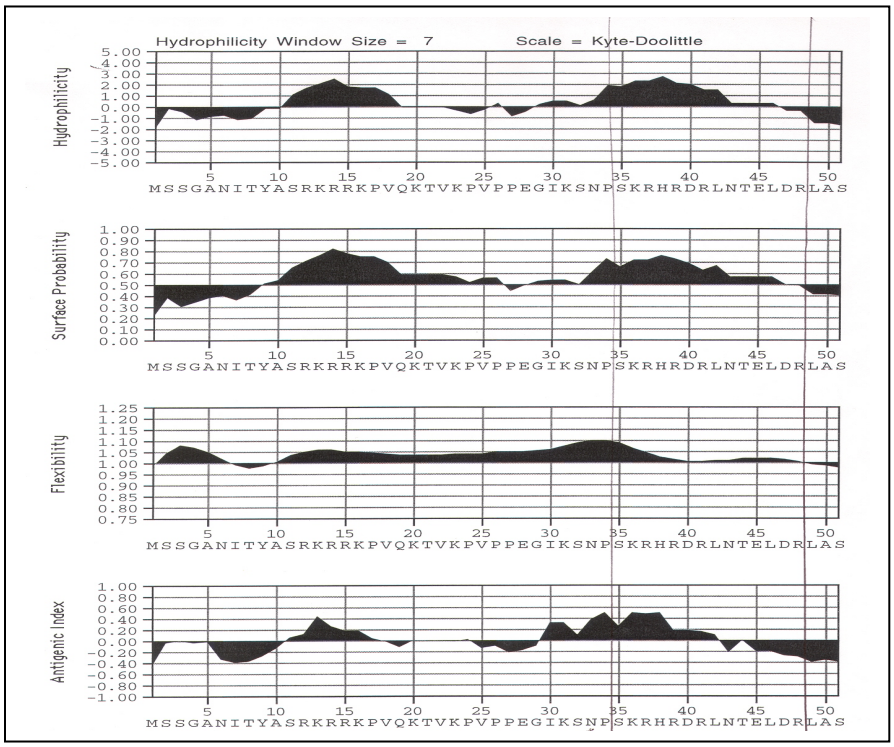


Figure 2. Protein tool box plot of selected the AhR amino acid sequence identifying the levels of antigenic index, flexibility, surface probability and hydrophilicity.

16.0 DISCUSSION

We have successfully isolated expressed aryl hydrocarbon receptor gene from the deer mouse and reversed transcribed a partial sequence to cDNA. The cDNA was used in real time quantification to determine relative expression of AhR in several deer mice tissue. Subsequently, computational analysis protein toolbox plots were used to characterize various segment of the isolated sequence for the selection of a 15 amino acid peptide. Based on hydrophilicity, surface probability, flexibility and antigenic index the following seqeuence - *(NH-serine-lysine-arginine-histidine-arginine-asparic acid-arginine-leucine-asparagine-threonine-glutamic acid-leucine-aspartic acid-arginine-cooh* - was selected as the best of three sequences that were identified for the generation of *Peromyscus maniculatus* arylhydrocarbon receptor antisera.

A specific anti-peptide antibody that is capable of distinguishing a speicifc protein from similar or related proteins is a very powerful technique for application in basic research and diagnostic applications (Yen, 2004). These antipeptides can be developed as new biomarkers of effects. We currently have the AhR antisera at the Institute of Environmental and Human Health. This resource will be shared with other members of the SERDP research team and researchers that are funded by SERDP upon request. Due to the limited quantity of the antisera for the deer mice AhR, sharing will be on a limited basis for external requests.

17.0 REFERENCE LIST

1. Dickerson R. L. and Frame, L (2004) Personal communication

2. Yen S. E. (2004). Anti-peptide Antibodies. Zymed Laboratories California.

APPENDIX

AhR sequence (Dickerson Personal communication 2004)

atggcggcgactacagctaacccagaaatgacatcagatgtaccatcgctaggttccaccattgcttctggaaaccctggacctg
ggattcaaggtggaggagctgttgtacagagggctattaagcgacgatcggggctggattttgatgatgatggagaagggaaca
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ccttttccgaatag

TITLE:	Reproductive toxicity of RDX in zebrafish
STUDY NUMBER:	ZEB-05-02
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
TEST SITE:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
ANIMAL TEST SITE:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
RESEARCH INITIATION:	August 2004
RESEARCH COMPLETION:	December 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Reynaldo Patiño
Co-Principal Investigator

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 17, 1989.

Submitted By:

_____ _____

Ryan Bounds
Quality Assurance Manager

Date

1. Descriptive Study Title:
Reproductive toxicity of RDX in zebrafish

2. Study Number:
ZEB-05-02

3. Sponsor:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. Testing Facility Name and Address:
Texas Cooperative Fish and Wildlife Research Unit
Texas Tech University
Box 42120, 218 Agricultural Science Building
Lubbock, Texas 79409-2120

5. Proposed Experiment Start and Termination Dates:

Start date:	August 2004
Termination Dates:	December 2005

6. Key Personnel:
Reynaldo Patiño, Co-Principal Investigator
Sandeep Mukhi, Study Director
George Cobb, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principal Investigator

7. Study Objectives/Purpose:
The goal of this proposed research is to generate a database for ecologically relevant, lethal and sublethal effects of RDX and its metabolites in an important group of aquatic vertebrates, the fishes. In our previous studies, we characterized the lethal (LC50) and sublethal effect of RDX on somatic growth and the bioaccumulation pattern of RDX in whole fish. In this study, we examined the sublethal effect of RDX on reproductive performance.

8. Study Summary
The objective of this study was to examine the effect of RDX on reproductive performance of zebrafish. Adult males and females were exposed to control, water, or two environmentally relevant concentrations of RDX, 0.5 and 3.2 ppm, for a period of 6 weeks. Male and female fish were exposed separately. Female fish tanks containing 8 females were the unit of replication for this study. Every two weeks, they were paired with 4 similarly-treated males and packed egg volume, egg fertilization rate and embryo hatching rate were determined. Mean packed-egg volume seemed to be increased in the 0.5-ppm group at 2 weeks of exposure but not at 4 or 6 weeks. No significant effects of

RDX on packed egg volume were noted at 3.2 ppm. Egg fertilization and embryo hatching rates were not affected at any RDX concentration during the exposure period. Overall, the results of the present study do not suggest deleterious effects on zebrafish reproductive performance when exposed to RDX at environmentally relevant concentrations. However, this study did not include measures of larval or juvenile health.

9. Test Materials:

Test Chemical name: Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)
CAS number: 121-82-4
Characterization: white powder
Purity: 99.9% pure as indicated by supplier
Stability: The chemical was found to be stable at least for 1 week in our test system
Source: Accurate Energetics (McEwen, TN, USA)

Reference Chemical name: Calcium Chloride
CAS number 10035-04-8
Characterization: coarse white powder or mixture with medium size granules.
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Magnesium Sulfate
CAS number: 100-34-99-8
Characterization: colorless crystals
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Potassium Chloride
CAS number: 7447-40-7
Characterization: white crystalline granules
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Sea Salts
CAS number: Not applicable
Characterization: an artificial salt mixture closely resembling the composition of the dissolved salts of ocean water.
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Aquarium Systems, Inc.

Reference Chemical name: Sodium Bicarbonate
CAS number: 144-55-8
Characterization: white crystalline powder

Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Sodium Chloride
CAS number: 7647-14-5
Characterization: white crystalline granules
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Ultrapure water with added salts needed by fish will be used as reference solution to which negative reference material or test material will be added for treatments.

CAS Number: Not applicable
Characterization: water quality will be tested by chemical analysis and pH will be monitored regularly.
Purity: ultrapure
Stability: stable
Source: Steam plant reverse-osmosis (RO) water that has been run through a carbon filter and a de-ionizer to convert it to ultrapure water was used in this study. To this RO water ROrite® (25 g /100 liter of RO water) was added to make suitable for zebrafish.

10. Justification of Test System:

The cyclic nitramine, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), is widely used as an explosive in commercial and military operations. It is believed that the large-scale manufacture, use, and improper disposal of RDX have led to contamination of soil and ground water by this compound and its metabolites (Sunahara et al. 1999). RDX in natural soil environments is metabolized to other compounds (Sheremata et al. 2001). RDX metabolites may include hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). RDX and its metabolites do not strongly adhere to soil particles, and there is a high potential for leaching from contaminated soils into surface waters. Among fishes, the number of species for which the acute (lethal) toxicity of RDX has been tested is small (Bentley et al. 1977).

In previous studies (DoD-SERDP, Phase VI), we examined the acute (lethal) toxicity of RDX (Mukhi et al., 2005b) as well as its sublethal toxicity on the growth and bioaccumulation in zebrafish (Mukhi et al., in review). The purpose of the present study (DoD-SERDP, Phase VII) is to examine the sublethal effects of RDX on the reproductive health of adult fishes. The model organism for the present study continues to be the zebrafish (*Danio rerio*). Zebrafish are frequently used in biomedical research and have the advantage of a wealth of information concerning its genetics and developmental biology. Also, the zebrafish genome sequencing project and the availability of commercial cDNA microarrays allow for the convenient application of current tools in molecular biology to this species. Further, zebrafish are easy and economical to maintain in the laboratory, and we have prior expertise and experience using this species for toxicological research.

11. Test Animals:

Species: *Danio rerio*, Zebrafish
Strain: Wildtype
Age: Adult zebrafish
Number: 72 females and 36 males (2:1 ratio of female to male)
Source: Purchased from local vendor

12. Procedure for Identifying the Test System:

The experimental units (aquarium) were labeled with the study protocol number, ACUC number, chemical and its concentration and the contact person name on it. There were three replicates for each treatment and each treatment was color coded for easy visual identification. Due to anticipated difficulty in identifying the males from the females after spawning, males were tagged with Visible Implant Elastomer (VIE) at the base of dorsal fin according to the manufacturer's protocol (Northwest Marine Technology, Shaw Island, WA, USA).

13. Experimental Design Including Bias Control:

Adult male and female zebrafish were exposed to RDX using a static renewal system according to contaminant exposure procedures previously developed in our laboratory (Mukhi et al. in review). The basic experimental design included control (no RDX), low (0.5 ppm) and high (5 ppm) nominal RDX concentrations. Each exposure was conducted in triplicates aquaria for females and single aquarium for males. The exposure period was 6 weeks. Once in 2 weeks during the exposure, spawning containers were placed in the female tanks and the 8 female fish in each tank will be combined with 4 males from the male exposure tank. Reproductive success was assessed by measuring packed-egg volume, fertilization rate and hatching rate. Cumulative packed-egg volumes were analyzed as previously described (Patiño et al., 2003).

Number of female fish required
8 females/replicate x 3 replicates x 3 treatments x 1 chemical = 72

Number of male fish required
12 male/replicate x 1 replicate x 3 treatment = 36

Total number of adult zebrafish required = 108

14. Methods

14.1. Chemical, Safety Procedures and chemical analysis

Hexahydro-1,3,5-trinitro-1,3,5-triazine (CAS Reg. No. 121-82-4) was obtained from Accurate Energetics (McEwen, TN, USA). The chemical was 99% pure and supplied in desensitized form, containing 20% water by volume. Standard solutions for RDX were obtained from Supelco (Bellefonate, PA, USA). RDX was stored in a specially designed bunker on campus and handled with utmost care to prevent spark or shock which could trigger explosions. RDX was transported from the bunker to the study facility in the desensitized form. Water samples from the treatment tanks were analyzed for actual RDX concentrations by high performance liquid chromatography (HPLC) according to Mukhi et al. (2005a).

14.2. Experimental animal and standard rearing condition

The use of animals in this study was reviewed and approved by the Texas Tech University Animal Care And Use Committee (Lubbock, TX, USA). Four-month-old, adult wildtype zebrafish (*Danio rerio*) were obtained from local vendor (Lubbock, TX, USA) and allowed to acclimatize to our laboratory condition for a period of three weeks before the spawning trials began. As the objective of this study was to evaluate the reproductive toxicity of RDX, male and female zebrafish were reared separately. Eight females or 15 males were randomly distributed into each of 9 or 3 10-gallon aquarium respectively. Animal husbandry procedures for this study were as described by Mukhi et al. (2005a). Briefly, each aquarium was filled with 30 L of system-water (25 g of ROrite/100 liter of reverse-osmosis supply water) and fitted with two hand-made internal biofilters. Each aquarium was marked at 15-L and 30-L volume level to facilitate 50% or 100% water exchange during experimentation. A water current through the filter was maintained by airflow via glass pipette. The tanks were added with stresszyme in weekly interval to maintain a biofilm in the biofilter. Water quality parameters were maintained at recommended levels for zebrafish (pH 6.5-8.0, 26-28.5 ºC, 12/12 light/dark cycle). If the pH in the treatment tanks fell below 6.5, appropriate volume of 5-M NaOH solution was added to aquarium to bring within the optimal range. Fish were fed either with adult frozen *Artemia* or Tetramin® flakes (Tetra Sales, Blacksburg, VA, USA) twice daily to satiation. Every evening, leftover food and fecal material were removed by siphoning. Temperature and pH was measured daily and dissolved oxygen, specific conductivity, salinity, unionized ammonia and nitrate was measured at least once weekly. Half of the water volume (15 L) was removed and replaced with clean system-water twice weekly.

Due to anticipated difficulty in identifying the males from the females after spawning, males were tagged with Visible Implant Elastomer (VIE) at the base of dorsal fin according to the manufacturer's protocol (Northwest Marine Technology, Shaw Island, WA, USA). Males were allowed to recover for at least 2 weeks after tagging before the start of spawning.

14.3. RDX exposure

Two environmentally relevant, nominal concentrations of RDX (0.5 and 5 ppm) were chosen for the experiment in addition to a control group. Female tanks were considered the unit of replication, and each treatment was conducted in triplicate for females (8 fish per tank). Males for each treatment were maintained together in a single tank (12 fish per tank). Therefore, the experiment consisted of 9 female-tanks and 3 male-tanks. A static renewal exposure procedure was followed as described earlier (Mukhi and Patiño, 2005). Briefly, every week, 50 percent of treatment water in each aquarium was renewed twice with preheated-aerated-fresh treatment water from the overhead tanks. Water samples (2-3 ml) were collected from each tank once a week before the second water exchange for verification of actual RDX concentrations in treatment tanks. Fish feeding and swimming behaviors were observed daily and any sign of abnormal behavior was recorded.

14.4 Measurement of Somatic Growth

Fish wet-weight was measured by placing the fish (all 8 females per tank or all 12 males per tank) in a pre-zeroed, 1-L beaker with water. The weight was taken one day prior to placement in the spawning chambers in order to minimize stress, which could affect spawning performance. Also, the weight was measured before the evening meal. As male tanks were not replicated, their weight was not used for statistical analysis.

14.5. Effect of RDX on packed-egg volume

The spawning procedure was as described as Patino et al. (2003). Briefly, plastic shoe boxes (30 cm long X 15 cm wide X 5 cm deep) coated with silicon were used as spawning chambers. The spawning unit consisted of an upper chamber for holding the fish and a lower chamber for collecting the eggs. Holes were drilled on the side walls of the upper chamber to allow air exchange to occur, and its bottom was replaced with a silicon-coated plastic mesh to allow the eggs to fall into the lower chamber. The day before spawning, fish were fed only the morning meal and a single spawning container was placed in each female fish tank. The 8 females from that aquarium and four males from the same RDX-treatment tank were placed into the spawning chamber. The following morning, fish were removed from the spawning container and returned to their original tanks approximately 2.5 hours after lights on. Fecal matter and other debris were removed from the egg slurry using pipettes and by rinsing 4 times with fresh zebrafish water. Packed-egg volume was determined by volume displacement in a graduated 5-ml glass cylinder.

The first spawning trial was conducted one week before the initiation of RDX treatment. The purpose of this spawning was to confirm spawning readiness and to synchronize reproductive cycles. The fish were then spawned once every 2 weeks after the initiation of RDX exposure. Therefore the packed-egg volume was measured at 1 week prior to exposure and 2, 4, and 6 weeks after initiation of exposure. One female was lost from one of the 5-ppm tanks following the first spawning; therefore, this replicate was eliminated from the study.

Rates of fertilization and hatching rate were also calculated. For this purpose, approximately 100 eggs from each treatment replicate were incubated in 500-ml beakers containing 300 ml of zebrafish water at 28 °C. After 6-8 hours of fertilization, unfertilized eggs became white (opaque) and were removed from the incubation beaker and counted. The fertilized, translucent eggs were counted once and kept in the same beaker until hatching. Fifty percent of the water in the beakers was replaced with fresh-preheated (28°C) system water. Three days after spawning, the number of unhatched eggs was determined in each beaker. Percent of hatching was calculated from the total fertilized eggs present in the beaker at the beginning.

14.6. Data analysis

The weight of female fish was measured as group-weight (8 fish), and the packed-egg volume was measured in each tank replicate. Thus, in all cases, sample size per treatment for statistical analyses is the number of tank replicates (n = 3). Unless otherwise noted, the effects of waterborne RDX (concentration and exposure time) on somatic condition, egg volume, fertilization rate and hatching rate were initially

assessed by two-way analysis of variance (ANOVA). Fertilization rate and hatching rate were subjected to arcsine transformation before analysis. The effects of RDX exposure on these endpoints at each exposure period were then analyzed with one-way ANOVA followed by Duncan's multiple range test (Statistica®, StatSoft, Tulsa, OK, USA). These analyses were performed at the level of significance of $\alpha = 0.05$. The average value for each parameter in the text and figure has been mentioned as the average of three replicates ± Standard Error. Behavioral observations were qualitatively documented and assessed.

15. Results

15.1. RDX in experimental tank

The average measured concentrations of RDX in the experimental tanks were 0±0, 0.5±0.1 and 3.2 ± 0.2 ppm in control, 0.5 and 5 ppm nominal concentration group, respectively.

15.2. Effect of RDX on weight

Two-way ANOVA (treatment X time of exposure) indicated that only treatment had an effect on weight of females ($p<0.05$); whereas time and interaction between treatment and time had significant effect on growth. Mean weight of females did not differ one week prior to RDX exposure ($p>0.05$) and RDX did not affect their weight at 2 and 6 weeks after the onset of exposure ($p>0.05$, 1-way ANOVA); however, mean weights differed at 4 weeks of exposure ($p<0.05$, 1-way ANOVA; Figure 1). The weight of females in the 0.5-ppm treatment group was significantly higher ($p<0.05$) than the 3.2-ppm group.

15.3. Effect of RDX on feeding and behavior

Fish aggregation behavior during feeding was recorded qualitatively. In the control and 0.5-ppm treatment groups, feeding activity was not affected at any point during the experiment. However, feeding activity was reduced in the 3.2-ppm female treatment group within one day of starting the exposure, and in the single male tank suppressed feeding activity was evident after one week. The effect on feeding behavior was temporary and only lasted until the end of the second week of exposure. However, males and females from the 3.2-ppm treatment group also showed aggressive (fighting and chasing) beginning after the second day of exposure, and this behavior persisted throughout the exposure (6 weeks).

15.4. Effect of RDX on packed-egg volume

Two-way ANOVA (treatment X length of exposure) indicated that treatment ($p< 0.05$) and interaction between the treatment and length of exposure ($p<0.05$), but not length of exposure ($p>0.05$) had significant effects on packed-egg volume. One-way ANOVA for treatments at each spawning period indicated that the treatment effect was significant ($p<0.05$) only at 2 weeks of exposure. RDX stimulated egg production ($p<0.05$, Duncan's multiple range test) in the 0.5-ppm treatment group at 2 weeks of exposure compared to the control and 3.2-ppm exposure group (Figure 2). Egg production seemed to decrease in the 3.2-ppm group relative to

controls, but this decrease was not statistically significant. Packed-egg volume in the 0.5-ppm treatment group subsequently decreased from its peak value at 2 weeks, and was not different from the control at 4 and 6 weeks of exposure.

Egg volumes for each treatment replicate (excluding the packed-egg volume before RDX exposure) were added to calculate the cumulative packed-egg volume in each treatment. Results of one-way ANOVA indicated that treatment had an effect on the cumulative packed-egg volume ($p<0.05$); RDX at 0.5 ppm caused a higher cumulative egg volume relative to RDX at 3.2 ppm, with a trend to be also higher than control values (Figure 3).

15.5. Effect of RDX on fertilization and hatching rate

Values for percentage fertilization and percent hatching were subjected to an arcsine transformation to achieve homogeneity of variances. Two-way ANOVA (treatment X length of exposure) showed no treatment or interaction effects ($p>0.05$) either on fertilization rate or hatching rate, but length of exposure had an effect on fertilization rate with a slight length-dependent increase being apparent in all treatment groups ($p<0.05$, Figure 4).

16. Discussion

Contamination of the environment with military waste, including RDX, has been reported in various parts of the world (Small and Rosenblatt 1974, Aller 1985, Walsh and Jenkins 1992). In water bodies, the concentration of RDX varies widely and may go up to 109 ppm (Ryon et al. 1984). These high concentrations of RDX may be causing toxic effects and adversely impacting the aquatic biota. To our knowledge, this is the first report of the effect of RDX on reproductive performance in fish. The concentrations chosen for this study (1 and 3.2 ppm verified concentration) are well within environmentally relevant concentrations.

Exposure to RDX has been shown to affect behavior and body weight in different species including zebrafish (Mukhi et al. in review). In an earlier study, we observed that RDX at 9.6 ppm affected feeding behavior temporarily. Similar results were obtained in the present study, where feeding behavior was temporarily affected by exposure of zebrafish to 3.2 ppm RDX. However, a novel finding of the recent study was the appearance of aggressive starting about 2 weeks after initiation of exposure to RDX; this behavior lasted until the end of the experiment (6 weeks). In an earlier study, we observed that the RDX at 1 ppm and 9.6 ppm reversibly reduced the weight of zebrafish after 4 weeks of exposure, with full recovery from this effect after 12 weeks of exposure. In the present study, we did not observe loss of weight in the 3.2-ppm treatment females compared to control at any point of exposure period. It is possible that the alteration in feeding behavior is not significant enough to cause a reduction in feed intake in the females. Information on the effect of RDX on reproductive performance is limited to few species.

RDX seems to be toxic to reproductive success in earthworm (*Eisenia Andrei*; Robidoux et al., 2002), enchytraeid worms (*Enchtraeus albidus*, *E. crypticus*; Dodard et al., 2005) and northern bobwhite (*Colinus virginianus*; Gogal et al., 2003). However, RDX showed no adverse reproductive effects in rats exposed to concentrations as high as 50 ppm

RDX/kg in the feed. In the present study, no deleterious effects of RDX on spawn volume were found; in fact, the volume of egg produced in the 0.5-ppm treatment group seems to have increased at 2 weeks of exposure. This observation suggests that RDX at low concentration (0.5 ppm in this experiment) has some stimulatory effect on egg production early during the period of the exposure. Moreover, the stimulatory effect of egg production was short lived. The biological relevance of this observation is uncertain at this time. Stimulatory effects of certain chemical contaminants on the reproductive performance of fishes have been previously reported; this effect has been described as "hormesis" (Calabrese and Blain, 2005).

Maternal exposure to RDX did not affect egg fertilization and embryo hatching rates. At the present time, we are unaware of information available for other species concerning effects of parental exposure to RDX on embryo development. There is also no information available to assess whether RDX can be transferred the mother to the embryo.

Overall, the results of the present study do not suggest deleterious effects on zebrafish reproductive performance of exposure to RDX at environmentally relevant concentrations. However, this study did not include measures of larval or juvenile health.

17. 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 the completion date of the study.

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19.0 Figures

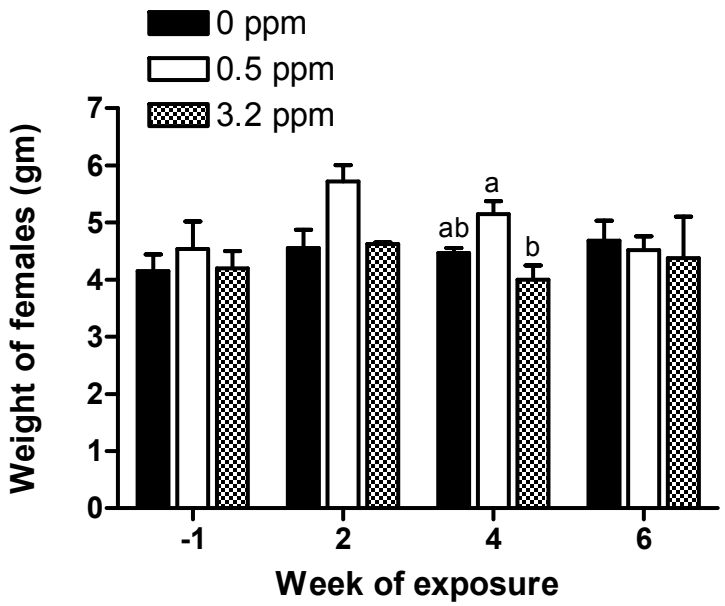

Figure 1: Effect of RDX on weight of female fish. Treatment with RDX, but not length of exposure, affected female weight (2-wat ANOVA). Bars associated with common letters are not significantly different (Duncan's multiple range test).

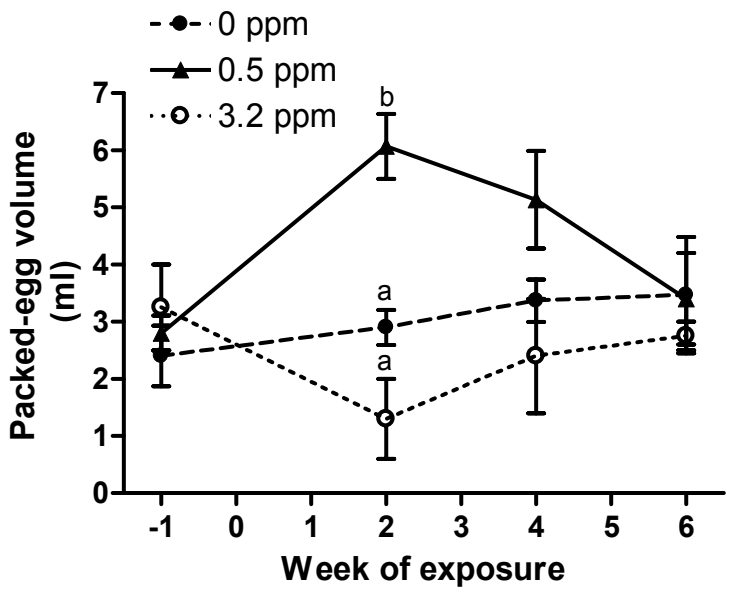

Figure 2: Effect of RDX on packed-egg volume. RDX stimulated egg production in the 0.5-ppm treatment group at 2 weeks of exposure (one-way ANOVA and Duncan's multiple range test; $p<0.05$). Although RDX reduced the egg production (almost by half) in 3.2 ppm group at 2 weeks, the effect was not significant.

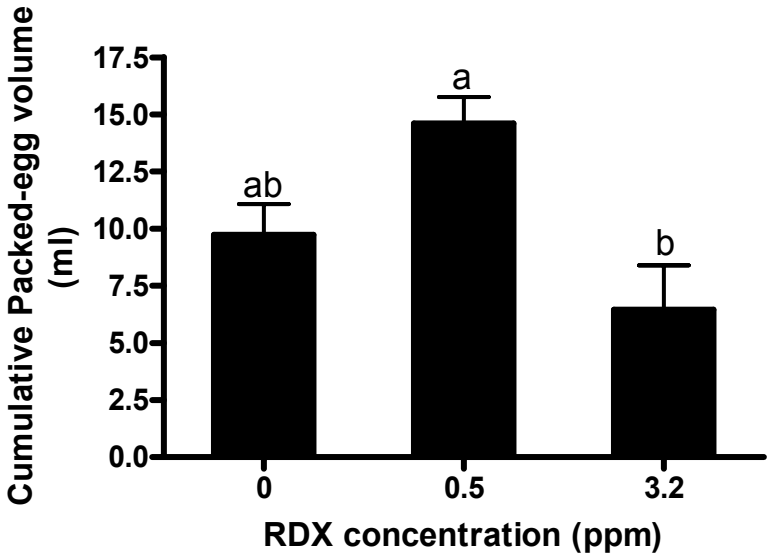

Figure 3: Cumulative effect of RDX on packed-egg volume. Cumulative packed-egg volume in the 0.5-treatment was significantly higher than 3.2 ppm group, whereas the cumulative packed-egg volume in the two RDX treatment group was not different from control (one-way ANOVA and Duncan's multiple range test; $p<0.05$).

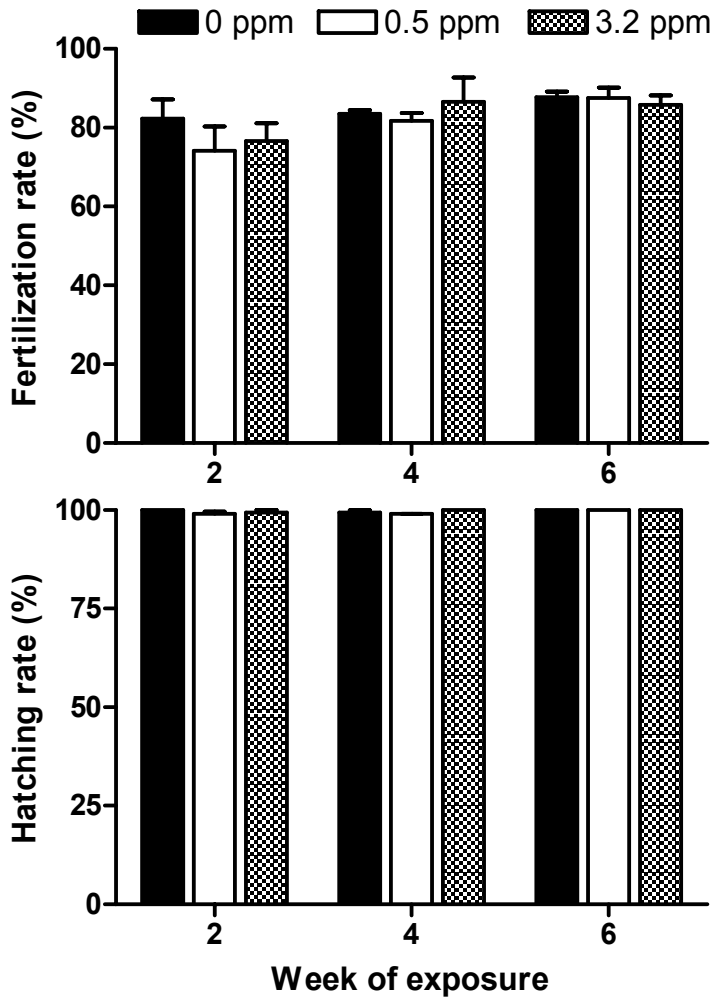

Figure 4: Effect of RDX on egg fertilization and embryo hatching rates. Parental exposure to RDX had no significant effect on these endpoints ($p>0.05$).

TITLE: Effects of explosive mixtures on Fathead Minnows and Larvae of *Xenopus laevis*

STUDY NUMBER: EXP-05-01

SPONSOR: Strategic Environmental and Research Development Program

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RESEARCH INITIATION: January 1, 2005

RESEARCH COMPLETION: December 31, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Ernest Smith
Co-Principal Investigator

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 17, 1989.

Submitted by:

_____ _____

Ryan Bounds
Quality Assurance Manager

Date

1.0 DESCRIPTIVE STUDY TITLE:
Effects of explosive mixtures on Fathead Minnows and Larvae of *Xenopus laevis*

2.0 STUDY NUMBER:
EXP-05-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
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4.0 TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University / Texas Tech University Health Sciences Center
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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: January 01, 2005
Termination Date: December 31, 2005

6.0 KEY PERSONNEL:
Mike Wages, Study Director
Ronald Kendall, Testing Facility Management/Principal Investigator
Ernest Smith, Co-Principal Investigator
Ryan Bounds, Quality Assurance Manager

7.0 STUDY OBJECTIVES / PURPOSE:
To determine the acute toxicity and effects of the mixtures of HMX, RDX, TNX, and TNT on growth and development of *Xenopus laevis* and fathead minnows (*Pimephales promelas*) larvae.

8.0 STUDY SUMMARY:
Xenopus laevis larvae were exposed to mixtures of RDX + HMX, RDX + TNX, and HMX + TNX in separate experiments. RDX and HMX were tested at saturated concentrations. Six concentrations of TNX were used in combination with RDX or HMX. *Xenopus* larvae were exposed to these contaminants starting at Nieuwkoop-Faber (NF) stages 8-10, and exposure was terminated at 96 hours. During the exposure and at termination, the number of dead and malformed embryos was counted. There were no effects of the combination of RDX + HMX on survival or development in this study. However, the combination of TNX +RDX and TNX+HMX resulted mortality, only at concentrations greater than 1000 ppm TNX plus saturated HMX. Similar results were observed for 1000 ppm TNX in combination with saturated RDX solution. No mortality was observed below 100 ppm TNX in combination with RDX or HMX. Based on the

lack of toxicity for the mixture of HMX and RDX (saturated solutions) and the information from the SAP at the summer 2005 meeting the evaluation of mixtures of these chemicals was not pursued any further.

Fathead minnow larvae were exposed to 7 concentrations (0.01, 0.1, 1, 10, 50, 100 and 1000 ppm) of TNX. At 10 days post-hatch, the larvae were exposed to TNX for 96 hours. During the exposure and at termination, the number of dead and malformed embryos was counted. TNX resulted in 100% mortality at 100 ppm and above. In comparison, 50 ppm resulted in 10% mortality. The significance of the mortality at 50 ppm was negated by a 20% mortality in the untreated controls. There was no mortality observed following exposure to saturated solutions of mixtures of RDX and HMX. Based on the lack toxicity at the point of saturation for HMX and RDX and the suggestion from the SAP at the summer 2005 meeting the evaluation of mixtures of these chemicals was not pursued any further for fathead minnow larvae.

9.0 TEST MATERIALS:

Test Chemical name: cyclotetramethylene-tetranitramine (HMX)
CAS number: 2691-41-0
Characterization: Determination of concentration in water samples.
Source: Aldrich Chemical Company

Test Chemical name: cyclotrimethylenetrinitramine (RDX)
CAS number: 121-82-4
Characterization: Determination of concentration in water samples.
Source: Aldrich Chemical Company

Test Chemical name: hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)
CAS number: 13980-04-6
Characterization: Determination of concentration in water samples.
Source: SRI International

Test Chemical name: trinitrotoluene (TNT)
CAS number: 118-96-7
Characterization: Determination of concentration in water samples.
Source: Aldrich Chemical Company

Reference Chemical name: FETAX medium will be prepared using de-ionized, carbon filtered water and reagent grade salts (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).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: Reverse osmosis and de-ionizer treated City tap water was used to prepare FETAX solution.

10.0 JUSTIFICATION OF TEST SYSTEM:

In natural systems, aquatic organisms are exposed to complex mixtures of contaminants. However, the toxicity of mixtures of explosive compounds has not been thoroughly examined in aquatic species. This is significant for DoD sites contaminated with explosives, because such contamination often consists of mixtures of RDX and HMX, and, when RDX breaks down, it may coexist with its metabolites (Hovatter et al. 1997, Sheremata et al.2001, Sunahara et al.1999). However, the toxicity of mixtures of explosive compounds and their metabolites has not been thoroughly examined in aquatic species.

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

Species: African clawed frog (*Xenopus laevis*) and fathead minnows (*Pimephales promelas*).

Strain: Outbred

Age: Larvae

Number: Approximately 2400 (*Xenopus*) and 200 (Fathead minnows)

Source: Xenopus were bred from captive stocks currently maintained in our laboratory and fathead minnow were obtained from a commercial vendor.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Glass Petri dishes were labeled, species name, animal use protocol number, project number, test system, and date of hatch.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Glass Petri dishes were labeled with treatment, species name, animal use protocol number, project number, test system, and date of hatch. Randomization was used to minimize bias.

14.0 METHODS:

14.1 Animal Selection and Receipt

Animals used were selected from in-house breeding colonies. Animals were selected for breeding if they were not previously bred within the last 60 days. Fathead minnows were obtained from a commercial vendor.

14.2 Assignment of Animals to Study Group and Identification

Larvae were placed into Petri dishes labeled with test chemical and concentration and identified by test group since identification of individual animals is not possible at this stage of development.

14.3 Acclimation

Xenpous larvae did not require acclimation. Fathead minnows were acclimatized for approximately 5 days.

14.4 Animal Husbandry

Animals were kept in water supplemented with 60 mg/L commercially available aquarium sea salts for adults (Goleman et al. 2001). Larvae were kept in FETAX solution, specifically formulated for the Xenopus larvae at this stage of development, according to ASTM (1998). One third of the FETAX solution was changed at least every other day. Aquaria were covered with plastic mesh to prevent escape. To breed frogs for egg production, adult males were injected with 300 and adult females were injected with 750 units of human chorionic gonadotropin dissolved in sterile water into the dorsal anterior lymphatic sac, and returned to tanks. After injection, one male and one female were placed in a 5.5 gallon aquarium with a false bottom (plastic-coated hardware cloth) to allow eggs to sink to the bottom without being eaten. Frogs were kept at a water temperature of 23º C (the preferred temperature of this tropical species) at a 12:12 h light;dark cycle. Water chemistry (pH, dissolved oxygen, ammonia, nitrate, nitrite, temperature) were monitored every other day, using a water quality meter (YSI, Inc.) and a spectrophotometic-based water quality kit (HACH, Inc.). Fathead minnows were kept in sea salt treated water without further modification.

14.5 Test Material Application

Test solutions consisted of RDX + TNX, HMX + TNX, and RDX + HMX. RDX, HMX, and RDX + HMX were dissolved in control medium (FETAX), and applied from stock solutions. Stock solutions consisted of saturated solutions of HMX or RDX. Eggs/embryo (Nieuwkoop -Faber [NF] stages 8-10, Nieuwkoop and Faber 1967) were placed into pre-cleaned Petri dishes and the hatched larvae were allowed to develop for 96 hours while being exposed to toxicants. Dishes were cleaned by washing according to SOP AQ-1-23 “Cleaning Glassware for Use with Xenopus laevis”, and all Petri dishes were baked at 250° C for 4 hours before use. For exposures, dishes were located in a Rubbermaid plastic tub in a controlled-temperature room. The arrangement of the dishes within the tub was randomized in order to avoid effects due to gradients in light and temperature in the laboratory, etc.

The overall experimental design consisted of range finding tests, in which the larvae were exposed to RDX + HMX, RDX + TNX, and HMX + TNX according to the following scheme:

TNX:

<u>Range finding tests</u>: 10 larvae/replicate x 2 replicates per treatment x 8 treatments

Treatment groups consisted of non-treated controls (FETAX), RDX-alone or HMX-alone (saturated solutions) and 6 concentrations of TNX combined with either RDX or HMX saturated solutions.

RDX + HMX
Range finding tests: 10 larvae/replicate x 2 replicate per treatment x 8 treatments
Treatment groups consisted of non-treated controls (FETAX), RDX-alone or HMX-alone (saturated solutions) and the saturated solution of RDX combined with HMX.

14.6 Food and Water Trace Contamination

Not determined.

14.7 Daily Observations

Each day, all Petri dishes were examined for dead and malformed embryos.

14.8 Euthanasia

At the end of the exposure, all animals were euthanized by immersion in 0.5 g/L MS222

14.9 Sample Collection

Tadpoles were collected at the end of exposure

14.10 Sample Analysis

TNX 1000 ppm was measured at 952.19 ppm.

RDX at water saturation was measured at 38.7 ppm.

HMX at water saturation was measured at 6.4 ppm.

15.0 PROTOCOL CHANGES / REVISIONS:

No protocol changes were made in this study

16.0 RESULTS:

Exposure to TNX at 0.01, 0.1, 1.0, 10, 100 and 1000 ppm in combination with either RDX or HMX resulted in toxic effects at concentrations above 100 ppm TNX. Similar results were observed for the combination of TNX above 100 ppm with HMX. The solutions of RDX and HMX tested in combination with TNX in this study were saturated solutions of RDX and HMX. Treated animals displayed stunted growth, edema, poor hatchability, severe malformation and mortality. In most cases the embryos did survive beyond 24 hours of hatching. Unlike the TNX, combination with either RDX or HMX, no toxic effects (neither mortality nor deformities) were observed when the animals were treated with the combination of RDX + HMX, even at the limit of RDX and HMX

saturated solutions. RDX and HMX were tested at 38.7 ppm and 6.4 ppm, respectively. This was determined to be at 100% water saturation at room temperature. It is not clear what toxic effect would be induced following longer duration of exposure to mixtures of these environmental contaminants.

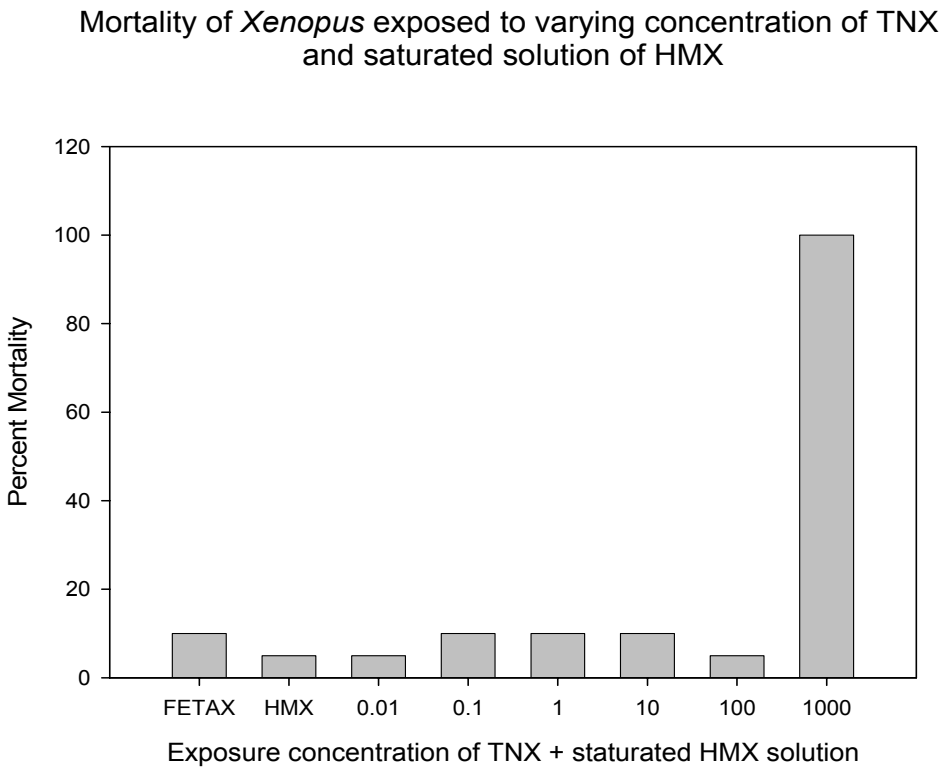

Figure 1. Mortality of Xenopus exposed to TNX +HMX.

Fathead minnow larvae were exposed to 7 concentrations (0.01, 0.1, 1, 10, 50, 100 and 1000 ppm) of TNX. The larvae were exposed to this contaminant starting at 10 days post-hatch and exposure was terminated at 96 hours. During the exposure and at termination, the number of dead and malformed embryos was counted. TNX resulted in 100% mortality at 100 ppm and above, while 50 ppm resulted in 10% mortality. The significance of the mortality at 50 ppm was negated by a 20% mortality in the untreated controls. There was no mortality observed following exposure to saturated solutions of mixtures of RDX and HMX. Based on the lack of toxicity at the point of saturation for HMX in combination with RDX and the suggestion from the SAP at the summer 2005 meeting the evaluation of mixtures of these chemicals was not pursued any further for fathead minnow larvae.

17.0 DISCUSSION:

The results presented above indicate that there is minimal hazard for RDX and HMX at environmentally relevant concentrations, at least for fathead minnows and *Xenopus*. TNX did show some acute toxicity, at levels above 100 ppm in combination with RDX or HMX. The teratogenic effects that we observed are more than likely associated with TNX since the exposure to RDX in combination with HMX did not affect the exposed animals even at the point of saturation. The "teratogenicity index" (TI = LC50/EC50), greater than 1.5 indicates a teratogenic hazard (ASTM 1998). The teratogenic hazard of TNX is 1.08, indicating no teratogenic hazard for *Xenopus* (Theodorakis, 2004). However, the concentrations of TNX in combination with RDX and MNX at which effects are seen are relatively high. Furthermore, TNX is typically found in the field in anaerobic environments. It would not be highly likely that *Xenopus* in the field would experience such concentrations. Thus, there does not appear to be an elevated hazard for this chemical for *Xenopus*. However, the question of the effects of these contaminants on native amphibian species that would be found at DoD contaminated sites requires characterization and evaluation. In addition, there are several sources of uncertainty for this hazard assessment, and these are as follows: 1) *Acute-chronic extrapolation*: It is currently unknown if there would be greater effects for longer exposures, beginning at the embryonic stage. It is also unknown if chronic exposures would lead to sublethal effects (e.g., effects on immunity, behavior, etc.), that would affect survival in the wild. 2) *Interspecific extrapolation*: The relative sensitivity of *Xenopus* compared to native frog species is currently unknown. 3) *Effects of biotransformation*: The test chemicals were exposed to young animals in this study, but older native species in the field are likely to metabolize these chemicals. It is not clear if the biotransformation effects would lead to less of the toxic compound in the animals.

18.0 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.

19.0 REFERENCES:

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TITLE: ENVIRONMENTAL MODELING

STUDY NUMBER: MOD-05-01

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RESEARCH INITIATION: October 1, 2004

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in compliance with Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Ken Dixon
Co-Principal Investigator

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 17, 1989.
Submitted By:

_____ _____
Ryan Bounds Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE:
ENVIRONMENTAL MODELING

2.0 STUDY NUMBER:
MOD-05-01

3.0 SPONSOR:
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Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
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4.0 TESTING FACILITY NAME AND ADDRESS:
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5.0 EXPERIMENT START AND TERMINATION DATES:
Start Date: October 1, 2004
Termination Date: December 31, 2005

6.0 KEY PERSONNEL
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Ryan Bounds, Quality Assurance Manager
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7.0 STUDY OBJECTIVES/PURPOSE
Modeling efforts utilized previously developed models to simulate the movement and effects of explosives. A suite of models has been developed to simulate the transport, uptake, and effects of explosives in terrestrial ecosystems (Figure 1). The emphasis in model development to date has been on uptake and distribution of explosives in mammal, bird, reptile, and plant species. Little information has been available on the effects of explosives on reptile species. The lab and field studies in this continuation provides data that can enhance the effects aspects of the models.

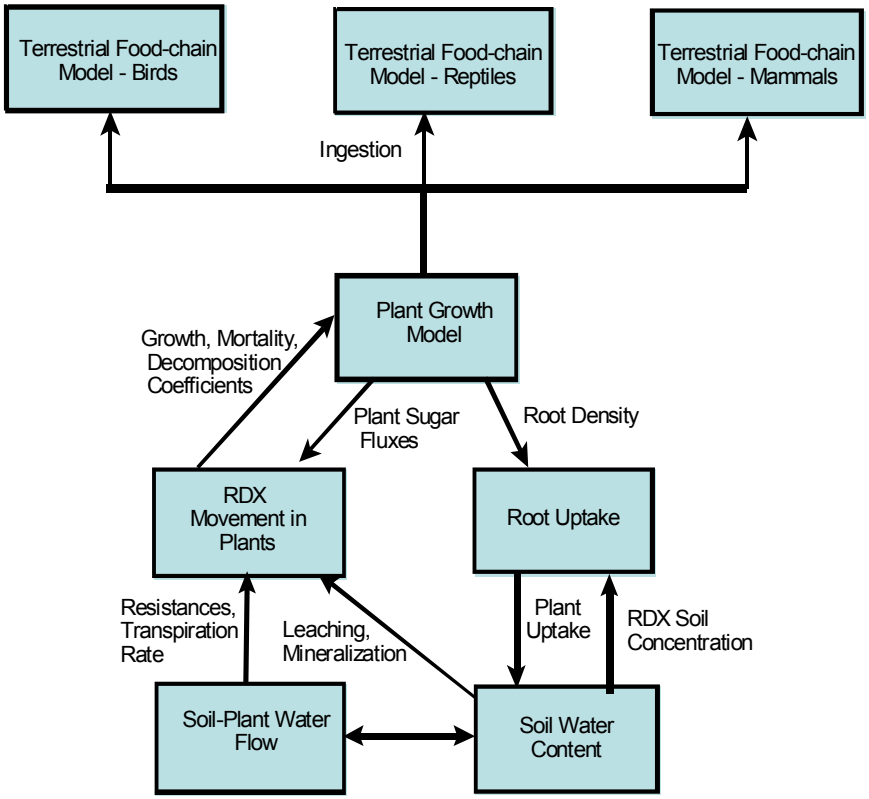

Figure 1. Flow diagram of explosives modeling suite

We also completed the implementation of the suite of models to provide for large-scale simulations, including estimates of risk for a risk assessment of explosives. This integrated suite of models will be used to assess explosives effects at different contaminated sites.

Small Mammal Model. The small mammal model was adapted from a similar model developed to predict the effects of perchlorate on thyroid activity (Dixon, et al. 2005). Mortality was added as a state variable in the model.

Bird Model. The bird model was adapted from a similar model developed to predict the effects of perchlorate on thyroid activity (Apodaca, et al. 2005). Food items for bird species and the explosives concentration in those items will be added to the model as those data become available from lab and field studies.

Plant Model. Little is known about explosives transport mechanisms in plants. Lab and field studies on explosives exposure in plants will provide data to incorporate more mechanistic transport processes in the plant models. Measured explosives concentrations in different plant

tissues will provide data for model calibration and validation.

8.0 METHODS

Small Mammal Model.

A physiologically based toxicokinetic (PBTK) model was developed to simulate the movement of explosives within small mammals. Contaminant movement was governed by a series of mass-balanced difference equations programmed in Matlab®. Model compartments and blood flow can be seen in Figure 2. Compartments in the small mammal model include blood, heart, brain, fat, kidneys, liver, gut wall, and gut contents (Figure 2). The primary environmental exposure pathway in small mammals is ingestion of explosive-contaminated food and water.

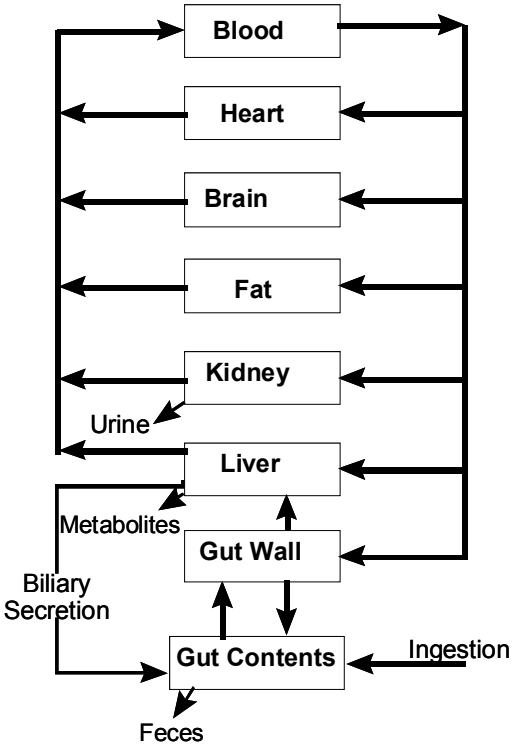

Figure 2. Flow diagram of small mammal PBPK model.

The amount of explosives distribution between venous blood and tissue concentration, $A_{i,t+1}$, is:

$$A_{i,t+1} = A_{i,t} + F_i\left(C_{vb} - \frac{C_i}{P_i}\right)$$

where,
$A_{i,t}$ is the amount of explosives in compartment i at time t, mg,
C_{vb} is the venous blood explosives concentration at time t, mg•L^{-1},
F_i is the blood flow rate into the compartment i, L•h^{-1},
P_i is the explosives partitioning coefficient for compartment i,

Explosives concentration in a compartment is the amount of explosives in the compartment divided by the mass of the compartment:

$$C_i = \frac{A_i}{m_i}$$

where m_i is the mass of compartment, g.

The amount of explosives in the gut, $A_{g,t+1}$, results from the rates of ingestion and elimination of explosives in the interval which can be described by the difference equation:

$$A_{g,t+1} = A_{g,t} + If_t + Iw_t + abs \cdot C_l - aks \cdot C_{gw} - akf \cdot C_g$$

where
C_l = explosives concentration in the liver, µg·g^{-1}
C_{gw} = explosives concentration in the gut wall, µg·g^{-1}
C_g = explosives concentration in the gut, µg·g^{-1}
$If_{i,t}$ = ingestion rate of explosives in food item i at time t, µg·g^{-1} h^{-1}
Iw_t = ingestion rate of explosives in drinking water at time t, µg·g^{-1} h^{-1}
abs = liver absorption rate constant, h^{-1}
aks = gut wall absorption rate constant, h^{-1}
akf = elimination rate constant, h^{-1}

The weight-specific mass ingestion rate of explosives in food, If_t, (µg·h^{-1}) may be written as

$$If_t = \sum_{i=1}^{m} p_i \times Cf_i \times \upsilon_i$$

where
p_i = proportion of total diet contributed by item i at time t
Cf_i = consumption rate of food item i, g·h^{-1}

v_i = explosives concentration in food item i , $\mu g \cdot g^{-1}$

Consumption rate is a function of body weight (USEPA 1993):

$$Cf = 0.398 \cdot W^{0.850}$$

where W = consumer body weight, g

Similarly, for ingestion of explosives in water, Iw_t ($\mu g \cdot h^{-1}$) is:

$$Iw_t = Cw \cdot \upsilon$$

where
Cw = consumption rate of water, $L \cdot h^{-1}$
v_i = explosives concentration in water, $\mu g \cdot L^{-1}$

Consumer body weight was modeled as simple exponential growth:

$$W = W_0 \, e^{b \cdot t}$$

where
W_0 = initial body weight, g
t = time, h
b = growth rate constant

Parameter estimation

Flow rates and tissue volumes were obtained from Brown, et al. (1997). Partitioning coefficients were calculated from data in Schneider, et al. (1978). Growth rate constants were estimated from lab data on growth of F1C deer mice dosed with varying concentrations of TNX in drinking water.

Bird Model.

A physiologically based toxicokinetic (PBTK) model was developed to simulate the movement of explosives within birds. Contaminant movement was governed by a series of mass-balanced difference equations programmed in Matlab®, similar to those in the mammal model. Model compartments and blood flow can be seen in Figure 3. Distribution was assumed to be flow-limited, i.e. chemical equilibrium existed between the tissues and blood leaving the compartment. An ingestion term was included to allow for the incorporation of multiple food sources of varying levels of toxicity.

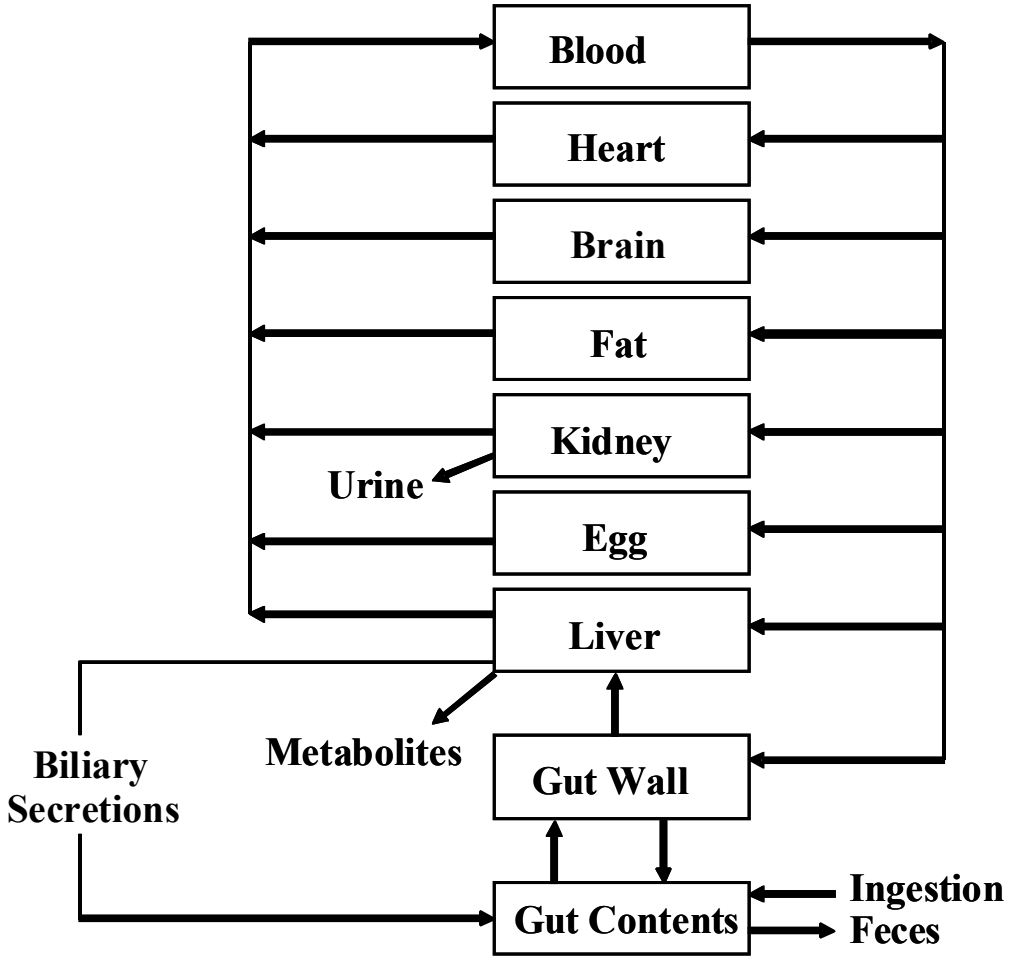

Figure 3. Flow Diagram of the PBTK model for explosives uptake in birds.

The previous model of RDX assumed that food consumption was a function of body weight (US EPA 1993). In our experiments with HMX, however, it appeared that bobwhites showed aversion to ingestion of food contaminated with RDX. Therefore, we modeled ingestion, *w*, as a

function of HMX concentration in food and body weight, *wt*, as a function of the mass of food ingested:

$$w = w_{\max}\left(1 - \frac{\nu(t - t_{\exp})}{560 \cdot \nu_{\max}}\right)$$

where
w_{max} = maximum ingestion rate
ν = HMX concentration in food, $mg \cdot kg^{-1}$
ν_{max} = maximum HMX concentration in food, $mg \cdot kg^{-1}$
t = time, h
t_{exp} = time of exposure

and

$$wt = 10^{(1.1765\log(w)+0.470)}$$

Reptile model.

A physiologically based toxicokinetic (PBTK) model was developed to simulate the movement of explosives within reptiles. Contaminant movement was governed by a series of mass-balanced difference equations programmed in Matlab®, similar to those in the bird model.

Ingestion in the reptile model differs from that in the bird model because of the less frequent feeding behaviour of reptiles. Consumption of insects, Nc_t, was assumed to be a Bernoulli random variable. The number of insects ingested was considered a Bernoulli trial in which an insect was ingested with probability p:

$$p(x) = \begin{pmatrix} 1-p & if\, x = 0 \\ p & if\, x = 1 \\ 0 & otherwise \end{pmatrix}$$

The assumption of a Bernoulli process was applied at an hourly time step in the model.

Plant Model.

The model simulates explosives uptake in terrestrial macrophytes and was programmed in Matlab using difference equations. To simulate and predict the uptake and transport of explosives in various terrestrial and aquatic plants, we developed new uptake and distribution components that are specific to explosives and modified the CERES model (Dixon, et al. 1978) by incorporating these new components (Figure 4). Additionally, an internal hydrological component was added to simulate environmental soil and water conditions.

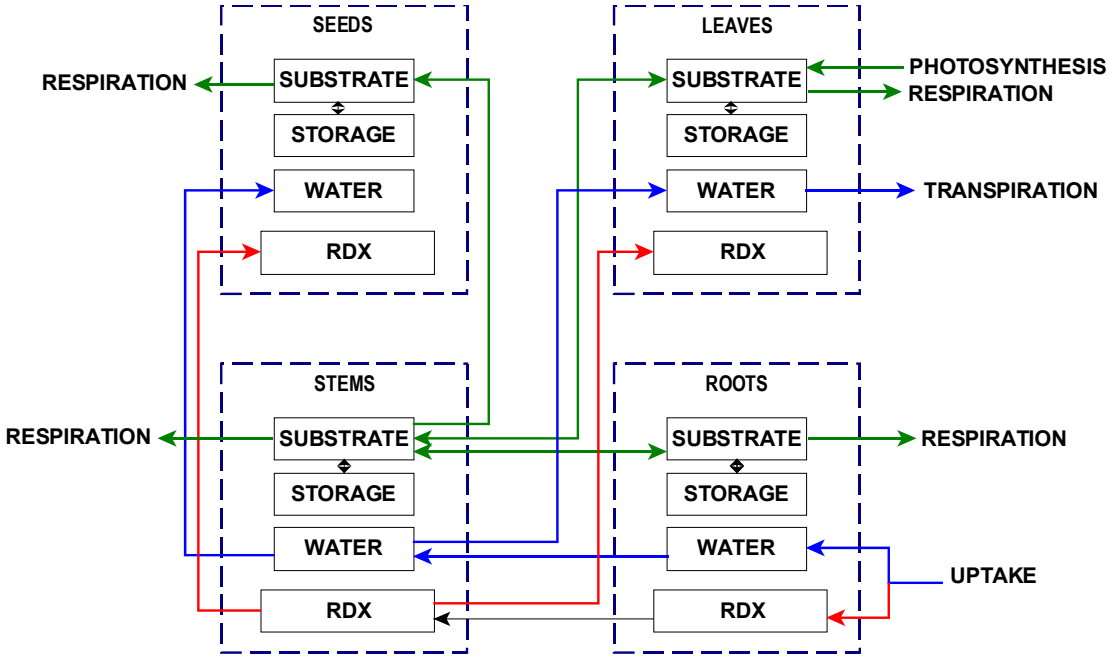

Figure 4. Flow diagram of plant uptake model

For the macrophyte model plant growth governing equations, we used those described by Dixon, et al. (1978).

The plant's water uptake is the product of the plant's ability to take up water, its leaf area, and the mass of water available to the plant in its growing soil horizon:

$$U_t^i = f \cdot L_t^{'} \cdot SM_A$$

where,

U_t^i = incremental water uptake at time t
f = water flow constant (h^{-1})
L'_t = Leaf Area Index
SM_a = Mass of water in Soil Horizon A ($g \bullet m^{-2}$ land area/hour)

Distribution of water and explosives between compartments is defined by the difference in water and explosives between compartments:

$$F_{ab} = \begin{cases} (S_a - S_b)/r_{ab} & t_1 < t \le t_4 \\ 0 & otherwise \end{cases}$$

where,
F_{ab} = flux from compartment a to compartment b ($g \bullet m^{-2}$ land area/hour)
S = amount of water in a given compartment ($g \cdot m^{-2}$ land area)
r_{ab} = water flux constant
t_1 = starting day of the growing season
t_4 = ending day of the growing season

The amount of explosives in individual compartments is defined by:

$$M_a = F_{ab} \cdot C_{\mathrm{RDX}}$$

where

M_a = mass of explosives in compartment a at time t ($\mu g \bullet m^{-2}$ land area)
F_{ab} = flux of water between the two involved compartments
C_{RDX} = RDX concentration in the incoming water

The ratio of the amount of explosives in the compartment to the biomass (wet weight) of the compartment determines the explosives concentration:

$$Q_{a,t} = \frac{M_{a,t}}{B_{a,t} + W_{a,t}}$$

where,
$Q_{a,t}$ = concentration of explosives in compartment *a* at time *t* (µg•g^{-1})
$M_{a,t}$ = amount of explosives in compartment *a* at time *t* (µg•m^{-2})
$B_{a,t}$ = biomass in compartment *a* at time t (g•m^2)
$W_{a,t}$ = mass of water in compartment a at time *t* (g•m^2)

Plant biomass is calculated by summing the soluble and insoluble photosynthate fractions (Dixon, et al. 1978):

$$B_a = S_a + ST_a$$

where,
$B_{a,t}$ = biomass of compartment *a* at time *t* (g•m^2)
$S_{a,t}$ = sugar substrate in compartment *a* at time *t* (g•m^2)
$ST_{a,t}$ = plant storage tissue in compartment *a* at time *t* (g•m^2)

For cattails, we added a term for uptake of explosives from standing water into plant stems:

$$M_{stem} = c \cdot (C_{\mathrm{RDX}} - Q_{stem}) \cdot B_{stem}$$

Model Assumptions:

- transport between leaves and stems occurs from the time of bud formation to the time of abscission.
- transport between stems and fruits occurs from the time of net photosynthesis to the time of abscission.
- transport between the stems and roots is assumed to occur throughout the year.

9.0 RESULTS

Small Mammal Model.

The earlier small mammal model was calibrated using data on the RDX distribution in different organs in the rat (Schneider, et al. 1978). The current model used laboratory data on chronic ingestion of TNX saturated drinking water at 1, 10 and 100 µg/L *ad libitum* by deer mice. The F1C generation showed a decreased rate of growth with increased TNX concentrations. The model predicted similar effects on growth (Figure 5). The lab data also showed accumulation of TNX in liver tissues in F1C deer mice exposed to 10 and 100 µg/L (Figure 6). Model predictions were within 95% C.I. of the mean (Figure 6).

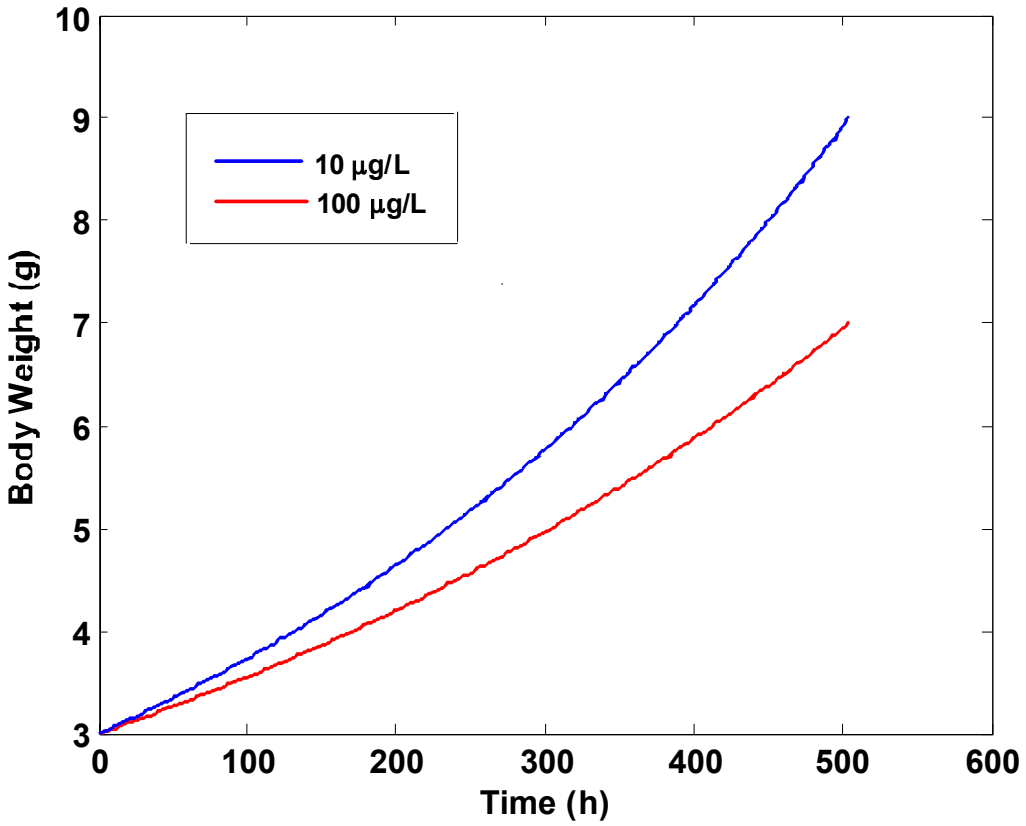

Figure 5. Predicted mean deer mouse body weights following exposure to TNX concentrations of 10 µg/L (blue) and 100 µg/L (red) in drinking water.

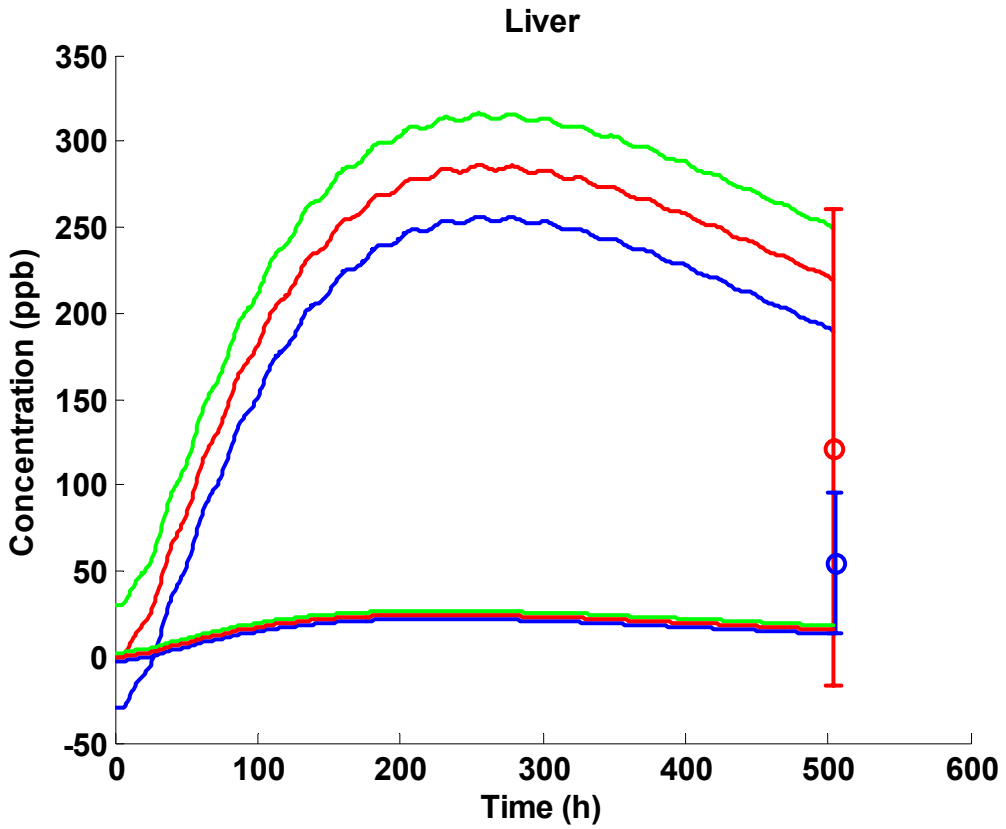

Figure 6. Observed and predicted liver TNX concentrations in deer mice. Predicted red lines are means and green and blue lines are upper and lower 95% confidence limits respectively. Red and blue circles with error bars are observed means ±S.E.

Note that the TNX concentrations increase over time with ingestion of contaminated drinking water and then decrease as body weight (and liver weight) increase.

Bird Model.

The previous model was calibrated using data from Gogal, Jr., et al. (2003). The model was calibrated further using data collected in the laboratory. In this lab experiment, quail were exposed to feeding mixtures of HMX ranging from 0 to 250 mg/kg. No birds died over the course of the feeding trial and no birds died in the simulation. Quail eggs had mean and standard error HMX concentrations of 1.48±0.30, 6.63±1.54, and 13.95±4.52 µg/g following ingestion of food at nominal concentrations of 25, 125, and 250 mg/kg, respectively. Predicted egg concentrations showed similar concentrations (Figure 7). Quail decreased food consumption and lost weight with increasing HMX exposure. At the 250 mg/kg exposure level, the average weight loss was approximately 40g. In the simulation at 250 mg/kg, predicted weight loss was of 40g occurred after 7 days of exposure starting at day 7 (hour 168) (Figure 8). Food consumption followed a similar pattern because body weight is a function of food consumption (page 12) (Figure 9).

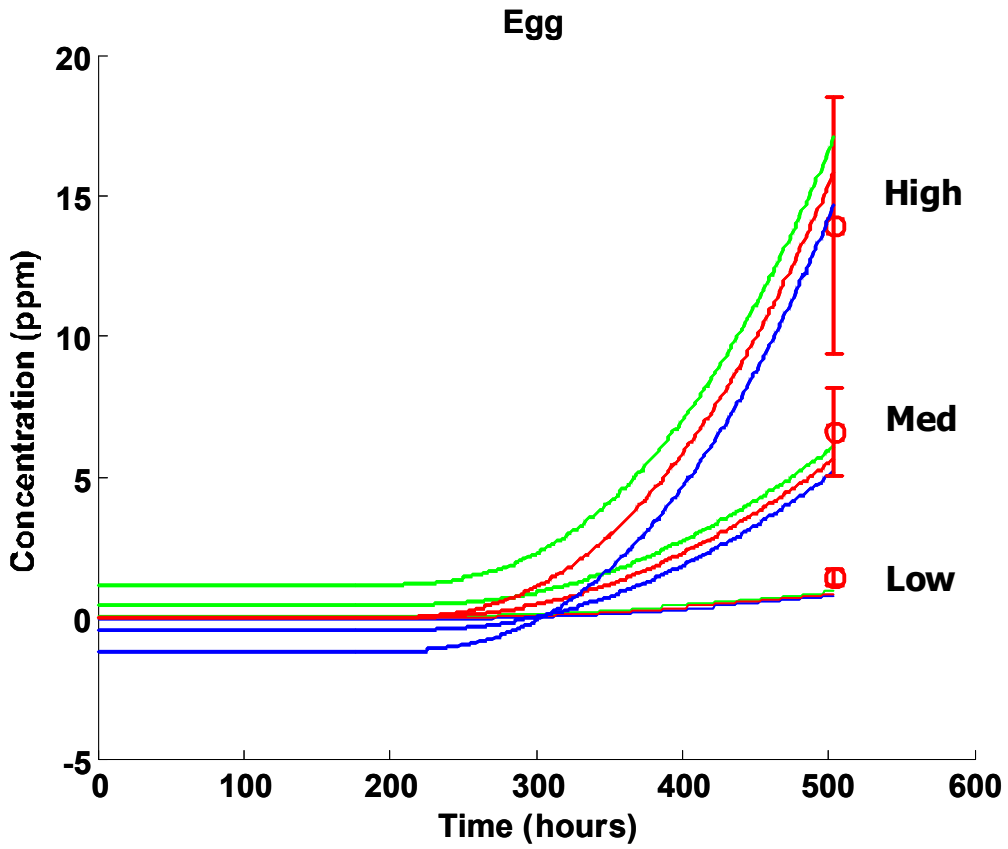

Figure 7. Observed and predicted HMX concentrations in bobwhite quail eggs resulting from ingestion of food with HMX concentrations of 25 (low), 125 (med), and 250 (high) mg/kg. Predicted red lines are means and green and blue lines are ± S.E.

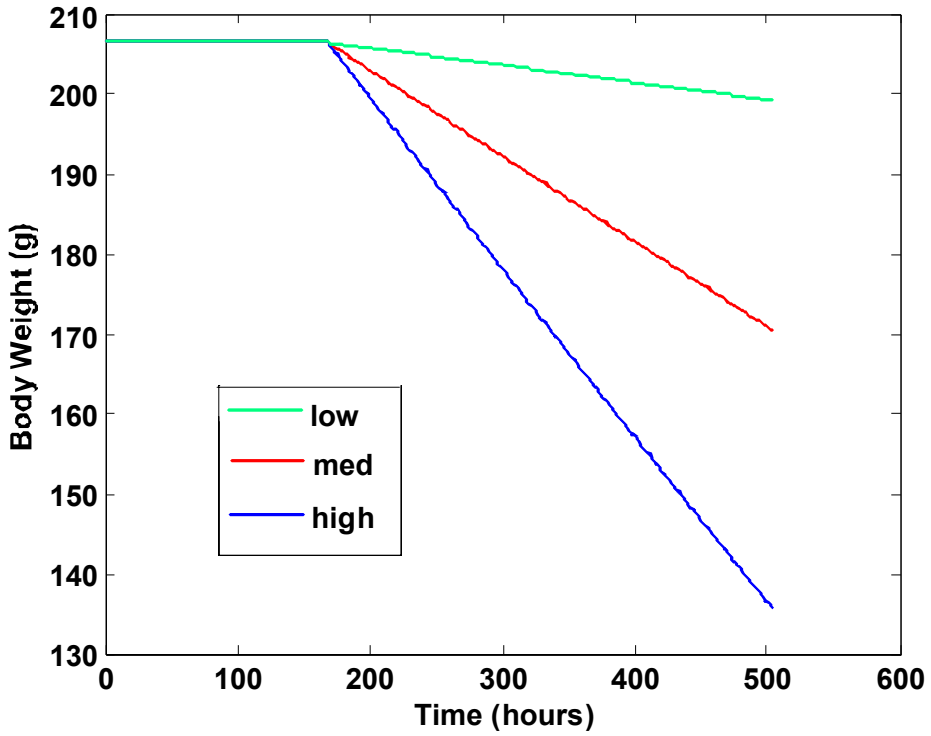

Figure 8. Predicted body weight (g) in a bobwhite with initial weight of 210 g following exposure to nominal HMX concentrations of 25 (low), 125 (med), and 250 (high) mg/kg in food starting at day 7 (hour 168).

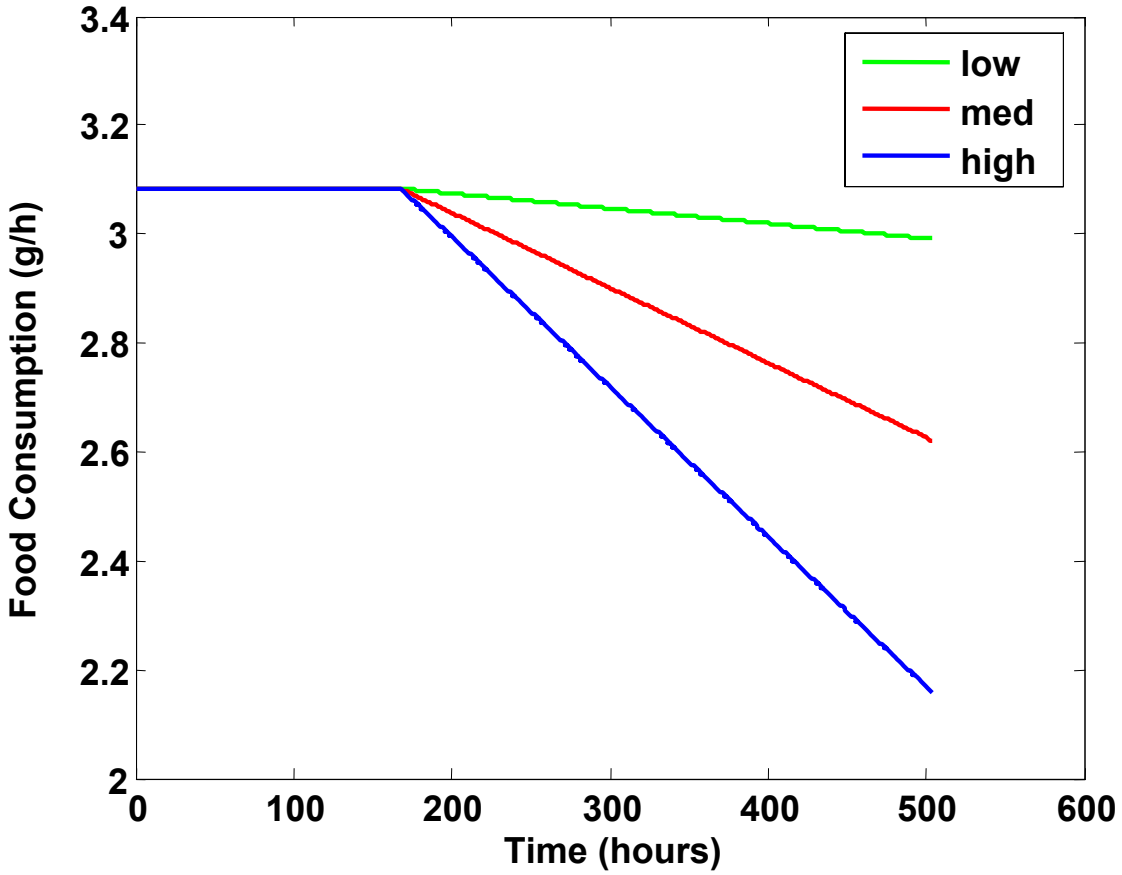

Figure 9. Predicted food consumption (g/h) in a bobwhite with initial body weight of 210 g following exposure to RMX concentrations of 25 (low), 125 (med), and 250 (high) mg/kg in food starting at day 7 (hour 168).

Reptile Model.

The model was calibrated using data collected in the laboratory. In this lab experiment, anoles were exposed to dosed crickets at a rate of 3 crickets per week. Crickets were dosed at 0, 500, and 1000 mg/kg. Using an estimate of an LD50 of >2000 mg/kg, no birds died in the simulation. Anole eggs had mean and standard error HMX concentrations of 0.637±0.048, 1.954±0.356, and 5.077±4.105 µg/g following ingestion of crickets by the adults at nominal concentrations of 0, 500, and 1000 mg/kg, respectively. Predicted egg concentrations showed similar concentrations (Figure 10).

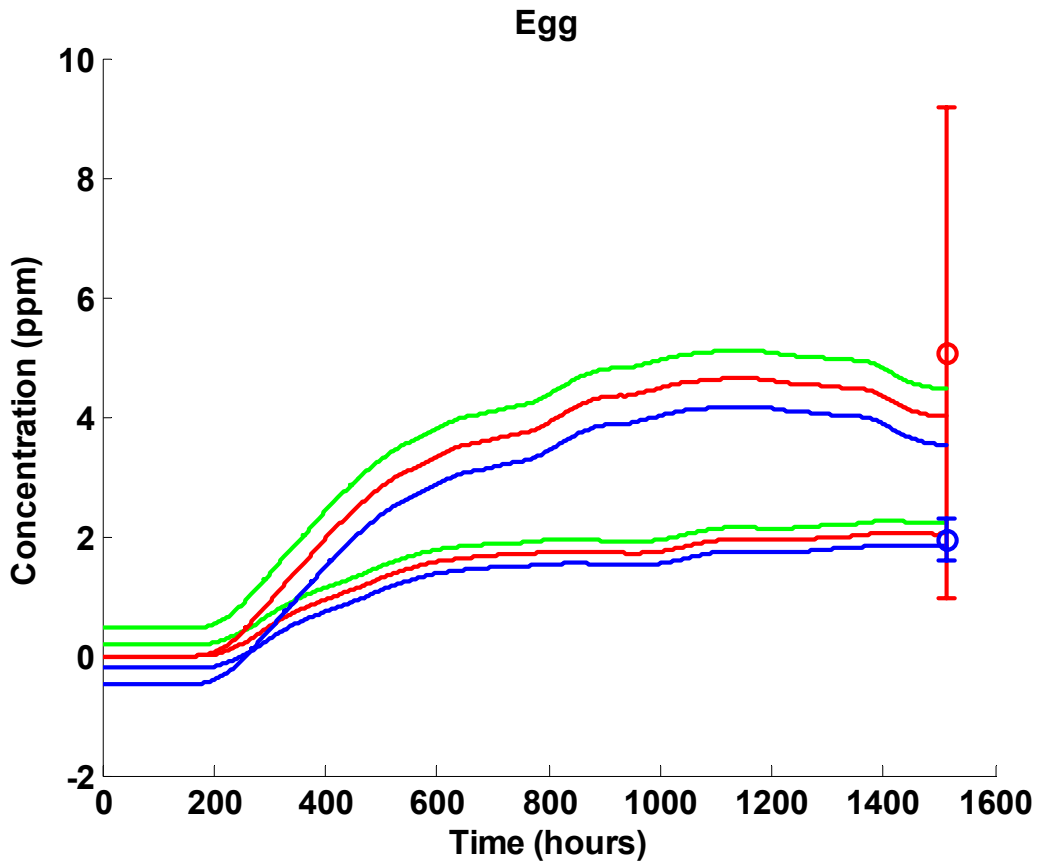

Figure 10. Predicted HMX concentration in anole eggs following exposure to HMX dosed crickets at concentrations of 500 and 1000 mg/kg starting at day 7 (hour 168). Predicted red lines are means and green and blue lines are ± S.E.

Plant Model.

The original plant model was calibrated using data on RDX uptake by tomato plants (Price et al. 2002). The current model used data on uptake of RDX in cattails in mesocosms following added influent at 1, 5, or 10 ppm RDX. Measured and predicted stem concentrations following 5 ppm influent showed similar values (Figure 11). The predicted explosives concentration in stems was adjusted to fit the observed data by reducing uptake from the water and reducing the flow into leaves and fruits. At the 10 ppm influent concentration, however, the model predicts a lower concentration than the reported mesocosm values (Figure 12).

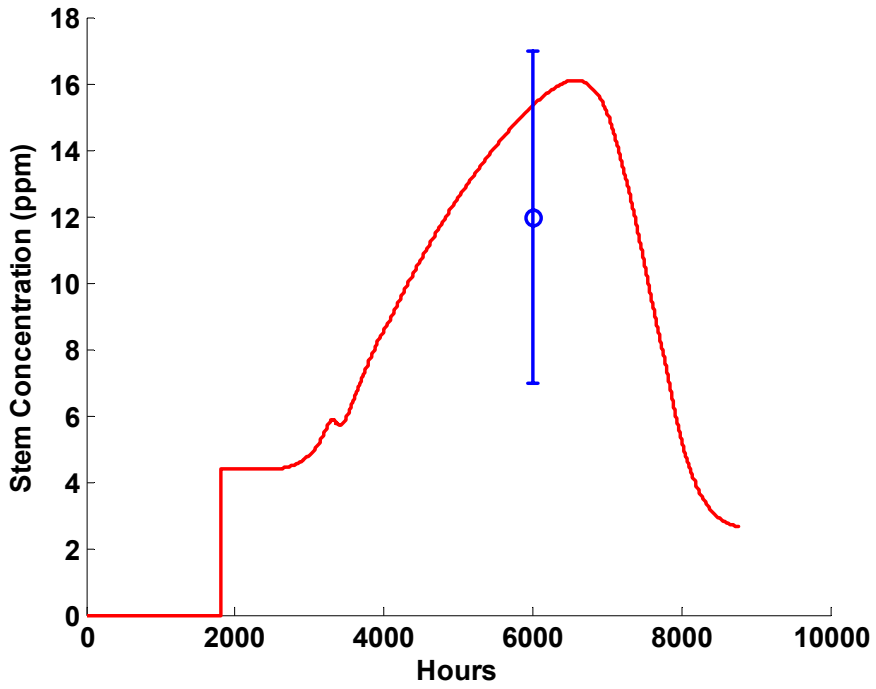

Figure 11. Observed and predicted RDX concentration in cattail stems (in red) following influent loading rate of 5 ppm. Observed concentration shown as mean ± standard error (in blue).

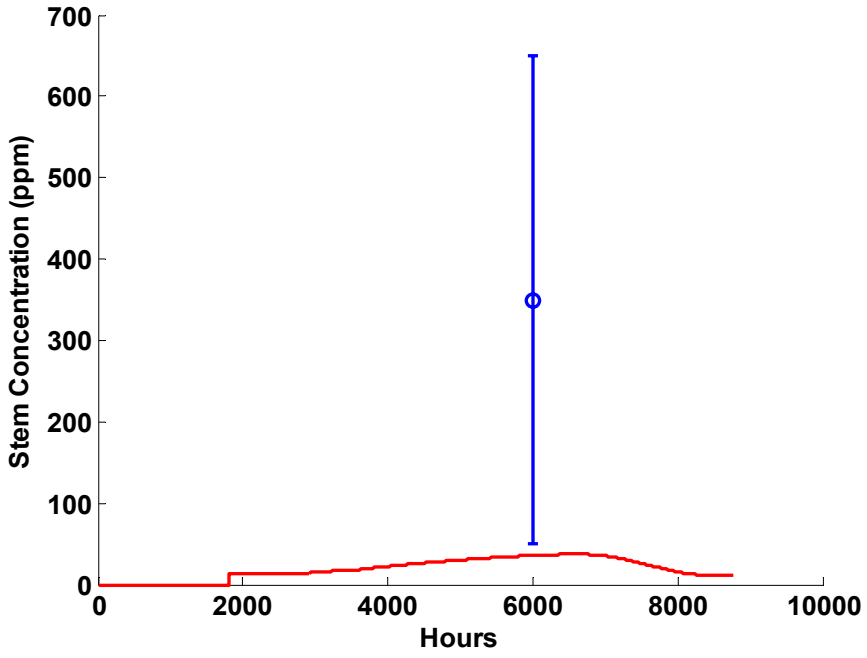

Figure 12. Observed and predicted RDX concentration in cattail stems (in red) following influent loading rate of 10 ppm. Observed concentration shown as mean ± standard error (in blue)

10.0 DISCUSSION

Small Mammal Model.

The predicted deer mouse liver concentrations of TNX fall within the 95% C.I. of the observed values. The variability of the observed data, however, is quite high. The C.I. of the higher dose rate (100 µg/L) completely contains that for the lower dose rate (10 µg/L). Further calibration of the model should await additional lab data.

Bird Model.

Calibration of the initial bird model was based upon RDX data from Gogal, Jr., et al. (2003). The current model was calibrated further using lab data on HMX with good agreement between observed and predicted values. Appropriate data on egg partitioning coefficients, excretion rates, and feeding avoidance are needed.

Reptile Model.

There was good agreement between observed and predicted concentrations of HMX in anole eggs. There was high variability associated with the high dose levels (1000 mg/kg) with the mean ± SE completely containing that of the lower dose level (500 mg/kg). This high variability demonstrates the need for larger sample sizes for future calibration efforts.

Plant Model.

The vascular plant model was developed under the assumption that water is the driving force behind the uptake and distribution of explosives in plants. Because the model under predicts tissue concentrations at the high influent loading rate (10 ppm), and the variability is quite high, additional data are needed for further calibration. The observed data suggest a nonlinear dose response of plant concentration to influent loading rate. The mechanism for this response needs to be identified before it can be incorporated into the model. Although parameter estimates were based on calibration with lab data, direct parameter estimation may improve the accuracy of the model predictions.

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TITLE: Developmental Response of Larval *Xenopus laevis* to TNX

STUDY NUMBER: TNX-05-01

SPONSOR: Strategic Environmental and Research Development Program
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901 North Stuart Street, Suite
Arlington, VA 22203

CONTRACT ADMINISTRATOR: The Institute of Environmental and Human Health
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ANIMAL TEST SITE: Texas Tech University
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RESEARCH INITIATION: April 28, 2005

RESEARCH COMPLETION: December 31, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in compliance with Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Angella Gentles
Co-Principal Investigator

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 17, 1989.

Submitted By:

_____ _____

Ryan Bounds Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE:
Developmental Response of *Xenopus laevis* to TNX

2.0 STUDY NUMBER:
TNX-05-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite
Arlington, VA 22203

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

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: April 28, 2005
Termination Date: December 31, 2005

6.0 KEY PERSONNEL:
Angella Gentles, Co-Principal Investigator
Mike Wages, Study Director
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principal Investigator and Testing Facility Management

7.0 STUDY OBJECTIVES / PURPOSE:
To assess gonadal development in *Xenopus laevis* in response to TNX exposure, evaluating histological end points of selected organs.

8.0 STUDY SUMMARY:
In this study, *Xenopus laevis* metamorphs were exposed to TNX for 30, 60, 90 and 120 days. The juveniles were euthanized in bicarbonate buffered MS-222. Subsequently they were weighed and their snout-vent length and hind limb length measured, and the gonads sectioned for histologic evaluation. TNX had no effect on the gross parameters measured in juveniles exposed to the chemical for 30, 60, 90, or 120 days. Histologic analysis of the gonads indicated that TNX has the potential to retard development of the gonads - the number of cohorts of sperm and spermatogonial cells were significantly different between control and treated groups.

9.0 TEST MATERIALS:
Test Chemical name: hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)

CAS number: 13980-04-6
Characterization: Determination of concentration in water samples.
Source: SRI International

10.0 JUSTIFICATION OF TEST SYSTEM:

This project is intended to evaluate risks of TNX exposure in developing *Xenopus laevis*. Xenopus were used because they are easily handled, permit large sample sizes, and are more economically feasible in a study of this nature. They are suitable models for assessing exposure risks in an ecological environmental setting.

This study cannot be substituted with culture or computer generated models. Culture and computer models cannot simulate physiological and in addition it would not provide pertinent scientific data for future use in risk assessment.

11.0 TEST ANIMALS:

Species:	*Xenopus laevis*
Strain:	Wild type
Age:	larvae
Sex:	Males and females
Number:	400
Source:	Xenopus Express

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Individual identification of the animals was not possible because of their size, however, each beaker 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.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The study consisted of 4 treatment groups, namely, control, 100ppb, 500ppb and 1000ppb TNX. There were 5 replicates for each treatment group and each replicate had 20 animals. The 90-day study had 4 replicates.

14.0 METHODS:

14.1 Test System acquisition, quarantine, and acclimation.

The juveniles were purchased from Xenopus Express (Tampa, Florida).

14.2 Assignment of Animals to Study Group and Identification

1. The frogs were allowed to acclimate for more than 7 days before the study was initiated. They were kept in FETAX medium in a large aquarium prior to the study. On the day exposure began, the frogs were removed with a net, then

weighed and their snout-vent length measured. They were then randomly placed in one of 20 beakers containing 2L of FETAX. After all the beakers had 20 animals, a pipette was used to add the appropriate amount of TNX solution to give the required concentration of TNX for exposure.
2.
3. The frogs were kept on a 12:12hr light :dark cycle and juvenile frog brittle from NASCO was supplied every other day. One half of the FETAX medium was changed every 3 days. Water quality was checked once per week. Record of dissolved oxygen, temperature, pH, conductivity and ammonia levels were measured and recorded.
4.

14.3 Test Material Application

Rates/concentrations: The frogs were exposed to TNX at concentrations of 0, 100, 500, and 1000 ppb.

Frequency: Test substance was supplied continuously in FETAX medium.

Route/Method of Application: The animals were exposed in a static renewal aquatic system.

14.4 Daily Observations

Animals were monitored daily for changes in general health.

14.5 Animal Euthanasia and Sample Collections

At the end of TNX exposure the tadpoles from each tank were removed using a fish net. They were placed in a beaker containing bicarbonate buffered MS-222 for euthanasia. They were weighed, and their hind limb and snout-vent length measured after which they were placed on dry ice and later stored in the -80C freezer or in Bouin's solution for 48hrs then transferred to 70% alcohol. The tissues were processed routinely for histology by dehydrating serially in increasing concentration of alcohol, clearing in xylene and infiltrating with paraffin followed by embedding in paraffin. The tissue was cut at 7µ and stained with hematoxylin and eosin. The gonads were inspected then for gross abnormalities.

14.6 Testosterone analysis

Testosterone was extracted from the frog tissue by homogenizing the whole body in 5ml phosphate buffered saline per gram of tissue. The homogenate was then centrifuged at 200g at 4C for 10 min. Then 1mls of the supernatant was removed and added to 2mls of ethylacetate. This mixture was then vortexed vigorously for 1hr. 1ml of the ethyl acetate was then removed and dried under nitrogen. Phosphate buffered saline (200 µl) was added to the dried sample. 50µL of each of these samples was analysed in duplicate according to the manufacturer's procedure. Briefly, the 1ml of radioactive testosterone was added to each sample

and standard in the antibody coated polypropylene tubes provided. This mixture was vortexed and incubated at 37C for 3hrs. The solution was then decanted and the tubes tapped dry. Radioactivity was measures on a gamma counter.

14.7 Evaluations

The following data were collected; mortality rate, snout-vent length, hind limb length and body weight. Histology was also done to assess the spermatogenic cell cycle in the gonads of animals exposed for 120 days. Testosterone levels were measured in the males.

14.8 Statistics

The endpoints listed in section 14.7 above were analyzed by ANOVA and Tukey's post-hoc test using MiniTab (Ver 3.0). The p value was set at $p < 0.05$.

15.0 RESULTS and DISCUSSION

Results

In this study, late metamorphic *Xenopus laevis* were exposed to TNX for periods of 30, 60, 90, and 120-days. At the end of each period the frogs were euthanized then they were weighed, and their hind limb and snout-vent length measured. The whole body level of testosterone was measured in male frogs, and the gonads of both sexes were assessed for changes in spermatogenic cells in animals that were exposed for 120 days. The 120-day animals were analyzed for whole-body TNX concentration. TNX was not detected in the control animals but was found to be 40, 228, and 615 ppb for the low, medium and high exposure groups, respectively.

Thirty-day Exposure

There were no significant differences in body weight, hind limb or snout-vent length. The absolute weight change and growth of hind limb over the period of exposure were also assessed. There were no statistical differences in any of these parameters.
See Fig. 1.

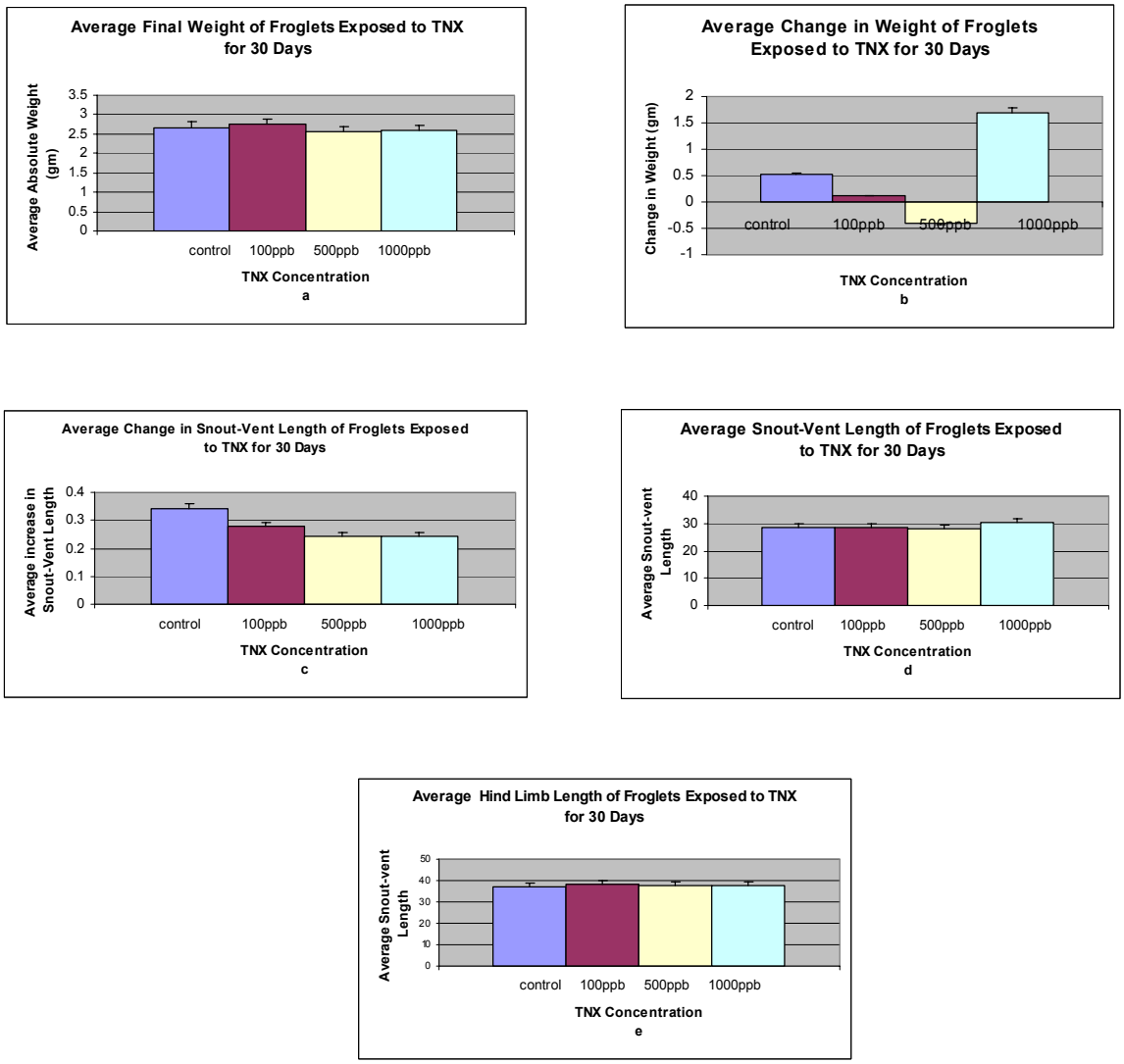

Figure 1. The effect of TNX on (a) body weight, (b) change in body weight, (c) snout-vent length, (d) change in snout-vent length, and (e) hind limb length in juvenile *Xenopus laevis* exposed for 30 days.

Sixty-day Exposure

There were no significant differences in body weight, or hind limb or snout-vent length. The absolute weight change and growth of hind limb over the period of exposure were also assessed. There were no statistical changes in any of these parameters. See Fig. 2.

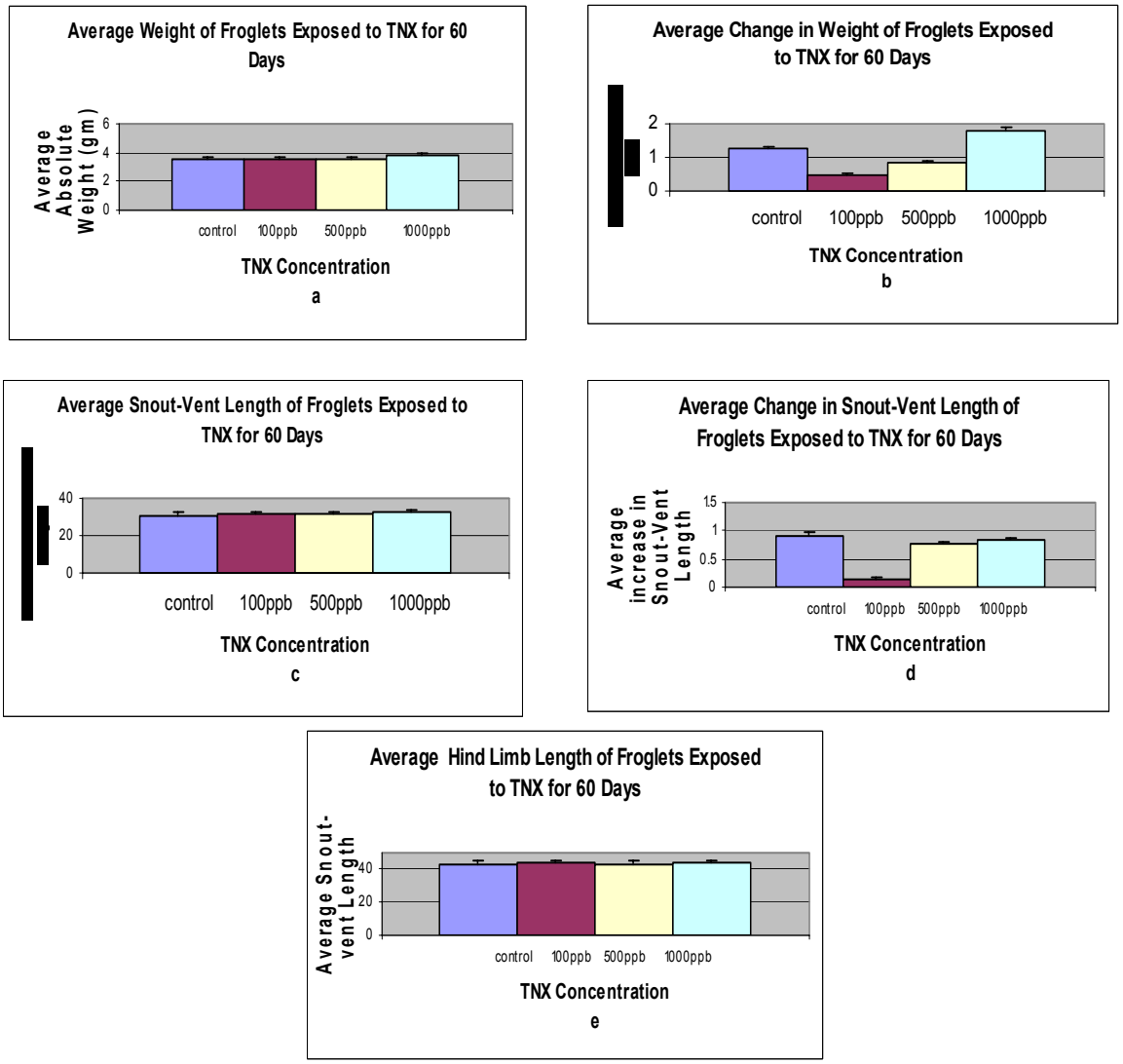

Figure 2. The effect of TNX on (a) body weight, (b) change in body weight, (c) snout-vent length, (d) change in snout-vent length, and (e) hind limb length in juvenile *Xenopus laevis* exposed for 60 days.

Ninety-day Exposure

There were no significant differences in body weight, or hind limb or snout-vent length. The absolute weight change and growth oh hind limb over the period of exposure were also assessed. There were no statistical changes in any of these parameters. See Fig. 3.

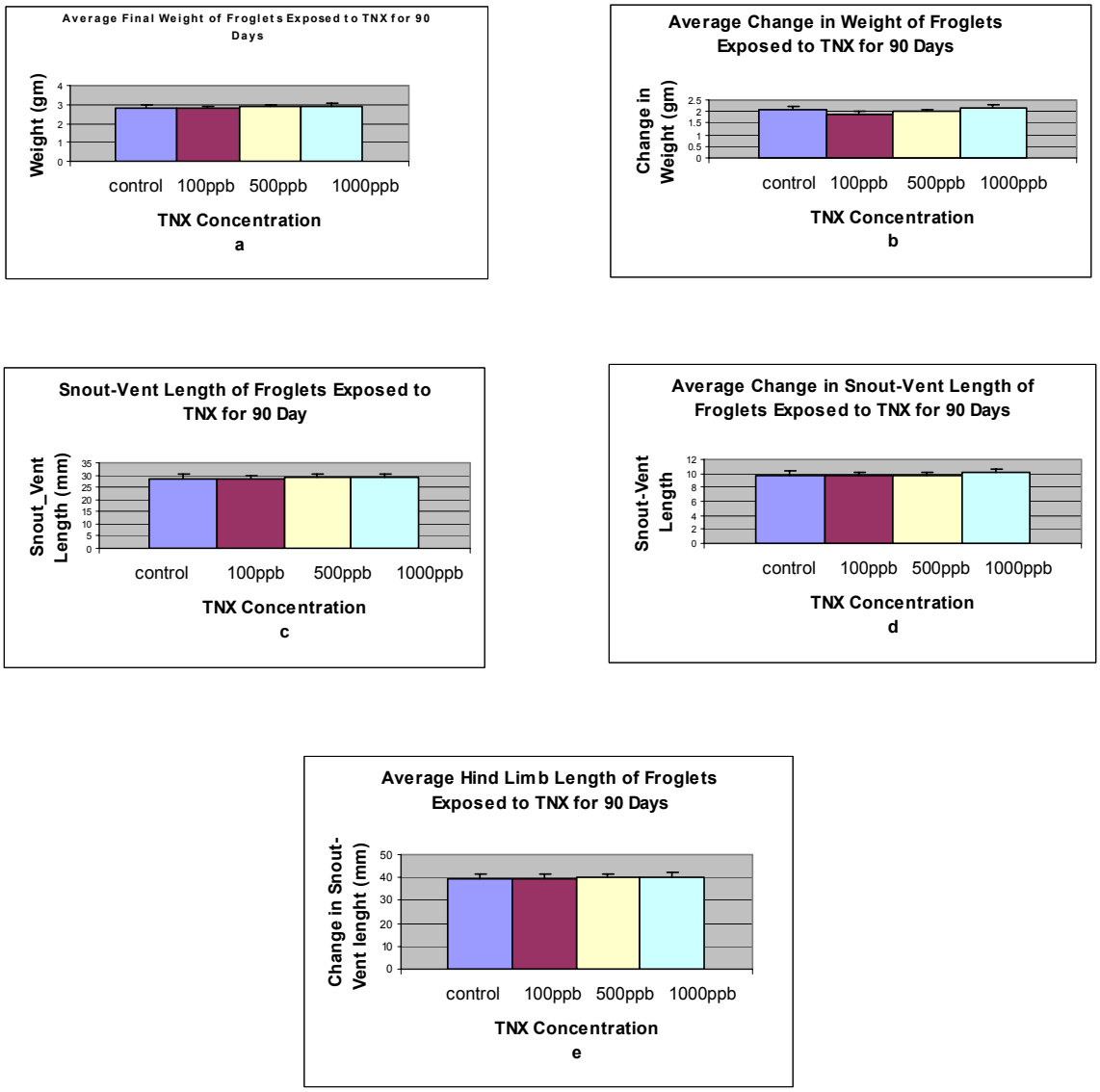

Figure 3. The effect of TNX on (a) body weight, (b) change in body weight, (c) snout-vent length, (d) change in snout-vent length, and (e) hind limb length in juvenile *Xenopus laevis* exposed for 90 days.

One hundred twenty-day Exposure

There were no significant differences in body weight, or hind limb or snout-vent length. See Fig. 4.

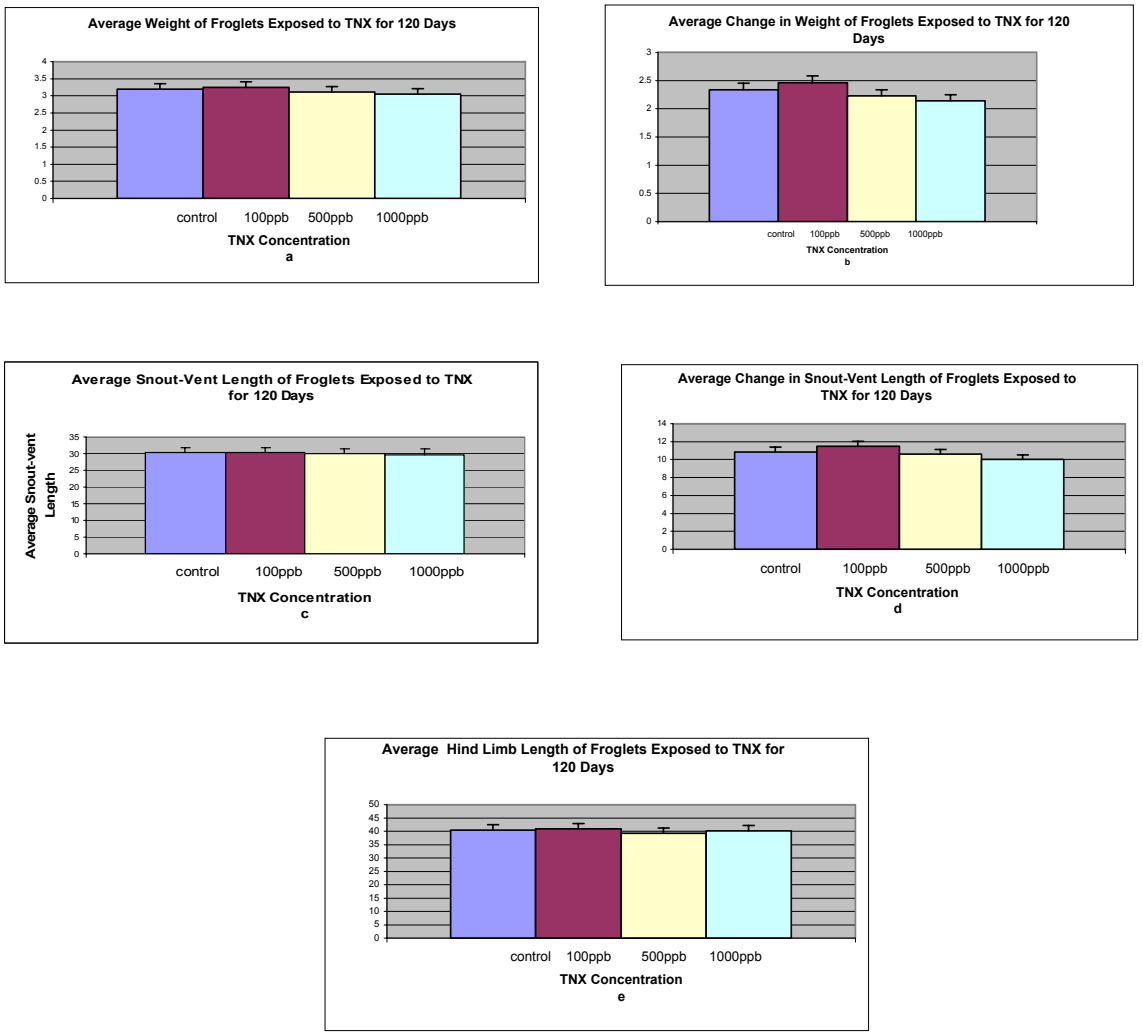

Figure 4. The effect of TNX on (a) body weight, (b) change in body weight, (c) snout-vent length, (d) change in snout-vent length, and (e) hind limb length in juvenile *Xenopus laevis* exposed for 120 days.

Exposure to TNX for 120 days resulted in a significant increase in the relative percentage of the number of cohorts of sperm and spermatogonial cells ($P < 0.05$). Relative to the control group, TNX did not cause any significant change in the clusters of spermatocytes, however; the 100ppb and 500ppb groups differ significantly from each other. Exposure to TNX did not cause any significant difference in spermatid clusters among the groups. Representative animals in each treatment group had testes with cells representing each stage of spermatogenesis, as well as some testes that were deficient in cells from the later stages of spermatogenesis. Testosterone was detected in only four animals. The ovaries were assessed qualitatively for developmental stage. There were no significant differences observed among the control and the treated groups.

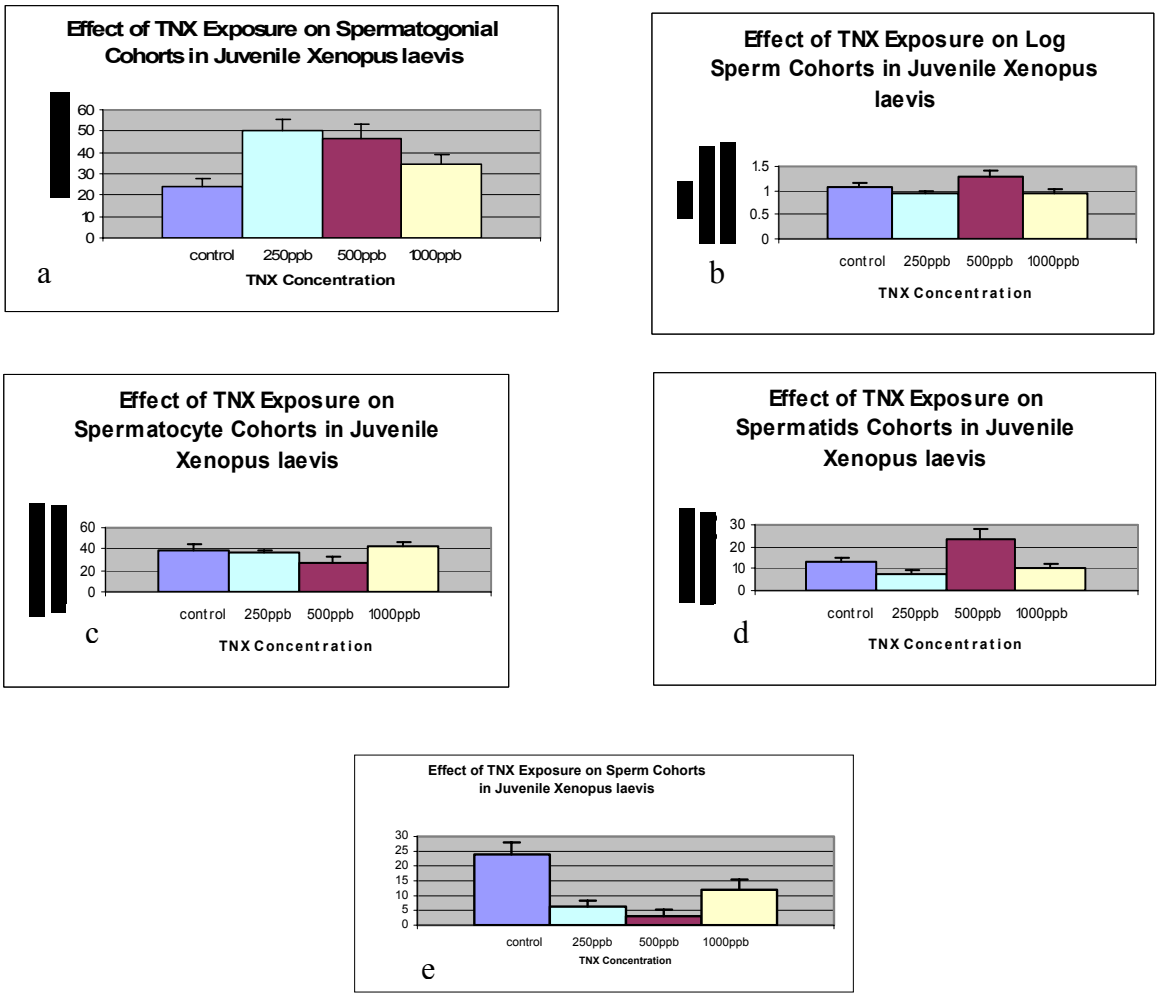

Figure 5. The effect of TNX on (a) percent spermatogonia, (b) log of percent sperm, (c) percent spermatocyte, (d) percent spermatid, and (e) percent sperm cohorts *Xenopus laevis* exposed for 120 days.

Discussion

The results indicate that the whole body concentration of TNX in frogs exposed to TNX for 120 days was concentration-dependent. In fact, the level of TNX in these animals was approximately a half that of the exposure concentration. It is uncertain if this unexpectedly high concentration of TNX in the animals was actually due to absorption or a result of external residual TNX from the medium taken up by the skin. Though there is some literature on bacterial aerobic and anaerobic degradation of RDX and some of its metabolites, it is still unknown whether TNX further metabolizes to other compounds. This large residual concentration in the body suggests that TNX is or might be absorbed faster than its excretion and/or metabolism. Interestingly, Smith et al., 2006 reported liver-levels of TNX that were approximately one half that of the concentration fed to mice over an extended period of time.

TNX at the concentrations used in this study, did not cause any changes in the body weight, snout-vent length or hind limb length of frogs exposed to the chemical for various periods of time. Considering the level of residual TNX found in the body of the frogs, it appears that TNX might be only slightly toxic. The report of Smith et al., 2006 is somewhat in support of this theory. Though the authors reported that the levels of TNX observed in the liver of mice were one half that of the concentration fed from conception until postnatal day 45, they also observed no response in gross parameter except in the postnatal day 21 animals. Animals treated with 10µg/L TNX had low body weight. This they ascribed to an indirect effect of TNX because the low body weight was seen only in nursing animals.

Based on the spermatogenic profile of these frogs, it would be expected that testosterone would be present in these animals. However, due to the small gonadal sample size whole testosterone evaluation was employed. Radioimmunoassay was used to evaluate the whole body levels of testosterone in male frogs. Testosterone was detected in 10% of the samples. We speculate that the non-detects were below the LOD and would require a more sensitive assay such as the GC/MS quantification assay.

The pattern of spermatogenic cells identified following TNX exposure – increase in the percentage of the number of cohorts of sperm and spermatogonial cells ($P < 0.05$)- would require long term reproductive and developmental evaluation to determine the effect on reproductive capacity of the exposed animals. It is also not clear if similar effect would be induced by TNX in native amphibian species. Relative to the control group, TNX did not cause any significant change in the clusters of spermatocytes, however; the 100ppb and 500ppb groups differ significantly from each other ($P = 0.047$). TNX did not cause any significant difference in spermatid clusters among the groups. It is uncertain if an increase in N (N=5) could affect the outcome of this result. It also appears that some of the spermatogenic cells are more sensitive to TNX exposure. However, a larger samples and longer duration of exposure would be necessary to fully characterize these effects.

Each treatment group had testes with cells representing each stage of spermatogenesis, as well as some testes that were deficient in cells from the later stages of spermatogenesis. However, testosterone was detected in only 10% of the animals evaluated and would appear to indicate that these animals are developing at different rates. Further support for this observation is that there

was a large variation in testicular development among animals within each treatment group. There were animals that had spermatogonial cells as the main cell type present and a few spermatocytes sparsely scattered among them. This was found mainly in animals with smaller body weights. The larger frogs had each type of germ cell present in its gonads. This characteristic has been observed in the American bull frog (*Rana catesbiena*) in our laboratory.

In summary, TNX had no effect on the gross parameters measured in juveniles exposed to the chemical for 30, 60, 90, or 120 days. Histologic analysis of the gonads indicated that TNX has the potential to retard development of the gonads - the number of cohorts of sperm and spermatogonial cells were significantly different between control and treated groups. Based the data from this study, TNX appears to be a potential reproductive toxicant to juveniles *Xenopus*. However, the population levels effects of exposure to TNX cannot be determined from the current study and would require further evaluation. In addition, this study should be repeated using native amphibian species at environmentally relevant concentration.

16.0 REFERENCES:

Smith, J.N., Pan, X. Gentles. A., Smith, E.E., Cox, S.B., and Cobb, G.P. 2006. Reproductive effects of hexahydro-1,3,5-trinitroso-1,3,5-triazine in deer mice (*Peromyscus maniculatus*) during a controlled exposure study**.**

17.0 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.

TITLE: HMX Toxicity in the Green Anole (*Anolis carolinensis)*

STUDY NUMBER: HMX-05-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

CONTRACT ADMINISTRATOR: The Institute of Environmental and Human Health
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TEST SITE: The Institute of Environmental and Human Health
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ANIMAL TEST SITE: The Institute of Environmental and Human Health
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RESEARCH INITIATION: July 2004

RESEARCH COMPLETION: December 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Scott T. McMurry
Co-Principal Investigator

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 17, 1989.

Submitted By:

_____ _____

Ryan Bounds Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE: HMX Toxicity in the Green Anole

2.0 STUDY NUMBER: HMX-05-01

3.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
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4.0 TESTING FACILITY: The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, TX 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: July 2004
Termination Date: December 2005

11.0 **KEY PERSONNEL**:
Dr. Scott T. McMurry, Co-Principal Investigator
Dr. Philip N. Smith, Co-Project Manager
Ms. Lindsey E. Jones, Research Assistant
Mr. Ryan M. Bounds, Quality Assurance Manager
Dr. Ronald J. Kendall, Principal Investigator / Testing Facility Management

12.0 **STUDY OBJECTIVES / PURPOSE:**

- To expand the database on HMX to include exposure and toxicity data on reptiles. We have found no data on reptiles.

- To quantify accumulation of HMX into developing eggs via contact with contaminated soil. Due to the life history of this animal, especially its tendency to oviposit in soil, offspring are likely to come into contact with HMX long before they are able to accumulate it dietarily.

- To attempt to quantify transfer of HMX from mother to developing embryo and its subsequent effects on the hatchling's development. Adult animals, though likely able to accumulate HMX via incidental soil exposure, are most likely to uptake HMX through the diet. The effect this exposure has on the mother, if/to what extent this accumulation is deposited in the egg, and what effect the accumulation has on the embryo will provide useful information for both risk assessors and managers dealing with HMX-contaminated sites.

13.0 TEST MATERIALS:

Test Chemical name: octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)
CAS number: 2691-41-0
Characterization: Explosive
Source: Accurate Energetics

14.0 JUSTIFICATION OF TEST SYSTEM:

The use and subsequent environmental contamination of energetic compounds is an ever increasing international concern (Talmage et al., 1999; USACHPPM 2001; ATSDR 1997). The real threat of these compounds from an ecotoxicological standpoint is how little is really known of their toxicity to the organism and the resultant effects to the individual's population and ecosystem. Perhaps one of the larger gaps in the knowledge of explosives is regarding reproductive toxicology.

One of the prime candidates for such an investigation is the green anole, *Anolis carolinensis*. The anole has been used extensively in the laboratory setting, is easy and cost-effective to maintain, and more importantly has been shown to imitate the sensitivities of birds and mammals, rather than those of other poikilotherms, perhaps allowing the data a broader range of extrapolation (Lovern et al, 2004, Hall and Clark, Jr., 1982).

Two studies were performed in an effort to define the role of HMX in reproductive toxicology of lizards. The first study used artificially contaminated nesting media as the source of contamination to assess accumulation of HMX into eggs at different combinations of time and concentration. Also, initial growth parameters were measured for all hatchlings. The second study was designed to assess HMX transfer from the diet of adults to eggs. In addition to assessing accumulation, we measured hatching rates of eggs and growth rate of hatchlings, and monitored adult anoles for signs of toxicity and signs of abnormal reproductive function.

15.0 TEST ANIMALS:

Species: green anoles (*Anolis carolinensis*)
Strain: green anoles (free-living)
Age: green anoles (hatching year or after hatching year)
Number: anoles (90)
Source: anoles (trapper from Louisiana)

16.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Anoles were placed into individual labeled 10-gallon glass aquaria containing the appropriate identification information for the animal on the front of the aquarium. Collected egg samples were placed in plastic cups with the appropriate identification information on it. All samples collected at the end of their term in the study were placed

in individually labeled bags/collection vials and were stored at -20°C until further analyses could be done.

17.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The egg exposure study was designed to quantify the accumulation of HMX from contaminated nesting material into the incubating egg. Four treatment groups were designed on a log scale of increasing dosages: control (0 HMX), low (20 mg HMX/kg soil), medium (200 mg HMX/kg soil), and high (2000 mg HMX/kg soil). Eggs from unexposed females were randomly placed into each of the groups so that no more than one egg from each female was placed in any one group. In turn, the eggs were randomly placed in the incubator to remove any possible position bias in the incubator.

The maternal exposure study was designed to quantify the accumulation of HMX from the mother to the offspring via maternal transfer. Five treatment groups were designed for this study, including an experimental control group (undosed crickets), a solvent control (polyethylene glycol-injected crickets), low (20 mg HMX/kg body weight), medium (250 mg HMX/kg body weight), and high (500 mg HMX/kg body weight). All female anoles were randomly placed in individual tanks and were divided into treatment groups using a randomized block design that was stratified so that each dose group had equal representation on each of the shelves. All animals were treated similarly throughout the study and separate utensils were used for all control groups so that unintentional exposure did not occur.

18.0 METHODS

18.1 Egg Exposure Studies (HMX transfer from soil to eggs)

18.1.1 Lizard Husbandry

Eggs for both the PSD study and the artificial nesting study were retrieved from an unexposed colony of *Anolis carolinensis* at the university that were trapped in southeastern Louisiana one month prior to the start of the study. The anoles were housed individually in 10-gallon glass aquaria with sphagnum peat moss substrate, half PVC pipe for hiding, dowel rod for thermoregulation, heat lamp, and access to UVB lamp that was turned on for two hours each day. Male and female anoles were paired for 24 hours once a month for breeding purposes.

18.1.2 Soil preparation

Standard HMX (CAS No. 2691-41-0; 99.0% pure; molecular weight = 296) and analytical grade acetone was obtained from Supelco (Bellefonte, PA, USA). Soil was mixed on May 31, 2005 to use in the artificial nesting study that would take place throughout the month of June. The soil mixture (75% sand and 25% organic potting soil, by volume) was put into a small cement mixer and was mixed for 30 min. After homogenizing, the soil was sifted through a 2 mm sieve. The soil was then separated into four groups (control, low, medium, and high) for spiking with an HMX/acetone solution.

Five kg of soil was spiked with 20 mg/kg HMX (low), 8.5 kg of soil was spiked with 200 mg/kg HMX (medium), 5 kg of soil was spiked with 2,000 mg/kg HMX (high), and 5 kg of soil was spiked with 200 mg/kg acetone (control). These values are considered an ecologically relevant representation of the range of concentrations found at military installations (Jenkins et al. 1999). Soil was sprayed with the appropriate solution, hand mixed, then sprayed again. The soil from each dose group was then put into a rock tumbler and tumbled for 40 min. to completely homogenize. The soil was then placed in a darkened hood overnight so that the acetone could evaporate. The next morning, the soil was mixed again and seven samples were taken randomly from each dose group for analytical verification of the concentration (Table 2). The samples were mixed with sodium sulfate, and the HMX was extracted using the Accelerated Solvent Extractor (ASE) (see analysis section below). The remaining soil was left for a second night to further evaporate the acetone. This soil was then put in large freezer bags and placed in a -20°C freezer until needed.

18.1.3 PSD study

This study was designed to assess the ability to use passive sampling devices (PSDs) to predict concentrations of HMX in eggs incubated in contaminated soil. Eighteen eggs and 18 PSDs (C18 sealed in whirl-paks) were housed individually in cups (eggs) or glass jars (PSDs) containing 100 g of medium HMX soil (283.37 mg/kg soil, SD = 51.19) and 10 mL of distilled water. Each egg was buried about one centimeter below the surface of the soil and cups covered with cellophane and secured with a rubber band. Egg cups and PSD jars were placed randomly in the incubator (maintained between 29C and 32C). Three eggs and their paired PSDs were taken from the incubator on days 1, 3, 6, 12, 18, and 30 and frozen, along with the soil from the container, at -20°C prior to analysis.

18.1.4 Nesting Study

This study was designed to assess the dose-dependent uptake of HMX by eggs incubated in soil during the normal incubation period. Beginning on June 9, 2005, unexposed eggs were randomly placed into one of the four treatment groups (control, low, medium, high), with care taken to have only one egg from each female in each group. Fifty-five eggs were collected for this study (14 control, 14 low, 14 medium, and 13 high). Eggs were placed in containers and treated exactly as described for the PSD study. Since the goal of this portion of the study was to look at uptake into the egg and embryo, all neonates were sacrificed within 24 hr of hatching. Measurements of the neonate included snout-vent length, whole body length, and weight, along with any visual observations. The hatchlings were then frozen at -20°C and analyzed for HMX residues.

18.1.5 Sample analysis

All samples (both soil and egg) were individually homogenized with liquid nitrogen and dehydrated with four to five grams of sodium sulfate using a small mortar and pestle. The samples were then extracted using a Dionex Accelerated Solvent Extractor (Model 200, Salt Lake City, UT, USA) using 100% acetonitrile (analytical grade, obtained from Supelco, Bellefonte, PA, USA) as the extraction solvent. The extraction procedure was as follows: 5 minute preheat, 5 minute heat, 5 minute static extraction at a constant temperature of 100°C and pressure of 1500 psi. The extracts (15-20 mL/sample) were

then purged from the cells into glass collection vials using nitrogen gas. The extracts were then diluted to 25 mL with acetonitrile using clean volumetric flasks. Five mL from each extract was taken for a stock solution and the remaining 20 mL was concentrated using a vortex evaporator until a final volume of approximately two mL was reached. The concentrates were cleaned up using a styrene-divinybenzene (SDB) cartridges obtained from Supelco (Bellefonte, PA, USA). Each SDB cartridge was conditioned twice with three mL of acetonitrile, after which the sample extracts were loaded, and the elutes were collected by aid of gravity into plastic 15-mL Falcon centrifuge tubes (Franklin Lakes, NJ, USA). The SDB cartridges were then rinsed with one mL acetonitrile three times, the elutes continuing to be collected. The final extract volume was adjusted to five mL with actonitrile. One mL of the final concentrated volume was then diluted with one mL of nanopure water (milli-Q water at 18.3 MΩ, Barnstead NANOpure infinity system, Dubuque, IA, USA) and filtered with through a 0.20 µm PTFE syringe filter into autosampler vials.

All samples were analyzed using liquid chromatography tandem mass spectrometry (LC-MS). The liquid chromatography portion used a Finnigan system, which included a vacuum membrane degasser, a gradient pump, and an autosampler (San Jose, CA, USA). Chromatographic separation was achieved using a Supelco RP C18 column (4.6 x 250 mm, 5-µm packing) (Bellefonte, PA, USA). Mass spectrometry analyses were conducted using a Thermo-Finnigan LCQ advantage ion trap mass spectrometer. Helium was used as the dampening gas for the ion trap and nitrogen was the sheath and auxiliary gas for the ion source. The LC-MS operation conditions are noted in Table 1.

The C18 from the passive sampling devices was extracted and filtered in the same way as the egg and soil samples. After using the vacuum manifold, the concentrated volume was adjusted to five mL. One mL of this concentrate was mixed with four mL of nanopure water and was filtered into the autosampler vial. These samples were analyzed using the HPLC.

LC Conditions		MS Conditions	
Mobile phase A:	Methanol	Mode:	Negative
Mobile phase B:	0.5 mM aqueous acetic acid	Ion spray voltage (KV):	3.5
A: B = 60:40 (v/v)		Sheath gas flow rate (L/hr):	44.0
Flow rate: 0.5 ml/min		Aux/Sweep gas flow rate (L/hr):	53.1
Injection: 25 µL		Capillary voltage (V):	- 6.3
		Capillary temp (°C):	140.0
		Multipole 1 offset (V):	1.7

	Lens Voltage (V):	25.6
	Multipole 2 offset (V):	7.0
	Multiple RF Amp (Vp-p, sp):	500.0

Table 1. Optimized LC-ESI-MS operation parameters (Pan et al., in press).

18.2 Maternal Exposure Studies

18.2.1 Anole housing and husbandry

Sixty-six adult female and twenty-four adult male green anoles were purchased from a trapper in southeastern Louisiana and housed at The Institute of Environmental and Human Health, Texas Tech University. Each anole was housed in its own individual ten-gallon glass aquarium, complete with sphagnum peat moss substrate, PVC pipe for hiding, wooden perch for thermoregulation, and plastic container with moist peat moss for oviposition. The aquaria were placed on metal racks around the room, with each animal having access to its own 40-watt heat lamp and the UVB bulb that was stretched across each row of four tanks. The UVB bulb was on for two hours every morning for vitamin B3 synthesis. All of the aquaria were misted via the Rainmaker I Expanded Fully Automatic Misting System (Ecologic Technologies, Inc., Pasadena, MD, USA) three times daily. The aquaria were cleaned at least once a week of fecal matter, dead skin, and cricket parts and were sterilized every six weeks with over-the-counter vinegar. The room was held at a 14:10 light:dark cycle to mimic the summer breeding season. A minimum of once per month the anoles were paired for 24 hours for breeding purposes.

The anoles were randomly assigned an identification number, the first anole out of the shipment being number one, the second being number two, and so on. Prior to any dosing with HMX, all anoles were given one large cricket each morning. To ensure proper nutrition, the crickets were given OrangeCube (Fluker Farms, Port Allen, LA, USA) every two days and were dusted with additional calcium powder once per week.

18.2.2 Dosing of adult females

Dosing of adult female anoles began on July 11, 2005. Each female was weighed on July 10, 2005 to get an intial pre-dose weight and to calculate the necessary dose for the first two weeks of dosing. The females were randomly assigned to one of the five dose groups (negative control (no solvent)), solvent control (polyethylene glycol), low dose HMX (20 mg HMX/kg body weight), medium dose HMX (250 mg HMX/kg body weight), and high dose HMX (500 mg HMX/kg body weight)) using a stratified block design, which was stratified by shelf so that each dose group had equal representation at each height around the room. The high dose was selected based on our data which indicated that no toxicity occurred below 2,000 mg/kg and pilot data that suggested rejection of crickets spiked with HMX at concentrations delivering a dose of 1000 mg HMX/kg body weight.

HMX was delivered to the anoles by injecting the appropriate amount of HMX/PEG solution into a live cricket, which was then offered to the anole as food three times per week for 12 weeks. Since the crickets often died within a few hours of injection, each female was offered a maximum of three dosed crickets throughout the day, until she either ate one of the crickets or it was determined that she refused to eat. If a female rejected her spiked cricket three times, she was offered an undosed cricket on the following day in order to minimize any problems with starvation. The anoles were fasted on the seventh day of the week to increase their chances of eating on the next dose day. The weight of each anole was recorded every two weeks and its dose adjusted accordingly. The HMX/PEG solution was mixed in a glass vial using magnetic stir bar and mixing continued throughout the dosing procedure to maintain the solution. The solution was drawn into a glass Hamilton syringe, a 28 gauge needle attached and then primed with solution. In an effort to validate this delivery method, each week for 10 weeks of the dosing study one cricket from each dose group was injected with the solution and immediately frozen for future residue analysis.

All female cages were checked daily for eggs. The first egg from each female during the first four weeks of dosing was immediately frozen at -20°C so that later analysis would show their concentration upon leaving the mother. All other potentially viable eggs were placed in a plastic cup with 9 g of vermiculite and 9 mL of nanopure water (milli-Q water at 18.3 MΩ) and the cup was secured with cellophane and a rubber band. The eggs were given 48 days in the incubator to hatch. Those that did not hatch were frozen at -20°C for later residue analysis. Those that did hatch were put in a clean five-gallon aquarium, set up exactly like the adult cages. These hatchlings were monitored daily and weight, snout-vent length, and whole length were recorded once weekly for 63 days (nine weeks), at which time they were sacrificed and placed in a -20°C freezer.

18.2.3 Analysis

Residue analysis on the un-hatched eggs was completed using the same method described above for the eggs from the artificial nesting study. The one exception to the above described method is that the eggs were rinsed with milli-Q water, dried, and weighed before homogenization. After extraction with the ASE, concentration, cleanup with the SDB cartridges, dilution, and filtering into the autosampler vials, the eggs were analyzed using the LC-MS. The same method described above for the LC-MS was used for these eggs (see Table 1).

19.0 RESULTS

19.1 Egg Exposure Studies

19.1.1 PSD Study

HMX accumulated in eggs and PSDs (Fig. 1). Accumulation of HMX into PSDs was variable, but did suggest a time dependent uptake by day 30. The spike in mean HMX concentration on day 6 is inconsistent with the remaining data and should likely be considered an artifact. Conversely, HMX accumulation into eggs was more consistent over time, and appeared stable through day 12, followed by a gradual increase through

day 30. With the exception of the PSD data on day 6, there appears to be reasonable correspondence between HMX concentrations in PSDs and eggs.

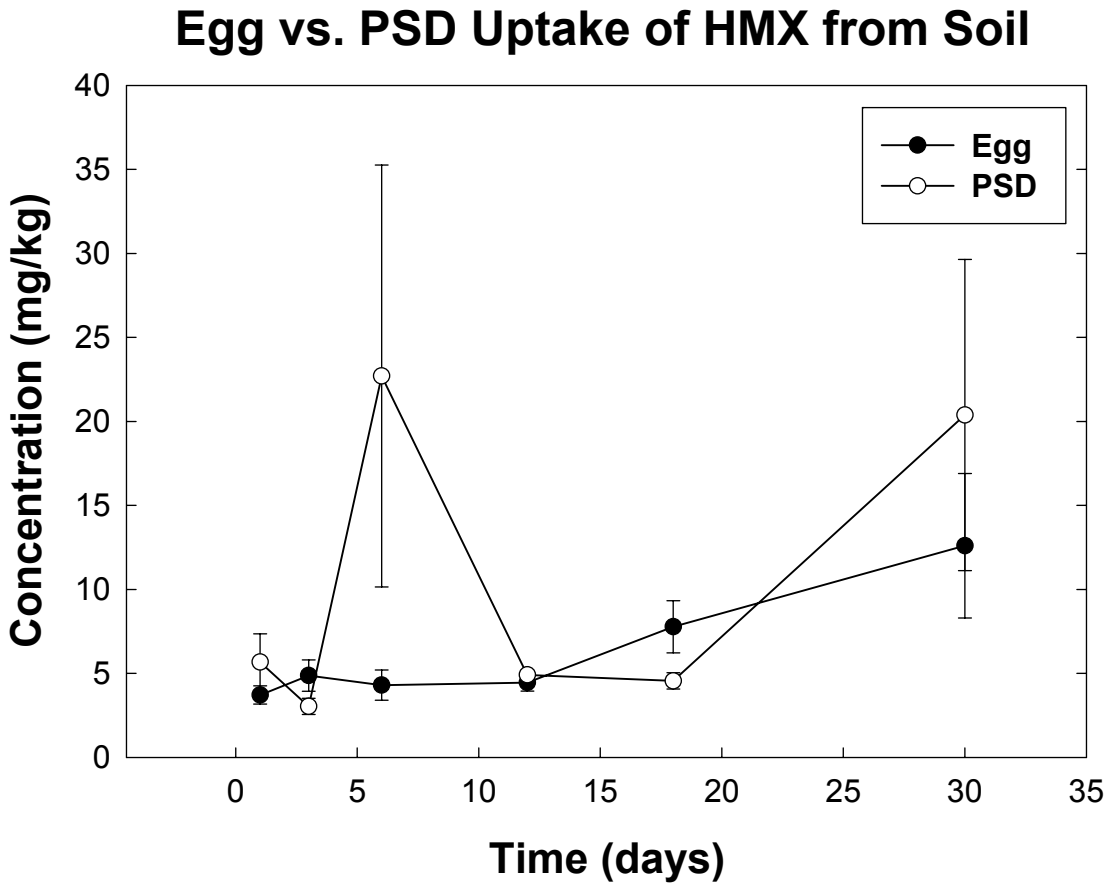

Figure 1. Mean (± SE) HMX accumulation from soil (at concentration of 283.37 ± 19.35) into the anoline egg and PSD over 30 days. Each time period had three replicates.

19.1.2 Nesting Study

HMX contamination of the soil used in the artificial nesting study was fairly consistent within each dose group. Aliquots from both the original stock of soil and from the soil used in the incubation study showed a relatively stable concentration of contaminant over time and among groups (Table 2).

Dose Group	Pre-Study Mean	SE	Post-Study Mean	SE
Control	0	0	0	0
Low	19.75725	0.6216	17.7215647	0.1937
Medium	283.36937	19.35	174.375139	3.3427
High	2244.1955	848.23	1760.69205	22.391

Table 2. Comparison of soil concentrations of HMX before and after use in the artificial nesting study.

Residue analysis on the eggs in the nesting study showed HMX accumulation into all eggs in the low, medium, and high groups following a dose-dependent response (Fig. 2). Of the 55 eggs incubated in this study, 27 hatched (7 control, 6 low, 9 medium, 5 high). None of the developmental parameters measured (body weight, snout-vent length, whole length, or incubation time) differed (P>0.05) among treatment groups (Fig. 3-6).

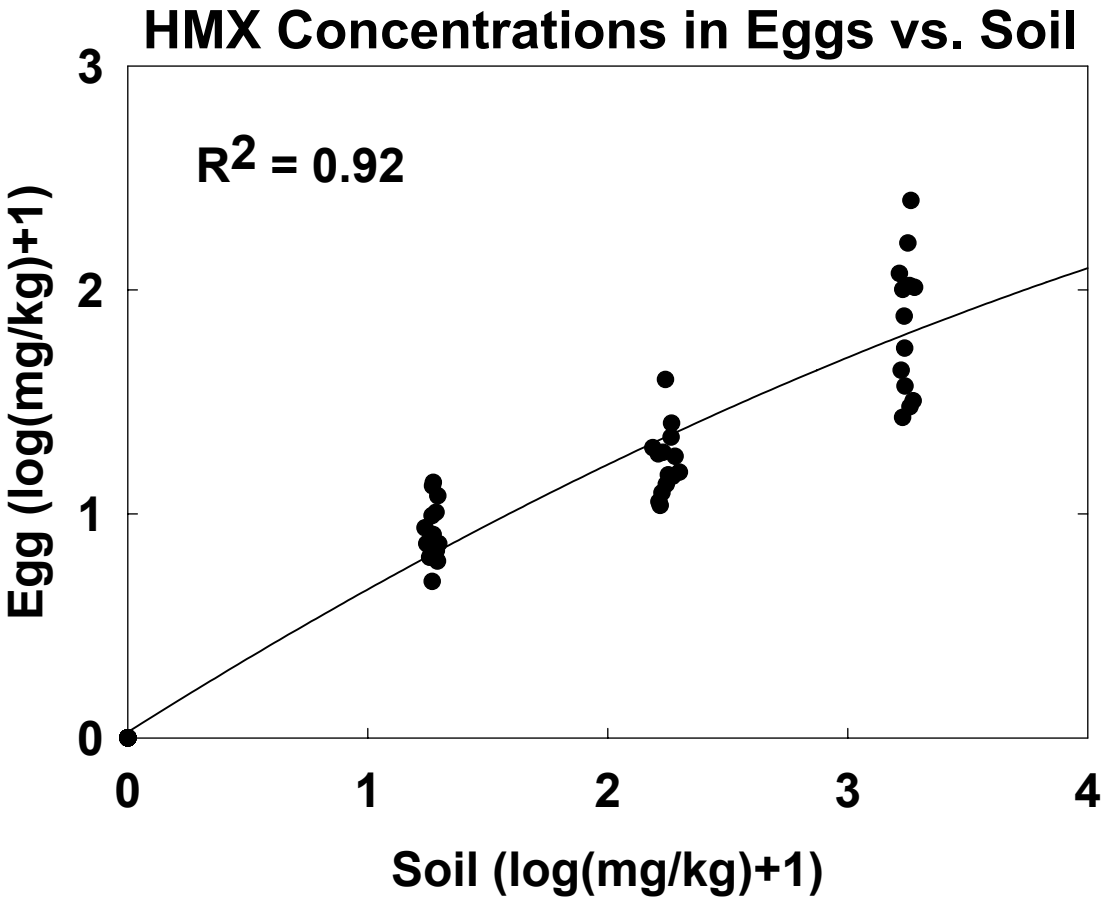

Figure 2. Comparison of HMX accumulation into the anoline egg at four nominal concentrations of HMX in soil: control (0 HMX; n=14), low (20 mg/kg HMX; n=14), medium (200 mg/kg HMX; n=14), and high (2000 mg/kg HMX; n=13).

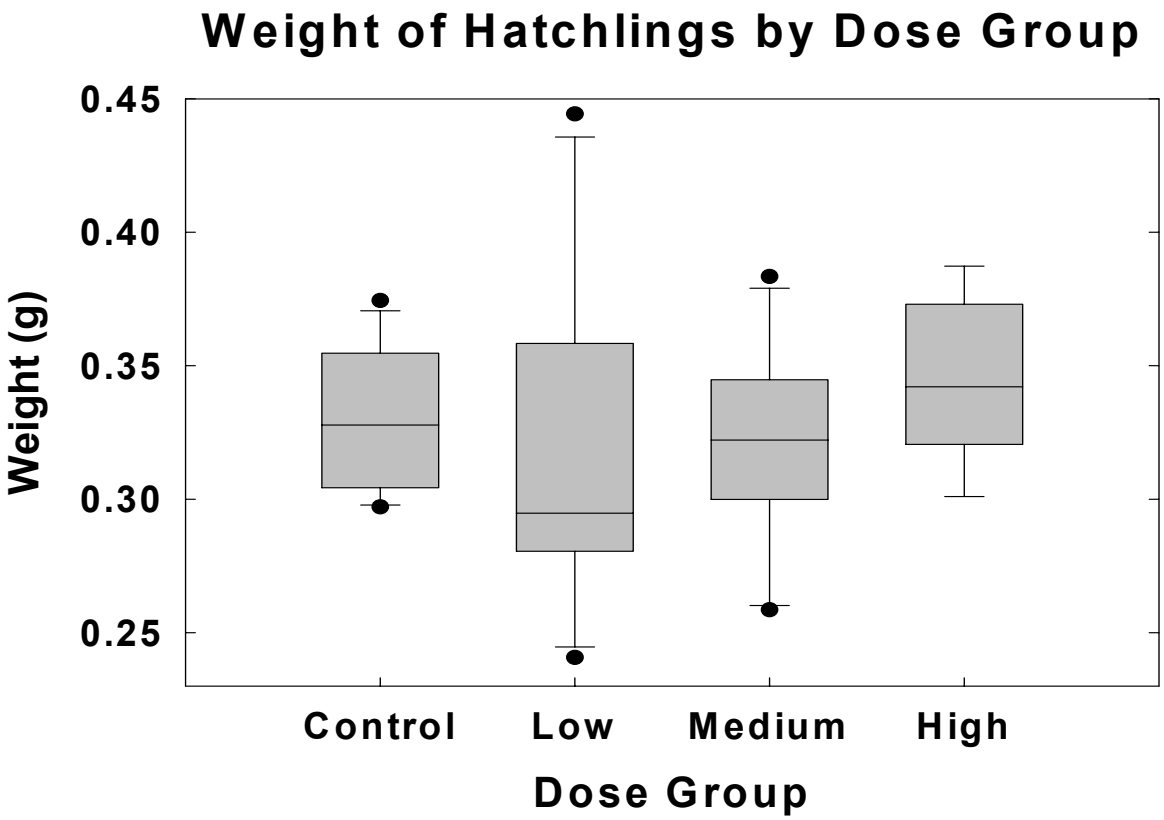

Figure 3. Comparison of hatchling body weight following chronic exposure to HMX via soil. Boxes represent first and third quartiles, lines represent medians, whiskers represent the largest/smallest observation that falls within a distance of 1.5 times the box size from the nearest hinge, and points represent outliers. Analysis of variance showed no statistical difference among dose groups ($p = 0.1792$).

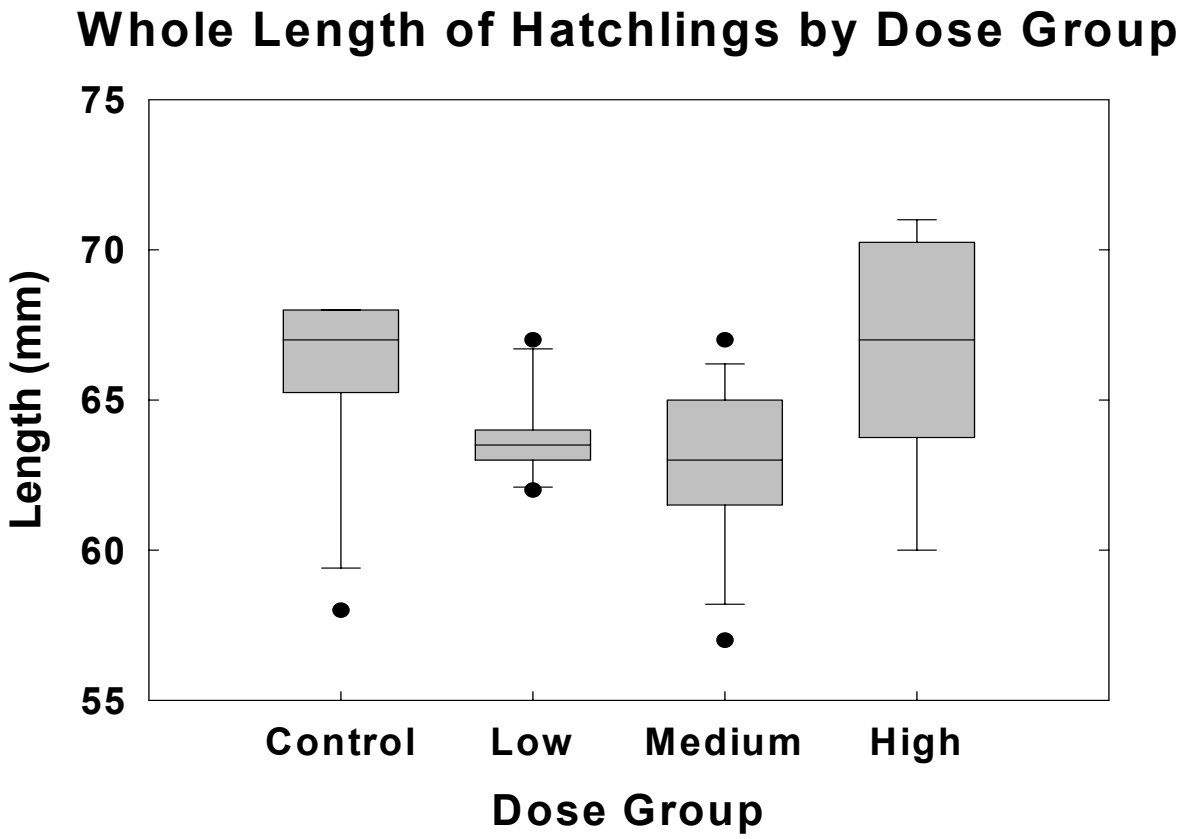

Figure 4. Comparison of hatchling body length following chronic exposure to HMX via soil. Boxes represent first and third quartiles, lines represent medians, whiskers represent the largest/smallest observation that falls within a distance of 1.5 times the box size from the nearest hinge, and points represent outliers. Analysis of variance showed no statistical difference among dose groups ($p = 0.1866$).

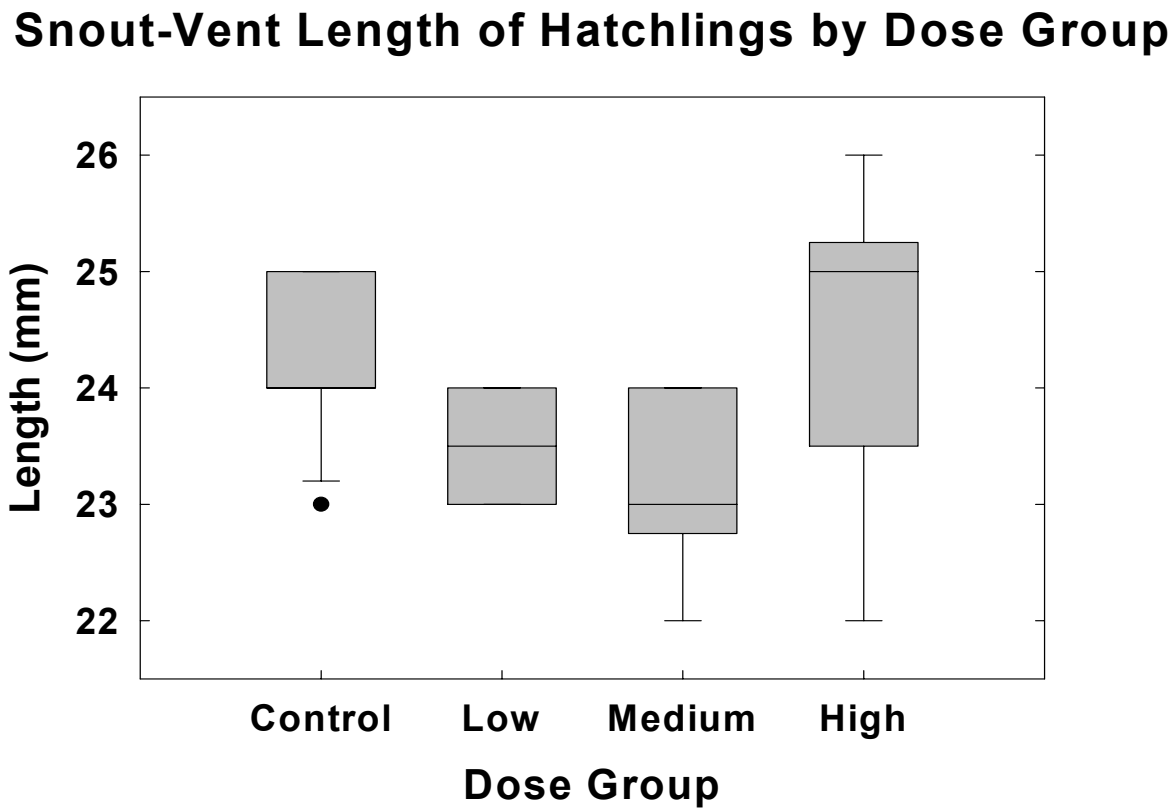

Figure 5. Comparison of hatchling snout-vent length following chronic exposure to HMX via soil. Boxes represent first and third quartiles, lines represent medians, whiskers represent the largest/smallest observation that falls within a distance of 1.5 times the box size from the nearest hinge, and points represent outliers. Analysis of variance showed no statistical difference among dose groups ($p = 0.0679$).

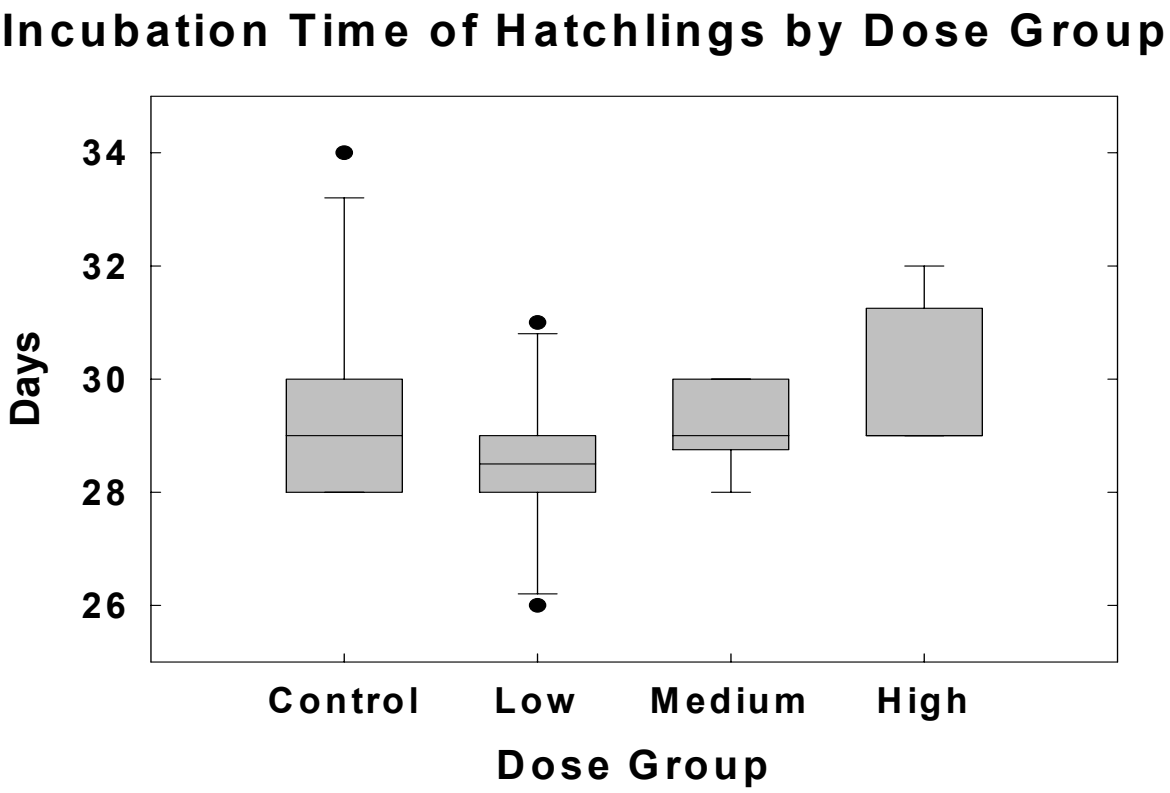

Figure 6. Comparison of hatchling incubation time following chronic exposure to HMX via soil. Boxes represent first and third quartiles, lines represent medians, whiskers represent the largest/smallest observation that falls within a distance of 1.5 times the box size from the nearest hinge, and points represent outliers. Analysis of variance showed no statistical difference among dose groups (p = 0.1017).

Dose Group	Number of Hatchlings	Number of Eggs Lain	% Hatching Success
Control	7	14	0.5000
Low	6	14	0.4286
Medium	9	14	0.6429
High	5	13	0.3846
Total	27	55	0.4909

Table 3. Comparison of hatching success across treatment groups of eggs exposed to HMX via contact with contaminated soil.

19.2 Maternal Exposure Study

19.2.1 Adult Females

On average, the female anoles consumed between 1.5 to 2.5 dosed crickets per week during the maternal transfer portion of the study (Figure 7). Though highly variable, the HMX treatment groups appear to consume fewer dosed crickets per week than do the control groups. This lowered food consumption seems to coincide with the patterns seen in the anoles' percent weight loss throughout the study (Figure 8). The three HMX treatment groups (low, medium, and high) consistently had a lower percentage of their original body weight than did either of the control groups. While this may seem like a confounding factor to the study, there was clear differentiation of actual administered dose amounts for each dose group (Table 4). Additionally, the percent recoveries from the dosed crickets frozen for method validation show that the expected concentrations administered to the anoles were accurate (Table 5), so that effects could justifiably be attributed to each dose group.

Consistent with the dose-dependent uptake of HMX into the anoles, a dose-dependent whole body concentration was seen at the termination of the study (Figure 9). The mean concentrations ± SE for the controls, low, medium, and high groups were ND, 1210.97 ± 509.80, 23012.88 ± 16724.97, and 63145.27 ± 25174.33, respectively. (One outlier was removed from the medium group for this analysis since its concentration (2.52e+6 mg/kg) was substantially higher than all other data points.) The high dose group (500 mg/kg) anoles had a whole-body concentration that was significantly higher than all other dose groups ($p < 0.001$). The medium dose group (250 mg/kg) anoles also had a residue concentration significantly different than the control groups ($p < 0.001$). There was no significant difference between the control groups and the low group (20 mg/kg).

With regard to the anoles' egg production rates, the findings are unclear as to what effect HMX might have on this system. As seen in Table 6, egg production rates are much lower in the high and medium groups than they are in the control groups even before dosing. The fact that the animals were placed in the room using a randomized stratified block design rules out the influence of any external variables in the animal room. Therefore the real reason for this discrepancy remains uncertain.

Average Number of Dosed Crickets Eaten per Week per Anole

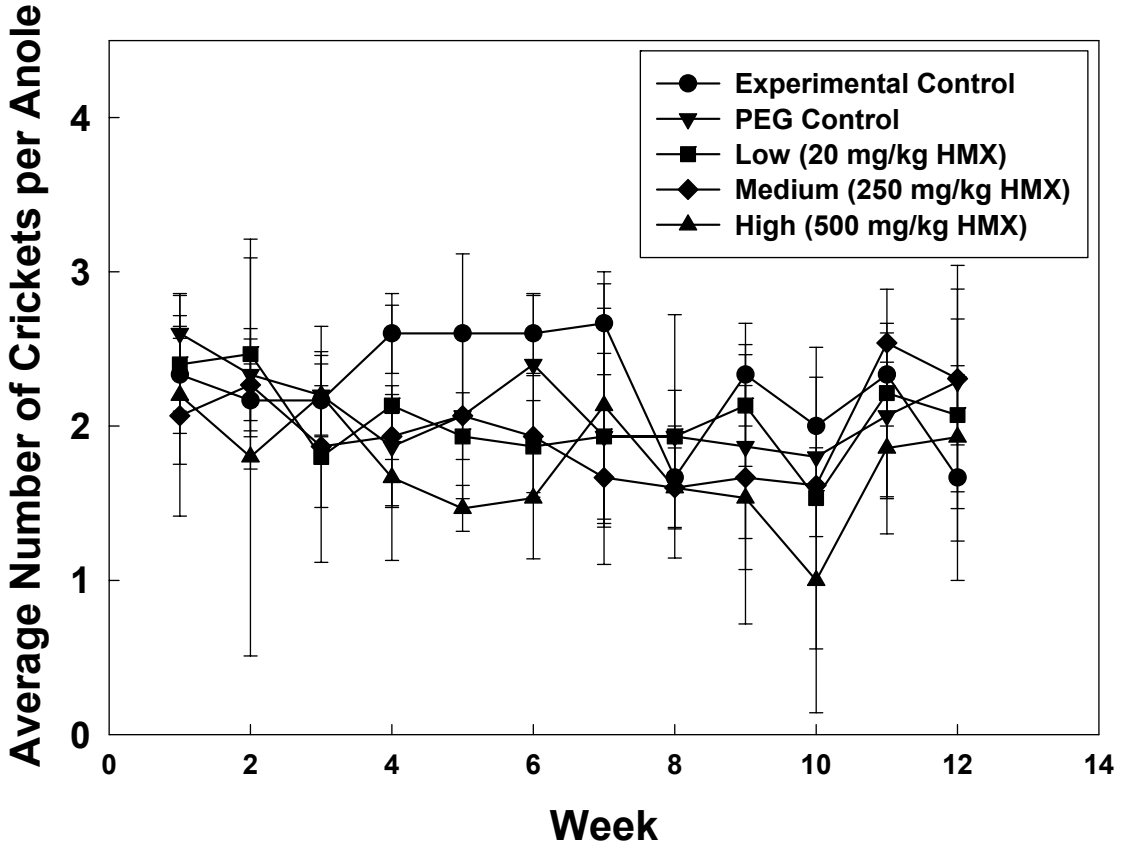

Figure 7. Mean (±SE) number of HMX-loaded crickets consumed per week by anoles in the experimental control (0 HMX or PEG), control (0 HMX; PEG injection), low (20 mg/kg HMX), medium (250 mg/kg HMX), and high (500 mg/kg HMX) dose groups.

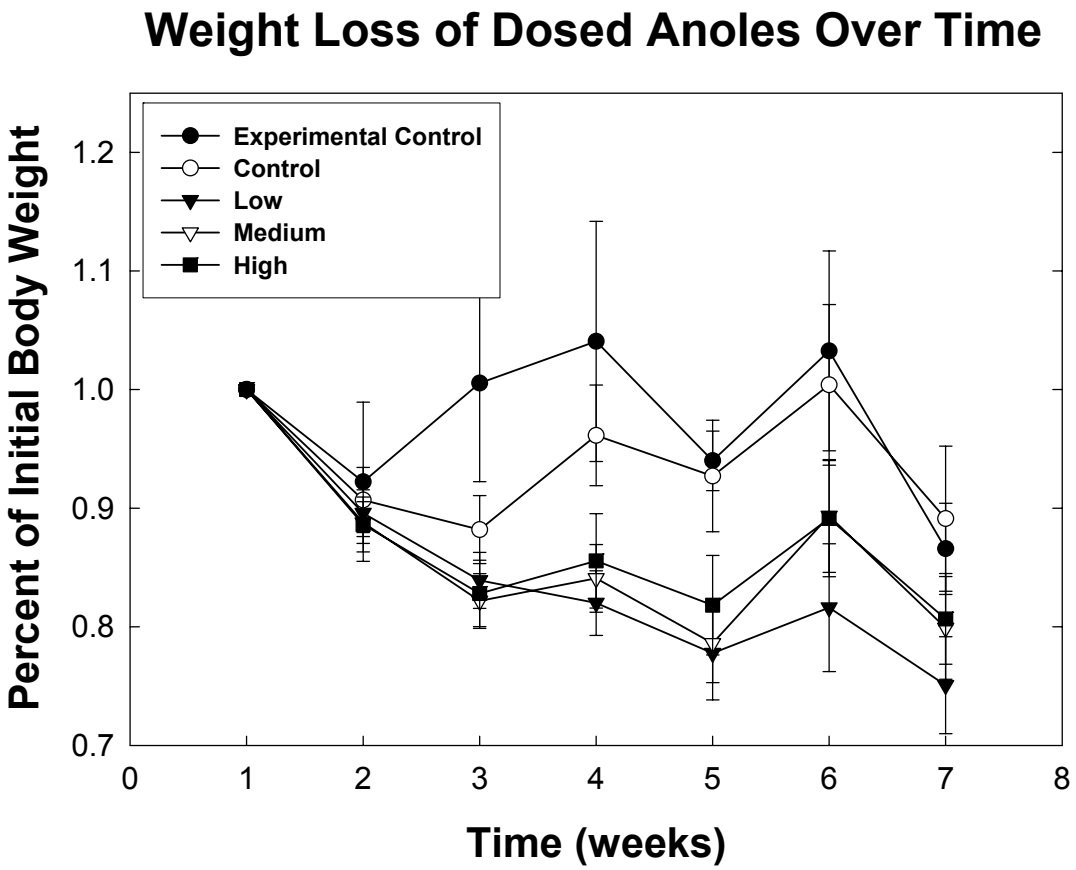

Figure 8. Mean (± SE) weight loss percentage of dietarily-dosed female anoles over time.

Dose Group	Average Dose per Week per Female (mg of HMX)	SE
Exp. Control	0	0
PEG Control	0	0
Low	0.108318684	0.010078154
Medium	1.252173037	0.111325656
High	2.308408542	0.16963382

Table 4. Comparison of the mean amount of HMX given to female anoles per week of the study.

Dose Group	Mean Percent Recovery	SE
PEG Control	ND	0.00
Low	103.28	9.79
Medium	114.26	26.50
High	91.59	12.36

Table 5. Comparison of the mean percent recovery of HMX in dosed crickets. Once per week for 10 weeks of the maternal dosing study one cricket was dosed and immediately frozen in order to validate the delivery method. The percent recovery for the PEG control group was not calculated since there was no detectable HMX in any of the control samples.

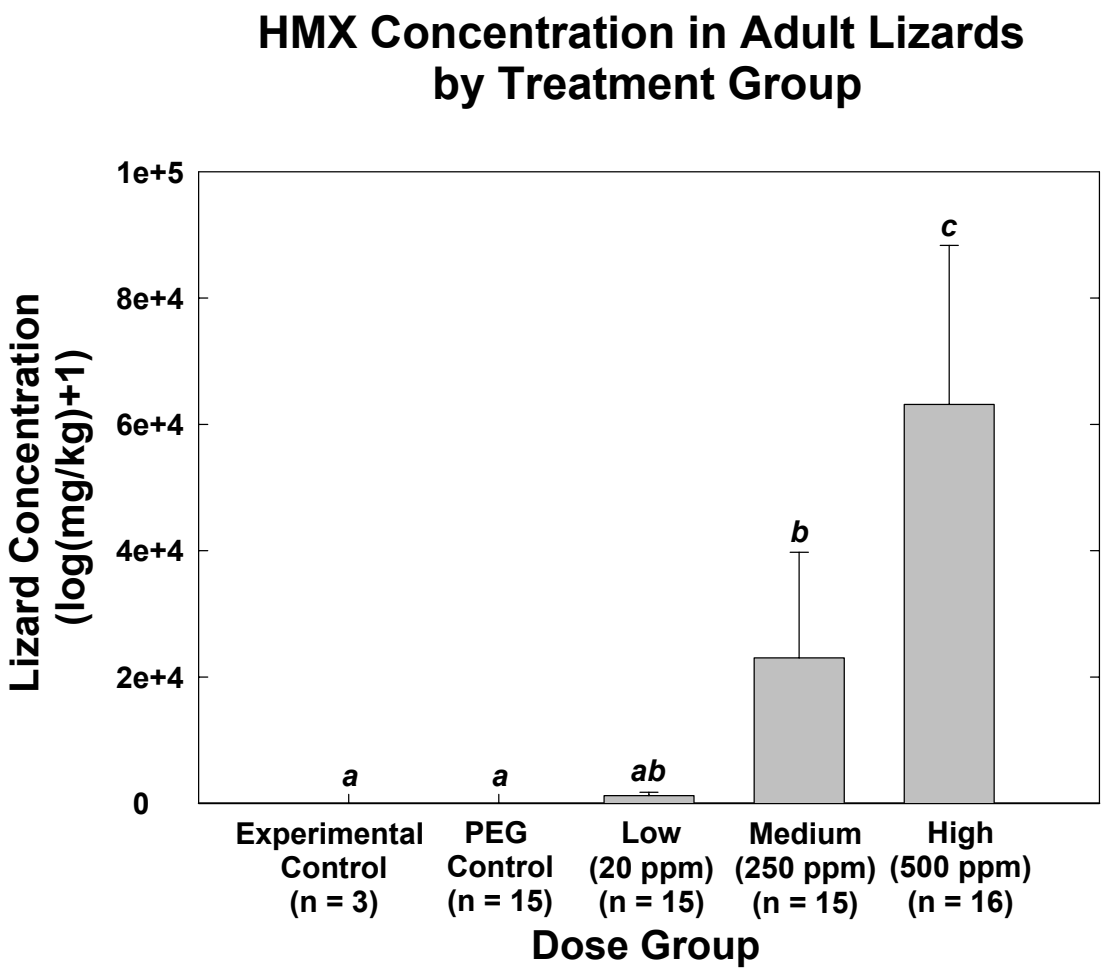

Figure 9. Mean whole body concentration (± SE) of treated female anoles at the end of the study, shown in mg HMX per kg body weight. Data shown excludes one female from the medium (250 ppm) group, whose concentration exceeded all other concentrations in the data set (concentration = 2.52e+6 mg/kg). Bars with different subscripts are different at $p < .001$.

	Maternal Dose Group (Total Eggs/Group; Eggs/Female/Week)									
	Experimental Control (n=3)		PEG Control (n=15)		Low (n=15)		Medium (n=15)		High (n=15)	
Total Number of Eggs Before Dosing	9	0.30	30	0.38	36	0.45	30	0.33	15	0.21
Total Number of Eggs After Dosing	13	0.36	40	0.26	39	0.25	28	0.23	27	0.21

Table 6. Comparison of the total number of eggs per dose group and the number of eggs per female per week.

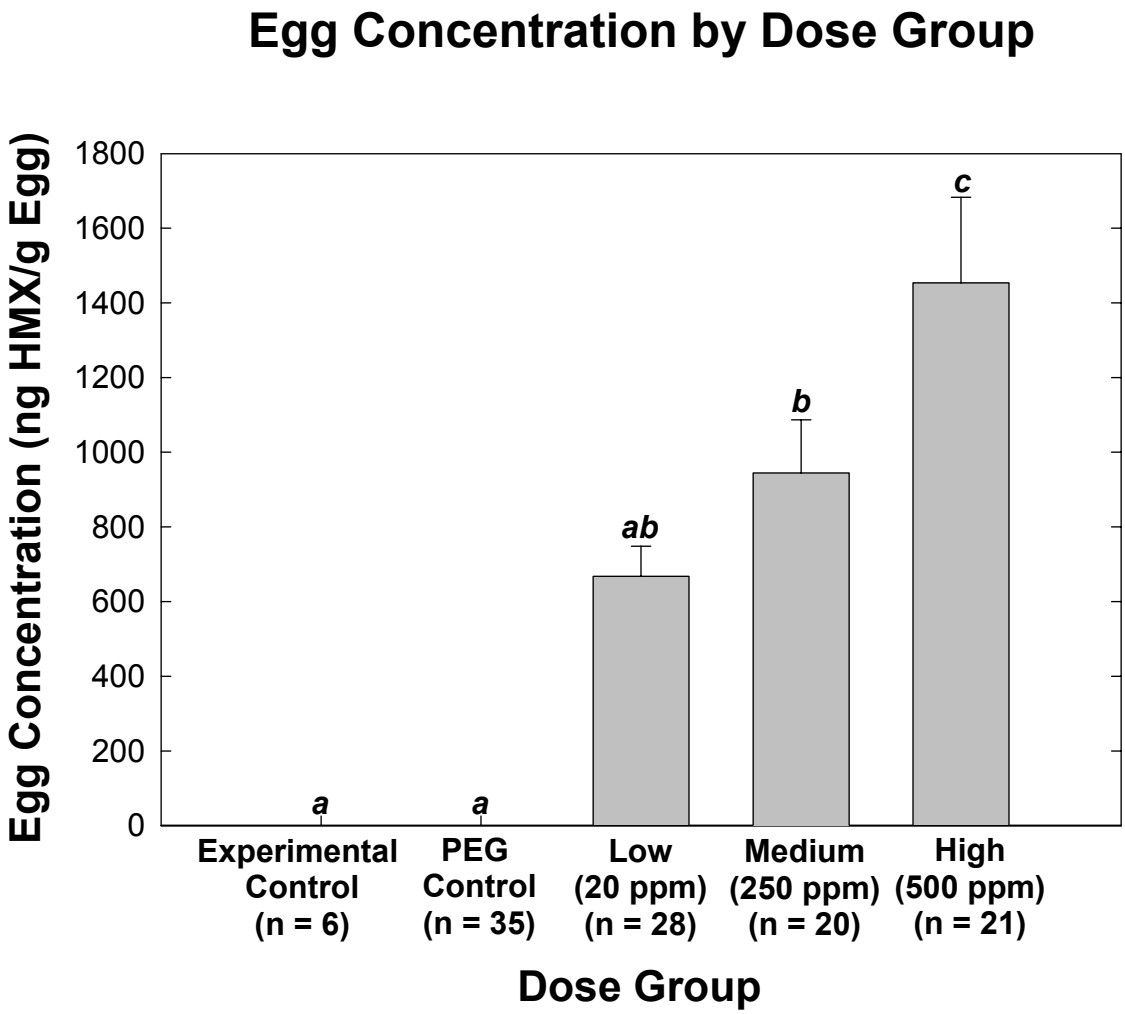

Figure 10. Mean egg concentration (± SE) of HMX as a result of maternal exposure to HMX in food. Bars with different subscripts are different at $p < .001$.

19.2.2 Eggs and Hatchlings

HMX was detected in eggs from all HMX-treatment groups (Figure 10). The deposition of HMX into the egg occurred in a dose-dependent fashion, with the high group having a significantly larger concentration than all other groups. The medium group eggs (maternal dose of 250 mg/kg) had a concentration that was significantly different than the control groups, but was not significantly different than the low group eggs (maternal dose of 20 mg/kg) ($p = < 0.001$). The weight of the eggs was not considered in this analysis, since a separate analysis of variance found the weights to be statistically similar ($p = 0.1212$). This dose-dependent trend is consistent with the trend seen in the maternal whole-body residues analysis (Figure 9).

With the exception of the experimental control group, the percent success of hatching seemed to be fairly consistent across the treatment groups (Table 7). When taken together, the anoles seem to have about a 37.9% success rate for hatchlings. However, when this is broken down into individual dose groups, it is interesting to see that the two groups with the lowest success rates are the control group and the high group (22.7% and 23.1%, respectively).

Dose Group	Number of Hatchlings	Number of Eggs Lain	% Hatching Success
Experimental Control	**9**	**13**	**0.6923**
PEG Control	**5**	**22**	**0.2273**
Low	**10**	**24**	**0.4167**
Medium	**6**	**15**	**0.4000**
High	**3**	**13**	**0.2308**
Total	**33**	**87**	**0.3793**

Table 7. Comparison of hatch success among HMX treatment groups.

The growth parameters of the hatchlings were analyzed using an analysis of covariance, allowing time to be a covariate (Figures 11 and 12). Both of the parameters (weight and snout-vent length) showed a significant interaction with the covariate, meaning that the relationship between the parameter and time changes between dose groups ($p = <0.001$, $p = 0.01965$, respectively).

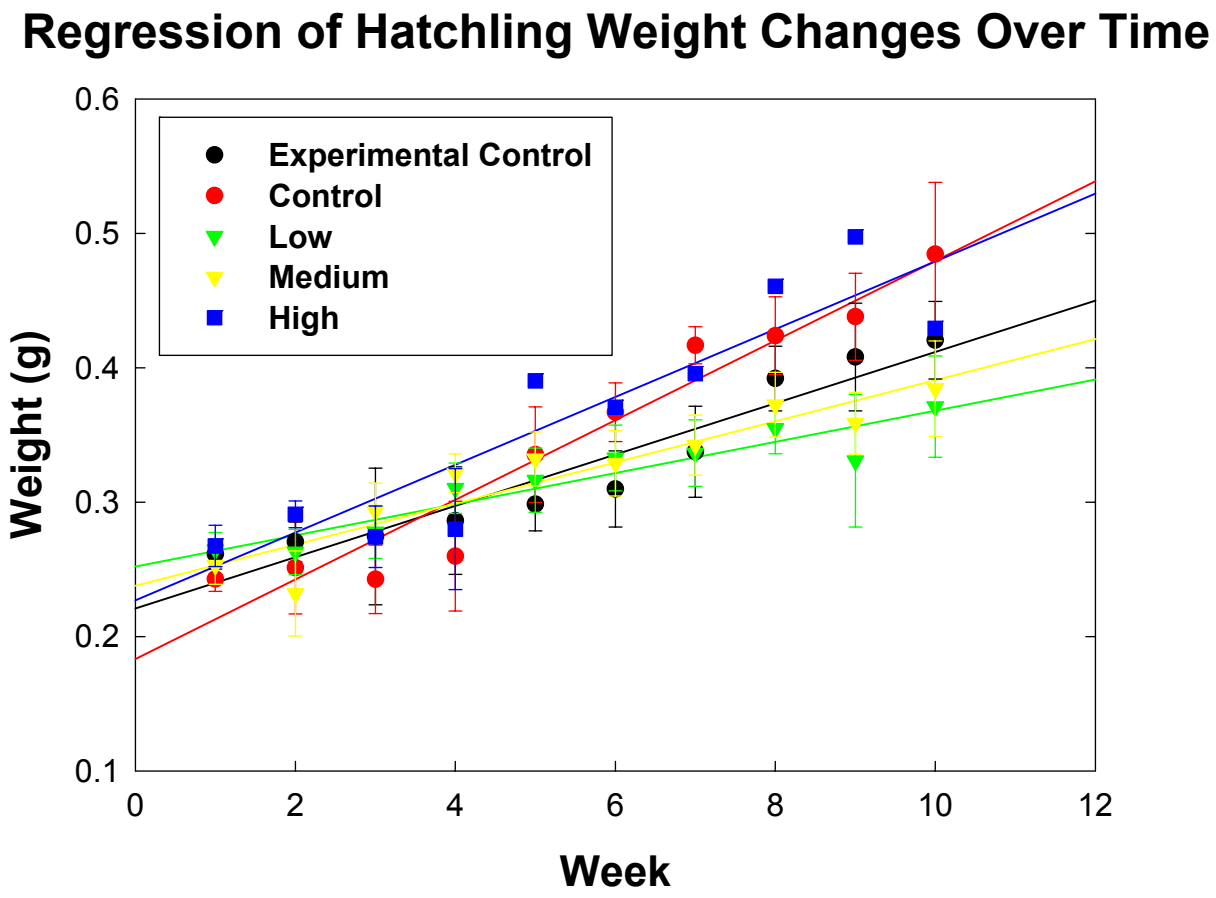

Figure 11. Comparison of the weight gaining trends of hatchlings exposed to HMX via maternal transfer. The interaction between the dose group and time is significant ($p = < 0.001$).

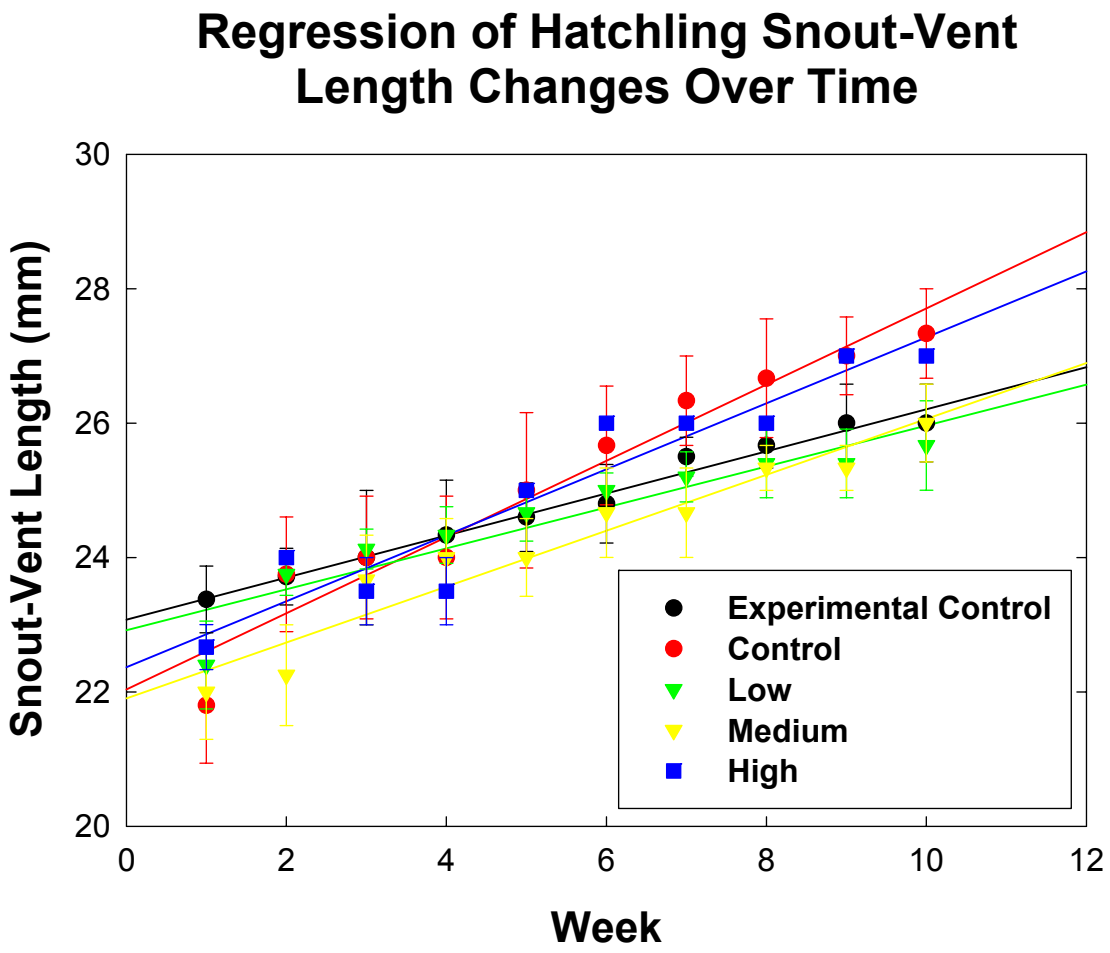

Figure 12. Comparison of the growth of the snout-vent length trends of hatchlings exposed to HMX via maternal transfer. The interaction between the dose group and time is significant (p = 0.01965).

20.0 DISCUSSION

20.1 Egg Exposure Studies

20.1.1 PSD Study

Although C18 PSDs have been shown to be good predictors of accumulation of contaminants into biota, their predictability in this study was unclear. This could be an artifact of either low moisture content of the soil or shortened exposure period. Other studies that have used C18 PSDs have used 75% moisture in the soil and have had exposure periods of up to 60 days (Zhang et al, 2006). Accumulation into the egg, on the other hand, seems to take place at a slow, steady pace.

20.1.2 Nesting Study

Results from the nesting study indicate that accumulation of HMX from the nesting media into a developing egg is possible. From the findings of the present study, it appears that this accumulation can be characterized in time, when all other conditions are held constant. Additionally, the HMX accumulation seen here indicates a dose-dependent trend. These results are considered to be quite reliable, as the concentration of the soil did not decrease appreciably throughout the term of the study.

The effect of this accumulation to the developing embryo is still somewhat uncertain. According to the statistical analyses of the developmental parameters measured (i.e., incubation period, weight, snout-vent length, and whole length), there are no significant differences between the treatment groups. Since no histopathological observations were made from the hatchlings, and their development post-hatching was not monitored, the true effects of the contamination cannot be known as yet.

20.2 Maternal Exposure Study

The present study examined the effects of repeatedly dosing the green anole, and via maternal transfer its offspring, with HMX. The findings suggest that the introduction of HMX to the anole's sole source of food causes a dose-dependent weight loss among treatments. Whether this weight loss is due to food repulsion or avoidance is unclear at this time.

Additionally, the findings suggest a dose-dependent accumulation of HMX in the body of the anole. While concentrations were extremely high, it is difficult at this point to determine what detrimental effects this had on the reproducing female. Of note is the several orders of magnitude difference from the concentration of the female to the concentration of the oviposited egg.

Finally, these data also suggest that HMX is able to enter the egg via maternal transfer in a dose-dependent manner. The effects that the accumulation of HMX may have on the developing embryo, at this point, appear to be minimal. The effect HMX has on hatchling success is still somewhat unclear, possibly an artifact of poor sample sizes across the treatment groups.

16.0 REFERENCES

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TITLE: **Evaluating Uptake of Incurred Explosives Residues**

STUDY NUMBER: **MRT-05-01**

SPONSOR: **Strategic Environmental and Research Development Program**
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

CONTRACT ADMINISTRATOR: **The Institute of Environmental and Human Health**
Texas Tech University/TTU Health Sciences Center
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TESTING FACILITY: **The Institute of Environmental and Human Health**
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Box 41163
Lubbock, TX 79409

TEST SITE:

RESEARCH INITIATION: **March 2004**

RESEARCH COMPLETION: **March 2005**

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

George P. Cobb
Co-Principal Investigator

Date

9.0 DESCRIPTIVE STUDY TITLE:
Evaluating Uptake of Incurred Explosives Residues

10.0 STUDY NUMBER:
MRT-05-01

11.0 SPONSOR:
Strategic Environmental and Research
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12.0 TESTING FACILITY NAME AND ADDRESS:

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13.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: March 2004
Termination Date: March 2005

14.0 KEY PERSONNEL:

Dr. George Cobb	Co-Principal Investigator
Mr. Nick Romero	Animal Care and Dosing
Mr. Jordan Smith	Animal Care and Euthanasia
Ms. Xiaoping Pan	Analyte Quantification
Dr. Kang Tian	Analyte Quantification
Dr. Ronald J. Kendall	Testing Facility Manager / Principal Investigator

15.0 STUDY OBJECTIVES / PURPOSE:

To determine the concentration of explosives and their metabolites in rodents following dosing of food containing incurred residues.

16.0 STUDY SUMMARY:

RDX and TNT are widely used explosives within the DOD. Many military installations have soil and/or groundwater contamination problems. These compounds are relatively tightly bound to organic matter in nature and are metabolized by soil microbes. We performed scoping studies wherein deer mice (*Peromyscus maniculatus*) are provided a

diet containing plant and earthworm material with incurred RDX, TNT and in all likelihood their primary metabolites.

17.0 TEST MATERIALS:

Test Chemical name: RDX
CAS number: 121-82-4
Characterization: Residues in plants cultivated in soils from Aberdeen Proving Grounds
Source: Waterways Experiment Station

Test Chemical name: TNT
CAS number: 38082-89-2
Characterization: Residues in plants cultivated in soils from Aberdeen Proving Grounds
Source: Waterways Experiment Station

18.0 JUSTIFICATION OF TEST SYSTEM:

The US Department of Defense desires knowledge regarding the trophic transport and disposition of explosives to assist risk assessors in their evaluation of ecological risks at military sites. For this reason a common species with wide distribution, the deer mouse (*Peromyscus maniculatus*) was selected. Dosing was conducted in a laboratory environment to control as many variables as possible.

19.0 TEST ANIMALS:

Species: *Peromyscus maniculatus*
Age: 45-90 day old
Number: 36
Source: University of South Carolina Peromyscus Stock Center
Sex: Male

20.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The study number was placed on the dosing room door and each cage contained information describing the study number, the mouse identification, sex and dose group. Each animal was also identified with a unique ear tag pattern.

21.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Effects and trophic transfer of TNT and RDX, respectively, on *Peromyscus maniculatus* (deer mouse) were determined by dosing mice with food contaminated with (a) TNT, and (2) RDX, incorporated into the tissues of *Lolium perenne* (perennial ryegrass) and *Eisenia fetida* (earthworm) in a study funded through ERDC-EL, Vicksburg, MS. Standard rodent chow was mixed with these powdered food stuffs. Dosing proceeded with as many animals as we calculated could be fed for 6 weeks with diets containing

decreasing percentages of each incurred residue type. We determined TNT, RDX, and selected metabolites in tissues and blood of the mice.

14.0 METHODS:

Animals: Sexually mature male deer mice were purchased from the Peromyscus Stock Center (Univ. of SC, Columbia, SC) and housed in an AALAC accredited facility at Texas Tech University. Mice were housed separately and maintained in environmentally controlled conditions of 18:6h light-dark cycle, 25° C, and relative humidity of 50%. Mice were acclimated for 1 week prior to dosing. Deer mice were randomly assigned to control and explosives contaminated food (see Table 1 for concentrations). Animals were weighed (±0.01g) before the initiation of the study. Access to food was provided on an *ad libitum* basis. Mice were dosed with powdered rodent chow (Purina Mills, St. Louis, MO, USA), amended with explosives residues from one of two sources, i.e., grass or earthworms

For each contaminant/food type combination, six animals were isolated for each dose group. The mice were housed as one per cage with aspen shavings as bedding. Environmental conditions were 12 h light: 12 h dark and temperatures of 21-24 ^{o}C. Each mouse received food and water *ad libitum*. All animals were exposed for 35 days to TNT, or 21 days to RDX. The weights of the animals and remaining food were also measured at regular intervals; i.e., at days -7, 0, 1, 7, 14, 21, 28, and 35 for TNT, and at days -7, 0, 1, 7, 14, and 21 for RDX. Every time the food stock was replenished in a cage, the weight of the food remaining before replenishment was recorded, and the food consumed calculated.

Preparation of food with incurred TNT or RDX: Plants and earthworms were cultivated in soils containing RDX or TNT by researchers at the Waterways Experiment Station. Each soil type was spiked with radiolabeled forms of RDX or TNT to allow better quantitation. Freeze-dried plant and earthworm materials were then shipped to TIEHH for testing.

Standard rodent chow was mixed with the plant and worm materials contaminated with TNT or RDX. Preparation was done by personnel from the food nutrition laboratory of the Animal Science Department at Texas Tech University, Lubbock, TX, USA. Each mixture was prepared by adding a preweighed amount of freeze-dried plant or earthworm material to an appropriate amount of powdered rodent chow (Table 1). Each food type was first hand-mixed with a stainless steel trowel for 5 minutes and subsequently mixed in a Hobart mixer (Hobart Corp; Troy, OH, USA) for 15 minutes. Doses were stored in air-tight plastic containers until use. Three trowels were available for hand-mixing to minimize reuse. Each trowel, mixing bowl and impeller was washed with water and acetone before and between uses. As much as 1.2 kg of food was available for each dose group. Each freeze-dried food amendment contained TNT or RDX as the primary contaminant. Radioactivity in the samples was used for dose confirmation (Table1), and the radioactivity increased linearly with dose.

General Observations: The general health of all animals was assessed daily by examination of the coat, activity, and food and water consumption. Body weights were recorded weekly and at necropsy. At day 30 animals were narcosed with CO_2. Animals were killed by cervical dislocation, and terminal blood samples were collected by cardiac puncture. Sera and liver were removed, weighed, and frozen (-20C) until chemical analysis. The accumulation of incurred TNT and RDX were evaluated after exposure.

Extraction of explosives and metabolites: Tissues were mixed with 8-10 g dried Na_2SO_4 using a mortar and pestle. A Dionex Accelerated Solvent Extractor (Model 200, Salt

Lake City, UT, USA) was used for all extractions as described in published methods developed in the Analytical Core of this research program (Pan et al., 2005, 2006) and are described here briefly. Each extraction cycle included 5-min preheating, and 5-min static extraction with 100% acetonitrile at 100°C and 1500 psi. Extracts were collected in glass vials and reduced to 1-2 mL using rotary evaporation in preparation for florisil and styrene-divinyl benzene cleanup (Pan et al., 2005). These steps removed large amounts of interfering compounds and pigments. The extract volumes were reduced to 1.5 mL under nitrogen using a N-EVAP^{TM} 111 (Organomation Associates, Inc. Berlin, MA, USA). The final volumes were adjusted to 2 mL with MilliQ water, filtered through a 0.20 µm membrane filter (Millipore, Bedford, MA, USA) into an autosampler vial, and stored until further analysis.

Blood samples were mixed with 7 mL of acetonitrile for every mL of blood. Mixtures were vortexed every 30 min for 2-3 h. Extracts were centrifuged at 3,500 rpm for 15 min. Supernatants were collected and purified using Florisil SPE cartridges. Extract volumes were reduced to 1-2 mL under nitrogen, and filtered through a 0.20 µm membrane filter prior to analysis.

Chemical Analyses: Chemical TNT and RDX concentrations were determined in blood using GC-ECD as developed in the analytical core for this research program (Zhang et al., 2005). An Agilent 6890 GC system (Palo Alto, CA) with automated 2 µl splitless injections was used with a 30m x 0.25 mm HP-5 column experiencing an oven temperature from 90 to 250 ^{o}C over a period of 13 min. The ECD was operated with an Ar/methane makeup of 40 mL min^{-1} and a constant current mode. Analyses were performed for TNT, RDX, MNX, DNX, and TNX. Detection limits of explosives in samples ranged from 0.2 to 2 ng mL^{-1} for the analytes used for calibration.

Explosives proved regularly to be below the detection limit of this method. Therefore, a more sensitive method using liquid chromatography-mass spectrometry (LC-MS) with selected ion monitoring (Pan et al., 2006) was also explored to measure the chemical levels. The latter method proved to be successful for the determination of TNT, RDX, and their known metabolites at the pg-level. Livers from the RDX portion of the study were evaluated by this technique.

5. RESULTS:

Given the low concentrations of toxicants incorporated into food, quantifying parent and metabolite concentrations was difficult. The chemical RDX-derived residues were determined in the blood of mice by GC-ECD. Concentrations were low with means ranging from 1.7 to 4.4 ng RDX ml^{-1} and 0.46 to 0.69 ng TNX ml^{-1} (Table 2). RDX derived residues in mouse liver were also determined by LC-MS (Table 3). Mean TNT, RDX, MNX, DNX and TNX concentrations in livers of mice receiving RDX treated food ranged from 0.95 to 20 ng/g but did not have concentrations that were different from

those found in control livers (P>0.44). Similarly, the TNT concentrations in muscle were low (Table 4) and treatment animals accumulated similar concentrations as did control animals.

Insignificant of TNT, RDX and RDX transformation products were accumulated during the study (Tables 2 through 4) indicating limited trophic transfer of TNT and RDX from food items to rodents. The possible reasons for this are the low doses used, rapid metabolism, or poor uptake via the rodent GI tract.

Table 1. Doses prepared by amendment of standard rodent chow with freeze-dried plant or worm material.

Explosive Dose	Food		
	% DW	[Explosive] (mg kg^{-1}) Nominal	Radiation Counts (millions)[a]
TNT			
Control	0	0	0
Plant, low	0.50	14.4	2.692
Plant, medium	1.49	42.9	7.262
Plant, high	4.04	116.3	14.199
Worm, low	0.42	4.1	3.287
Worm, high	1.32	12.9	7.680
RDX			
Control	0	0	0
Plant, low	0.46	15.0	7.130
Plant, medium	0.91	29.6	23.686
Plant, high	1.89	61.4	61.369
Worm, low	0.35	15.2	4.516
Worm, high	1.0	43.4	16.143

a- each food type was spiked with radiolabeled explosive before receipt at TIEHH to allow analytes to be detected more readily.

Table 2. TNT and RDX-residues in *P. maniculatus* blood following ingestion of contaminated food (N=6 unless specified in footnotes).

Exlosive/ Dose	Explosive concentration in blood (ng mL^{-1})[a]		
	TNT Dosing	RDX Dosing[b]	
	TNT	RDX	TNX
Control	0	0	0
Plant, low	6.87 (6.40-12.53)[c]	3.35 (1.02-11.02)	0.52 (0.33-0.80)

Plant, medium	9.08 (7.09-12.70)	4.37(0.60- 31.69)	0.53 (0.21-1.31)
Plant, high	31.11(23.23-53.3)[d]	2.64 (1.52- 4.58)-	0.57 (0.25-1.28)
Worm, low	9.93 (6.40-17.80)	4.28 (0.92-19.77)	0.46 (0.30-0.71)
Worm, high	8.05 (5.38-12.53)	1.74 (1.06-2.84)	0.69 (0.32-1.48)

[a]- corrected mean (95% confidence interval), t=2.571,
[b]- MNX and DNX were not detected
[c] N=4, t=3.182
[d] N=5, t=2.776

Table 3. Mean RDX and metabolite concentrations in *Peromuscus maniculatus* liver following ingestion of residues incurred in food. (N=6).

Explosive/ Dose	Explosive concentration in liver (ng g^{-1})[a]				
	TNT	RDX	MNX	DNX	TNX
Control	2.09 (1.22-3.72)	8.99 (3.21-27.69)	8.48 (3.31-23.91)	2.66 (0.49-16.00)	2.02 (0.59-7.69)
Plant, low	5.59 (1.40-18.95)	9.01 (2.38-37.54)	15.11 (7.14-35.24)	19.38 (9.91-41.73)	1.12 (0.43-3.11)
Plant, medium	2.37 (1.69-3.66)	7.30 (1.21-48.37)	6.04 (1.70-21.41)	6.95 (1.14- 46.79)	1.26 (0.44-3.99)
Plant, high	3.37 (1.50-7.43)	10.41 (3.28-36.43)	7.79 (3.51-17.25)	4.34 (0.80- 25.97)-	1.90 (0.49-7.98)
Worm, low	11.27 (2.37-48.07)	15.22 (5.97-42.74)	10.08 (5.78-19.38)	10.75 (2.22-57.15)	1.70 (0.36-8.97)
Worm, high	4.12 (2.35-7.63)	10.41 (2.36-50.56)	12.40 (6.08-27.92)	11.72 (2.21-68.39)	0.93 (0.31-3.06)

[a]- mean (95% confidence interval), t=2.571

Table 4. Chemical TNT residues in *P. maniculatus* muscle following ingestion of TNT contaminated food. (N=6).

Explosive/ Dose	Explosive concentration in liver (ng g^{-1})[a]
	TNT
Control	17.16[a] (8.63-36.7)
Plant, low	79.83 (28.34-244.34
Plant, medium	70.06 (28.37-188.40)
Plant, high	31.51 (12.41-85.81)

Worm, low	40.63 (19.26-93.20)	
Worm, high	15.49 (7.02-36.35)	

a- mean (95% confidence interval), t=2.57

DISCUSSION:

This study did not demonstrate significant uptake of TNT or RDX into mouse tissues. It is entirely possible that the dosing concentrations were too low for intake to exceed metabolic capacities. The maximum dose represented only a few percent of contaminated food in the diet. This dose should be increased to evaluate accumulation of these contaminants in the terrestrial environment.

6. REFERENCES:

Pan X, Zhang B, Cobb GP**.** 2005. Extraction and analysis of cyclonite (RDX) and its nitroso-metabolites in animal tissue using gas chromatography with electron capture detection (GC-ECD). *Talanta*. 813-824.

Pan X, Zhang B, TA Anderson, Cobb GP. 2006. Determination of N-nitroso metabolites of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in soils by pressurized liquid extraction (PLE) and liquid chromatography-electrospray ionization - mass spectrometry (LC-ESI-MS). *J. Chromatog. A.* 1007:2-9.

TITLE: Environmental fate and transfer of RDX in Constructed Wetlands

STUDY NUMBER: WET-05-01

SPONSOR: Strategic Environmental and Research Development Program
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TESTING FACILITY: The Institute of Environmental and Human Health
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RESEARCH INITIATION: December 1, 2004

RESEARCH COMPLETION: December 31, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

W. Andrew Jackson Date
Co-Principal Investigator

1.0 DESCRIPTIVE STUDY TITLE:
Environmental fate and transfer of RDX in Constructed Wetlands

2.0 STUDY NUMBER:
WET-05-01

3.0 SPONSOR:
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4.0 TESTING FACILITY:
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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: December 1, 2004
Termination Date: December 31, 2005

21.0 **KEY PERSONNEL:**
Dr. W. Andrew Jackson, Co-Principal Investigator / Study Director
Dr. Todd Anderson, Co-investigator
Mr. Darryl Low, Student Researcher
Dr. Ronald Kendall, Principal Investigator

22.0 **STUDY OBJECTIVE/PURPOSE:**

To evaluate the fate of RDX within wetland systems, develop new tools for monitoring RDX in saturated sediments at cm resolution, and to assess exposure and other processes. Natural wetlands frequently serve as interception points for discharging groundwater or surface run-off to waterways. Important fate processes include sorption, plant uptake, microbial transformation, and associated fate of daughter products.

23.0 TEST MATERIALS:

Test Chemical name: Hexahydro-1,3,5-trinitro-1,3,5-triazine
CAS number: 121-82-4
Characterization:
Source: SRI International

24.0 JUSTIFICATION OF TEST SYSTEM:

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has been found on numerous military bases at firing ranges, storage facilities, and other sites exposed to explosives and other energetics. As there may be a significant environmental risk posed by these compounds and non-point source cleanups are challenging and economically taxing, installed wetland systems are now being considered. The use of low impact passive technologies such as natural wetland systems to remove RDX from surface water and groundwater may be a promising alternative. In addition, wetland systems are often a critical interface of exposure for contaminants. This can occur either as interception points of non-point source runoff (e.g. firing ranges) or by groundwater discharges.

RDX has been a major explosive used by the United States military for over half a century. Widespread and severe contamination has been found at a number of sites across the country. Biodegradation of RDX has been achieved by a number of methods. Anaerobic degradation has been shown to have the greatest degradation potential while aerobic degradation occurs at a much slower rate. The most common degradation pathway is the oxidation of RDX's three nitro groups into nitroso groups then ring cleavage. Previous studies have observed the uptake of RDX by reed canary grass and RDX degradation within the plant (Just and Schnoor 2004).

Explosives persistence and bioavailability in the environment is dependent on a number of temporally variable factors. Contaminated surface water is the most commonly examined source, however, other important sources include near surface contamination in saturated and unsaturated systems. Contaminated surface water is likely to have temporally variable explosive concentrations due to variations in precipitation, groundwater discharge, and biological stability of explosives in the ecosystem in question. Persistence of explosives in contaminated surface and subsurface soil is dependent on infiltration rates, plant uptake/transformation, temperature, percent water saturation, and substrate availability. While many of these factors are site specific, a more rigorous understanding of the relationship between high explosive concentrations in sediments and bioavailability is required. One method which is ideal for studying bioavailabily in sediments is diffusion chambers or peepers.

Dialysis samplers or "peepers" are diffusion-based samplers, which can be used to determine the vertical distribution of soluble constituents in sediments or soil

porewater in the saturated zone. Dialysis sampling techniques have been utilized in numerous geochemistry studies beginning with the original design by Hesslein (1976). Examples include recent studies of heavy metal geochemistry in sediments (Shi et al., 1998), nitrogen discharges from groundwater to rivers (Doussan et al., 1998), vertical profiles of anions, cations and gases in peats (Steinmann and Shotyk, 1997) and seasonal changes in nutrient concentrations in marshes (Jackson and Pardue, 1997). Recently, dialysis samplers have been utilized to detect discharge of VOC contaminated groundwater to surface water at various sites (Vroblesky and Robertson, 1996; Vroblesky et al., 1996; Vroblesky and Hyde, 1997). The technique has also been utilized to assess fate processes for VOCs in wetland sediments at the Aberdeen Proving Ground (APG) in Maryland (Vroblesky et al., 1991; Lorah et al., 1997; Lorah and Olsen, 1999). Steinmann and Shotyk (1997) and Teasdale et al. (1995) describe the procedures for using dialysis samplers in detail. Some basic theory has also been developed for membrane type and design as a function of equilibration time (Brandl and Hanselmann, 1991; Webster et al. 1998; Harper et al., 1997).

Solid Phase Micro-Extraction (SPME) fibers are passive equilibrium sampling devices that are negligible depletion and non destructive (Monteil-Rivera et al., 2004; Condor et al, 2003). SPME fibers are biomimetic devices that only absorb bioavailable compounds and concentrate them to higher concentrations than the surrounding matrix. SPME method is highly sensitive, simple, rapid, and economical compared to other current technologies. SPME fibers exhibit a linear uptake relationship which makes it a powerful tool to monitor trace contaminants in the environment. The effectiveness for

a particular fiber depends on the coating material and the surface area available for absorption of the contaminant (Mayer et al, 2003). SPME fibers are proven to be effective for monitoring TNT, HMX, RDX, and their breakdown byproducts (Monteil-Rivera et al., 2004; Condor et al., 2003; Barshick and Griest, 1998).

25.0 TEST PLANTS:

Species: Graceful cattails (*Typha laxmanil*)

26.0 EXPERIMENTAL DESIGN

RDX in Wetlands

Four mesocosms (Figure 1) were constructed (dimension of 2 ft L × 1 ft W × 2 ft H) with multiple sampling ports (3, 9, 15, 18 inches below the water sediment-water interface). At the bottom of the microcosm, there was a 3 inch sand layer to distribute and drain the flow. Above the sand, the mesocosms were filled with 15 inches of wetland substrate medium composed of a peat/sand/peat moss mixture (2:2:1 by weight). Physical characteristics of the mesocosms are listed in Table 1.

The mesocosms were operated on a 12/12 hour light/dark cycle in a laboratory setting at The Institute of Environmental and Human Health with constant temperature and humidity. Downflow mode was established at a flow rate of 10 mL/min. A standing water depth of approximately 2 inches was maintained by adjusting the effluent tubing height. Initially, the mesocosms were fed with water containing 25 ppm Cl^-, 5 ppm NO_3-N, and 20 ppm SO_4^{2-} for two months to establish flora and bacterial growth.

The wetlands were operated in two phases to examine the effect of RDX and nitrate levels on RDX biodegradation. Phase I explored the ability of the mesocosm to degrade RDX at 5 ppm NO_3-N when RDX loading was 1 ppm, 5 ppm, and 10 ppm (Table 2). Phase I began with a loading rate of 1 ppm RDX was introduced to the system. The mesocosms were then sequentially challenged with the increased RDX concentrations of 5 and 10 ppm. The system was operated for approximately 12 weeks for each loading rate. After Phase I finished, a desorption period was maintained for six weeks with no RDX loading. In addition, the plants were removed from one of the two mesocosms.

Phase II explored the effect influent nitrate concentration and plants have upon RDX degradation. RDX loading rate was set at 1 ppm, while the nitrate concentration was decreased from 5 ppm used in Phase I to 1 ppm NO_3-N (Table 2). The system was maintained for 12 weeks until the nitrate was lowered again to operate at 0.1 ppm NO_3-N for 12 weeks. The effect of nitrate was examined by comparing the degradation rates

between the nitrate loading rates. Only the planted mesocosm was used to determine the effect of nitrate. Including the nonplanted mesocosm when comparing with previous loadings would introduce a new variable that could affect degradation rates.

Passive Samplers to Monitor RDX

Diffusion Sampler -A stainless steel 30 cm long 1.25cm diameter prototype diffusion sampler was designed with 10 1ml wells spaced at 2.5 cm intervals. The wells have a LW/D ratio of 1cm. The diffusion sampler can be used in two ways. Traditionally as a diffusion sampler with water in the wells or to hold SPME fibers which can be used to estimate pore water concentration if previously calibrated to the compound/s of interest. Both methods give estimates of the dissolved concentration of contaminant in the pore water. The sampler was inserted into the mesocosm following several weeks of constant RDX loading. The sampler was removed after two weeks of equilibration. Either the water or SPME fiber depending on the test were removed and analyzed as described below. Concentrations were compared to filtered pore water at various depths.

SPME Fibers-Four types of 50 cm custom made SPME fibers were purchased from Supelco: 100 µm Polydimethylsiloxane (PDMS), 70µm Carbowax- Polydimethylsiloxane (CW-PDMS), 65 mm divinylbenzene/ Polydimethylsiloxane (DVB/PDMS), and 70µm Carbowax- divinylbenzene (CW-DVB). RDX solution of 0.01 M (2200 ppm) was diluted using DDI water to yield RDX concentrations of 250 ppb, 500 ppb, 1000 ppb, and 2200 ppb. The four types of fibers were cut into 2.0-cm pieces and each fiber was placed in a 50 mL glass vial containing 25 mL of RDX solution. One combination of fiber- RDX concentration was duplicated for the four fiber types and four different RDX concentrations. The fibers were allowed to equilibrate with the RDX solutions for 7 days.

27.0 METHODS:

Water Sample- Water samples were tested for energetic concentration (ClO_4^- or RDX, TNX, DNX, MNX) and anions and dissolved organic carbon (DOC). Water samples from ClO_4^- challenged mesocsoms were filtered with 0.2 µm nylon filters and analyzed for perchlorate and anions using ion chromatography (described below) and analyzed for TOC using a combustion analyzer. RDX challenged mesocosm samples were filtered using 0.2 µm Teflon filters, the filtered samples were analyzed for anions using ion chromatography, for TOC using combustion analyzer, and for RDX were extracted and analyzed using HPLC (described below).

Plant Samples -Plant material was rinsed with DI water to remove surface contamination. Surface moisture on plant tissue was dried by blotting with paper towels. Plant material was then cut into small pieces and homogenized using a mortal and pestle. Wet plant material (~1g) was transferred to a 15 mL vial, extracted with 10 mL 100 % acetonitrile, thoroughly mixed using a vortex mixer for at least 3 min after which samples were sonicated for 1h, and centrifuged for 10 min at 3500 rpm.

The sonicated mixture was then passed through a florisil cartridge and placed on a 24-port manifold (Supelco, Bellefonate, PA, USA). Before loading samples, florisil cartridges were conditioned with acetonitrile (2 x 5-ml). Filtrates were collected into 10-ml graduated centrifuge tubes. And, the florisil cartridge was rinsed 3 times with small amounts of acetonitrile (3 x 1-ml). Then, the sample was concentrated to 0.5-1.0 mL under nitrogen using a N-EVAPTM111 nitrogen evaporator (Organomation Associates, Inc. Berlin, MA, USA). The final volume was adjusted to 1 mL in the graduated centrifuge tube. The 1 mL extract was finally filtered through a 0.25 µm membrane filter (Millipore, Bedford, MA, USA) and was collected into a GC vial prior to GC analysis.

Analytical:

Water Samples- HPLC was used to analyze water samples. An HPLC (Hewlett-Packard HP 1100) was interfaced with the HP ChemStation software and equipped with a binary pump G1312A, an ultraviolet detector and an autoinjector with a 50 µL loop. The detector was operated at excitation wavelength of 254 nm. Separations were performed with a reverse-phase C18 column (Supelco, Bellefonate, PA).

For energetic compounds RDX, TNT, MNX, TNX and DNX, the mobile phase consisted of 50% acetonitrile and 50% ultra-pure water. All solvent flow rate was 1 mL/min, and the injection volume was 25 µL. Chromatography was performed at room temperature (about 25°C). At least three calibration standards were run with each batch of samples to span the expected range of toxicant in samples. Water blanks were also run with each set of samples.

Plant Extracts-A Hewlett Packard 6890 series gas chromatograph (GC) was employed to analyze RDX, MNX, DNX, and TNX. The GC equipped with a HP 6890 autosampler and an electron capture detector was controlled by HP 6890 series ChemStation from Hewlett-Packard (Agilent, Palo Alto, California, USA). Separation was performed on a capillary DB-5 column (30 m x 0.25 mm x 0.25 µm). The GC oven temperature was initially held at 90°C for 3 min, increased to 200° C at a rate of 10° C/min, and then raised to 250° C at 25° C/min, and finally held at 250° C for 5 min. The Injector temperature was kept at 170 °C. The detector temperature was 270°C. The injection volume was 2-µl. The carrier gas was helium (99.9999% pure) at a constant flow-rate of 20 mL/min. The makeup gas for ECD detector was argon methane at a combined flow rate of 60.0 mL/min. The ECD was operated in the constant current mode.

SPME Fibers-The fibers were recovered from each vial and were placed in 10 mL glass vials (Supelco Clear Screw Top with Phenolic Cap, PTFE/Silicone Septa) containing 5 mL HPLC-grade (99.9+%) acetonitrile for 24 hours. The acetonitrile was transferred to 15 mL PYREX vials. The 10 mL Supelco glass vials were rinsed with 1 mL of acetonitrile and the rinse was added to the original 5 mL. The 6 mL of the acetonitrile was blown down to less than 0.5 mL using nitrogen gas. The final sample was brought to 0.5 mL and transferred to HPLC vials. The 15 mL pyrex vials were rinsed with 0.5 mL of DDI water and the rinse was added to the sample in HPLC vials. The samples were analyzed for RDX by HPLC-UV method with a MDL of 5 ppb.

13.0 RESULTS

Fate of RDX in Wetland Systems

Effect of Influent RDX Concentration on RDX Degradation-The ability of constructed wetlands as a RDX degradation device was evaluated by varying the influent RDX concentration to the wetlands and observing the mesocosm's ability to deal with the higher loading rates during Phase I operations. To evaluate to the ability of the wetlands to degrade RDX, the change in RDX concentration across the depth of the mesocosm was used. The averages of the RDX and anion concentrations over the exposure period were used to plot concentration profiles and determine degradation kinetics (Figure 2). As the RDX influent concentration increased, both the percentage of RDX removed and the first order biodegradation rate decreased. The biodegradation rate of RDX decreased from 5.95 to 0.77 d^{-1} as RDX concentration increased from 1 to 10 ppm RDX (Table 3). Likewise, the nitrate degradation rate decreased from 11.59 to 1.85 d^{-1} as the influent RDX concentration increased. The ratio of nitrate degradation rate to RDX degradation rate is 2.01 ± 0.38. As the ratio of the two degradation rates remained constant regardless of RDX concentration, it is possible that the high RDX loading is having an inhibitory effect on the growth of RDX degrading organisms. This also may be due to a lack of substrate availability or non-1st order degradation.

Effect of Nitrate on RDX Degradation-The effect of nitrate on RDX degradation in the mesocosm was evaluated by varying the influent nitrate concentration while maintaining a RDX loading rate of 1 ppm during Phase II operations. Nitrate influent levels were 5 ppm, 1 ppm, and 0.1 ppm NO_3N. Nitrate was reduced to near or below detection limit at a depth of 7.5 cm and below detection limit at a depth of 22.5 cm (Figure 3). The RDX degradation rate varied from 5.95 to 1.55 d^{-1} while the nitrate degradation rate dropped from 11.59 to 0.93 d^{-1} (Table 3).

Effect of Plants on RDX Degradation-After the initial loading study observing the effects of influent RDX concentration on degradation rates, the plants from one of the mesocosms were removed. This was done to determine the effect the graceful cattails (*Typha laxmanil)* have upon RDX degradation. The influent RDX concentration remained at 1 ppm during the exposure while the nitrate was decreased to 1 and 0.1 ppm during Phase II operations. The RDX degradation rates between the plant and unplanted mesocosms were not significantly different. At 1ppm and 0.1 ppm NO_3-N, RDX degradation rate decreased from 1.55 to 0.92 d^{-1} and 2.23 to 1.88 d^{-1} respectively due to the removal of plants (Figure 5). The nitrate degradation rate increased from 6.71 to 7.87 d^{-1} and 0.93 to 3.22 d^{-1} for 1ppm and 0.1ppm NO_3-N respectively with the removal of plants although both mesocosms had no detectable nitrate in the effluent (Table 3).

Plant uptake of RDX-Plants were seen to uptake RDX in amounts proportional to influent water RDX concentration (Figure 5). However, when RDX concentrations were reduced, the plants retained a large amount of RDX disproportional to water

concentrations. There is a semi-log relationship between the influent water concentration and plant concentration. This relationship does not apply to plants that were exposed to a high initial concentration of RDX but later reduced. After the RDX loading was decreased from the maximum concentration of 10 ppm RDX, plants continued to show high levels of RDX. When RDX concentrations were reduced to 1ppm RDX, the plant concentrations remained an order of magnitude larger than when they were first exposed to 1ppm RDX levels.

RDX byproducts were found in the cattails and increased as influent RDX concentrations increased. This may be due to greater concentrations of RDX byproducts broken down by microbes present in the root zone of the plants. It is also possible that the cattails are breaking down the RDX that is taken up. While there appears to be a relationship between the RDX influent concentration and concentration of RDX byproducts inside the cattails, the small concentrations present and the variable nature of RDX byproducts make further analysis difficult.

RDX byproducts-RDX byproducts MNX, DNX, and TNX were all seen in both mesocosm water and inside the plants (Figure 6). As mentioned previously, RDX byproducts are less stable than RDX and RDX may have multiple degradation pathways that do not involve all 3 major degradation byproducts. This is consistent with the lesser concentrations of each of the subsequent byproducts. MNX, the first degradation byproduct was seen at shallower depths in the mesocosm and in greater amounts than any other byproduct. DNX presence was similar to MNX but had lower concentrations. Finally, TNX was rarely seen during the course of the experiment. Significant TNX was only seen at high influent RDX concentrations at the deepest parts of the mesocosm. As there is typically MNX and DNX in the effluent of the mesocosm, it is probable that greater amounts of TNX would be present as the hydraulic retention time increased.

Use of Passive Samplers to Monitor RDX Fate in Saturated Systems

Due to significant construction delays of the sampler only one preliminary experiment was completed in time for this report. Other experiments are ongoing and the complete evaluation of both methods for monitoring RDX in pore water will be complete by May.

The PDMS fiber did not absorb RDX at any concentration thereby is unsuitable as a passive sampling device for RDX. The other three fibers had a linear relationship between bulk solution concentration and RDX mass absorbed by the fibers (Figure 1). CW-DVB seems to be the best fiber for monitoring RDX in bulk liquid solution with a regression coefficient of 0.998 between residual RDX mass in fiber and RDX concentration in the bulk solution. Fibers CW-PDMS and DVB-PDMS had a regression coefficient of 0.98 and 0.96 respectively. This is consistent with the results observed in a study conducted by Barshick and Griest (1998).

Actual RDX concentration in the fibers and partition coefficient for RDX between water and the fibers were calculated. To determine the actual RDX concentration in the fibers the volume of individual fibers were estimated using fiber thickness and length of fiber (20 mm). RDX concentration in individual fibers was determined as the ratio of residual RDX mass in each fiber and the volume of fiber (Table 1). Partition coefficients (K_P) for the fibers were determined according to equation 1 and listed in Table 4.

$$K_P = \frac{S(ppb)}{C(ppb)}$$

Where S is RDX concentration in the fiber and C is the bulk liquid RDX concentration.

The fibers with polar carowax coating had a better absorption of RDX than the fibers with non polar poly(dimethylsiloxane) (PDMS) coating. The best partition coefficient for RDX from bulk liquid solution was with fiber CW-DVB (K_P= 3.10±0.07) followed by fiber CW-PDMS (K_P= 3.02±0.09), and fiber DVB/PDMS (K_P= 2.88±0.13). The SPME fiber CW-DVB was found to be the best fiber type for monitoring RDX in the environment.

14.0 DISCUSSION

Wetlands have the potential to remove RDX to below detection levels at moderate loadings (~1ppm) and significantly reduce (<.5ppb) RDX even at loading rates exceeding 10ppm. These results also suggest that exposure of ecosystems to high or prolonged concentrations of RDX in anaerobic environments (e.g. sediments) can produce at least temporary build up of break down products (MNX, DNX, and TNX) although as seen before, substantially less daughter products are produced compared to the amount of RDX transformed. Plants did not appear to directly impact overall RDX loss from solution. However, plants would be critical in maintaining a long term supply of organic carbon in the wetlands. In addition plant uptake may pose an increased exposure risk to surface ecosystems beyond that predicted from water analysis alone. RDX concentrations were generally highest in the plant compartment and release/transformation of RDX was slow following termination of RDX exposure. Finally, the ability to use SPME fibers to monitor RDX in saturated environments seems promising although more appropriate fibers may be required. This work is ongoing and should be complete by May. Finally, the most important implications of this work include the need to perform detailed compartment evaluations of RDX in the environment and the high potential to use constructed wetlands as passive interception treatment technologies for either non-point source runoff or surfacing groundwater.

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14.0 FIGURES AND TABLES

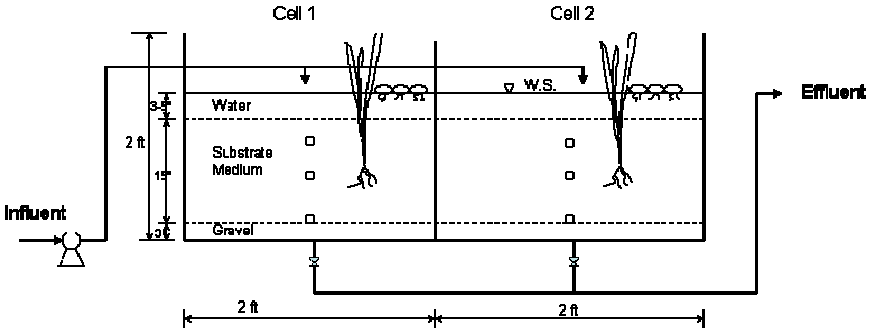

Figure 1. Diagram of Mesocosms

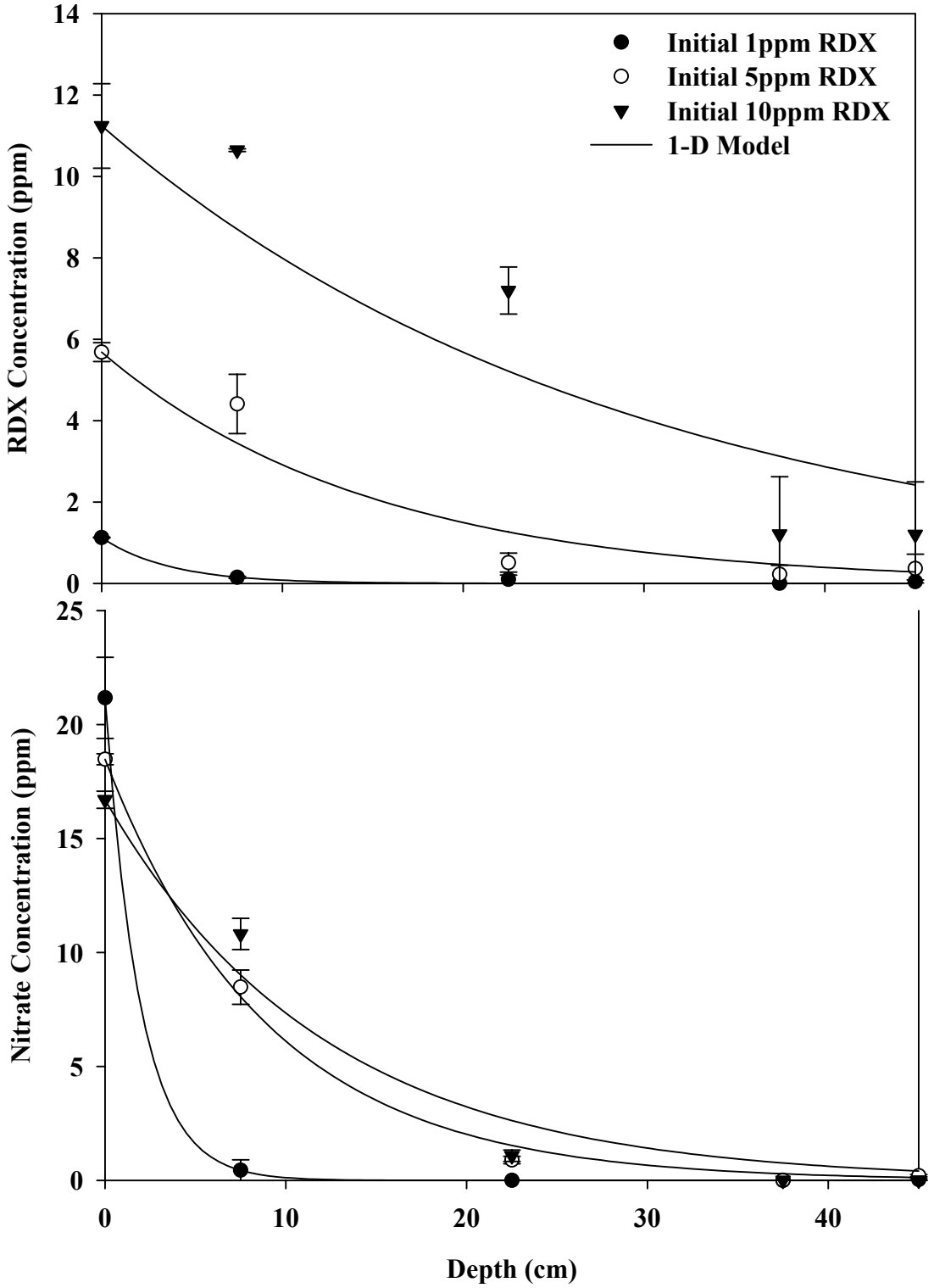

Figure 2: RDX and Nitrate concentrations across wetland depth for various RDX loading rates and constant nitrate loading (5mg/l-N).

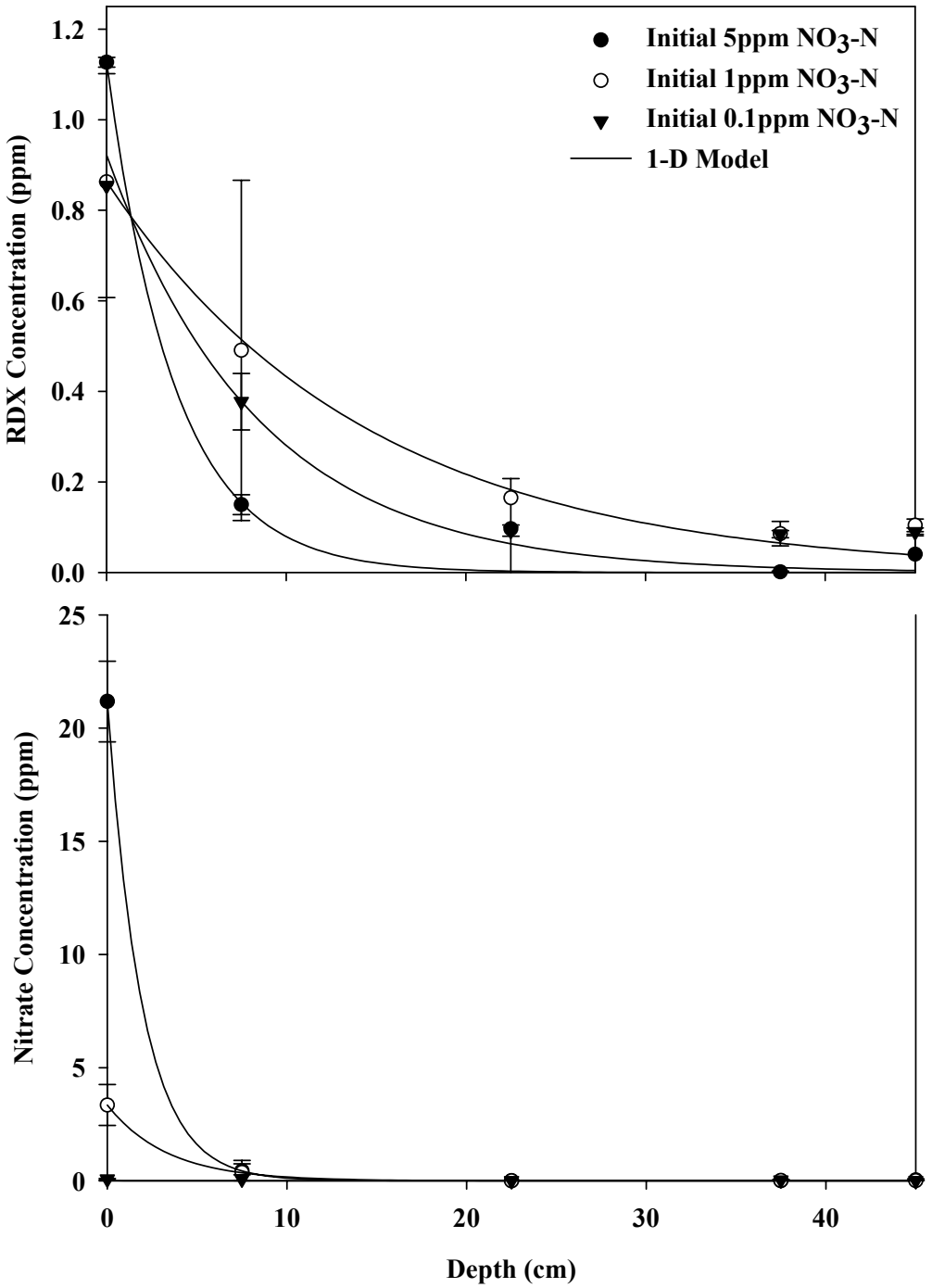

Figure 3: RDX and Nitrate concentrations across wetland depth for constant a RDX loading rate (1ppm) and variable nitrate loading (0.1-5 mg/l-N).

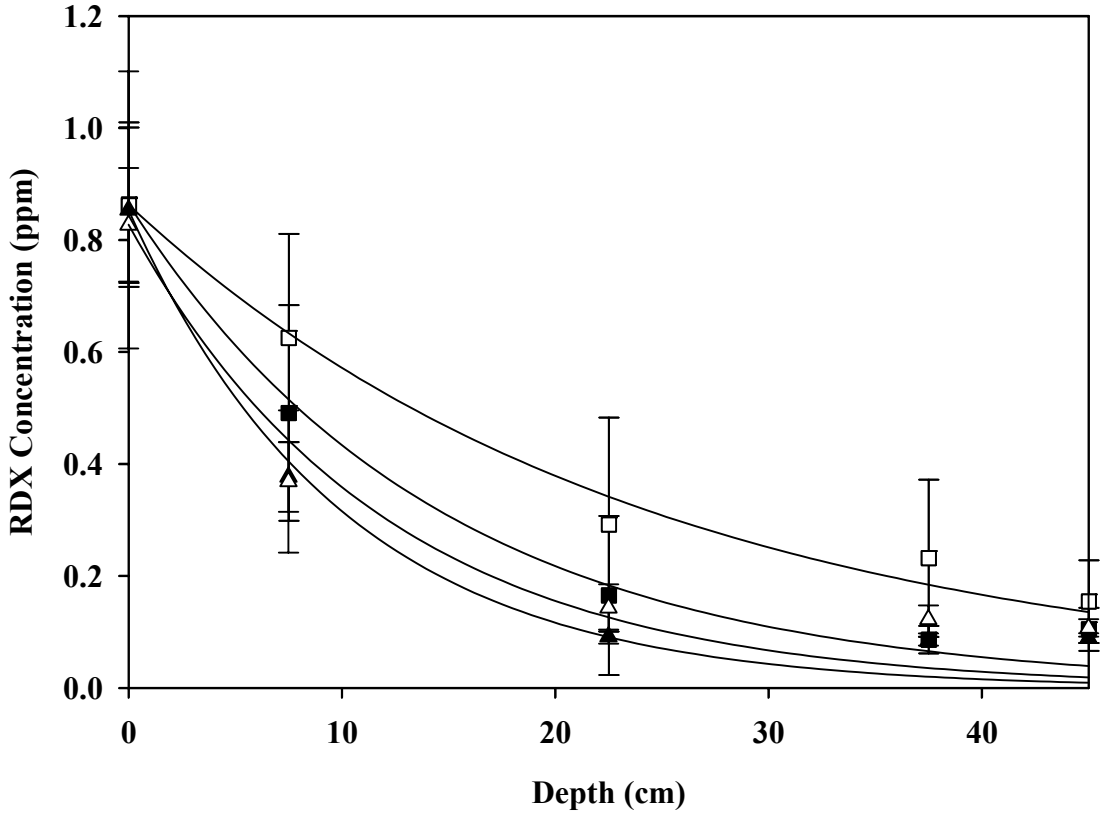

Figure 4: Effect of Plants (■,▲) on RDX fate in mesocosms for constant RDX loading (1ppm) at NO_3^- loading of 1 (■,□) and 0.1 (▲,Δ) mg/l-N.

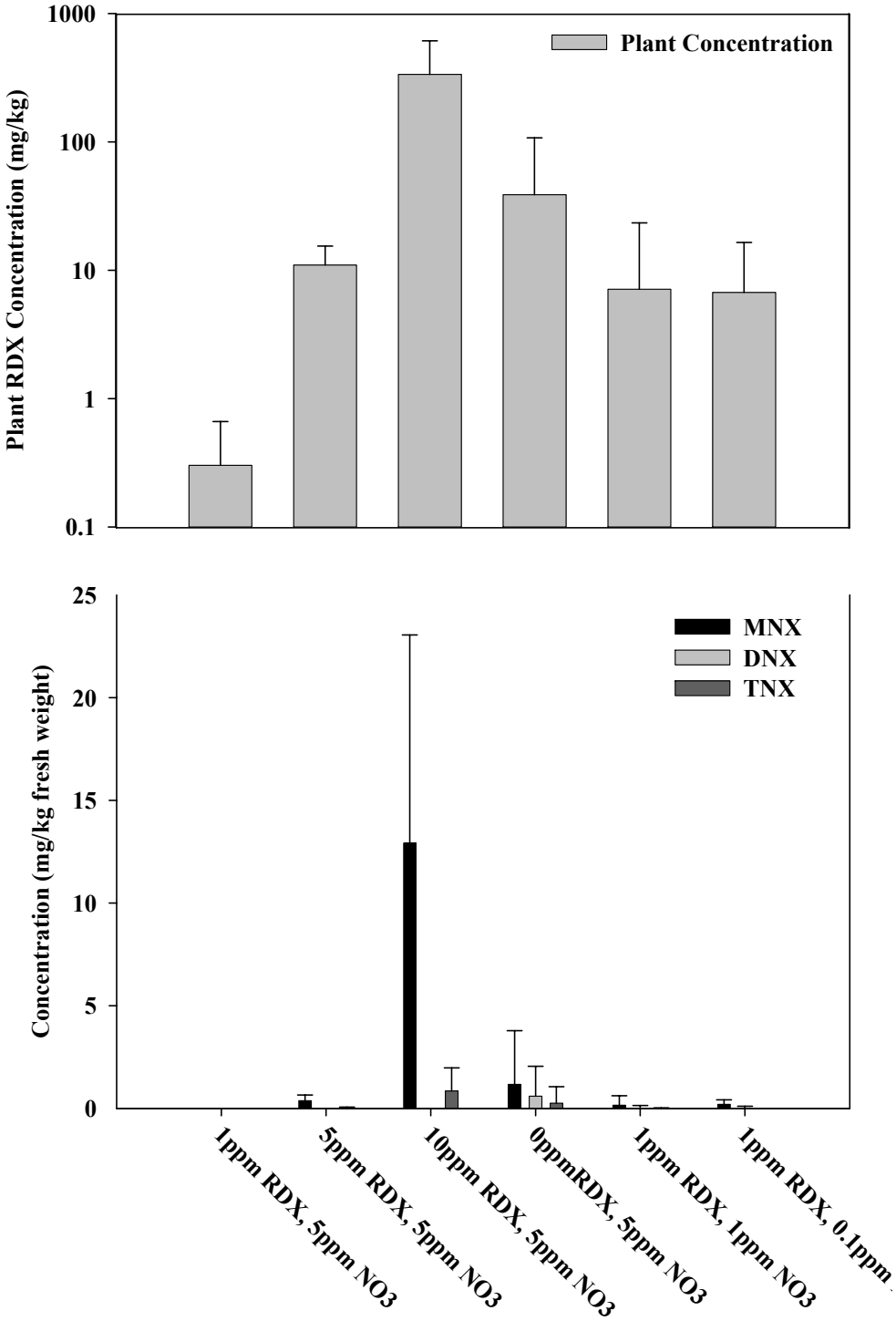

Figure 5. Plant concentrations of RDX, MNX, DNX, TNX for sequentially applied loading rates of RDX and NO_3 over time.

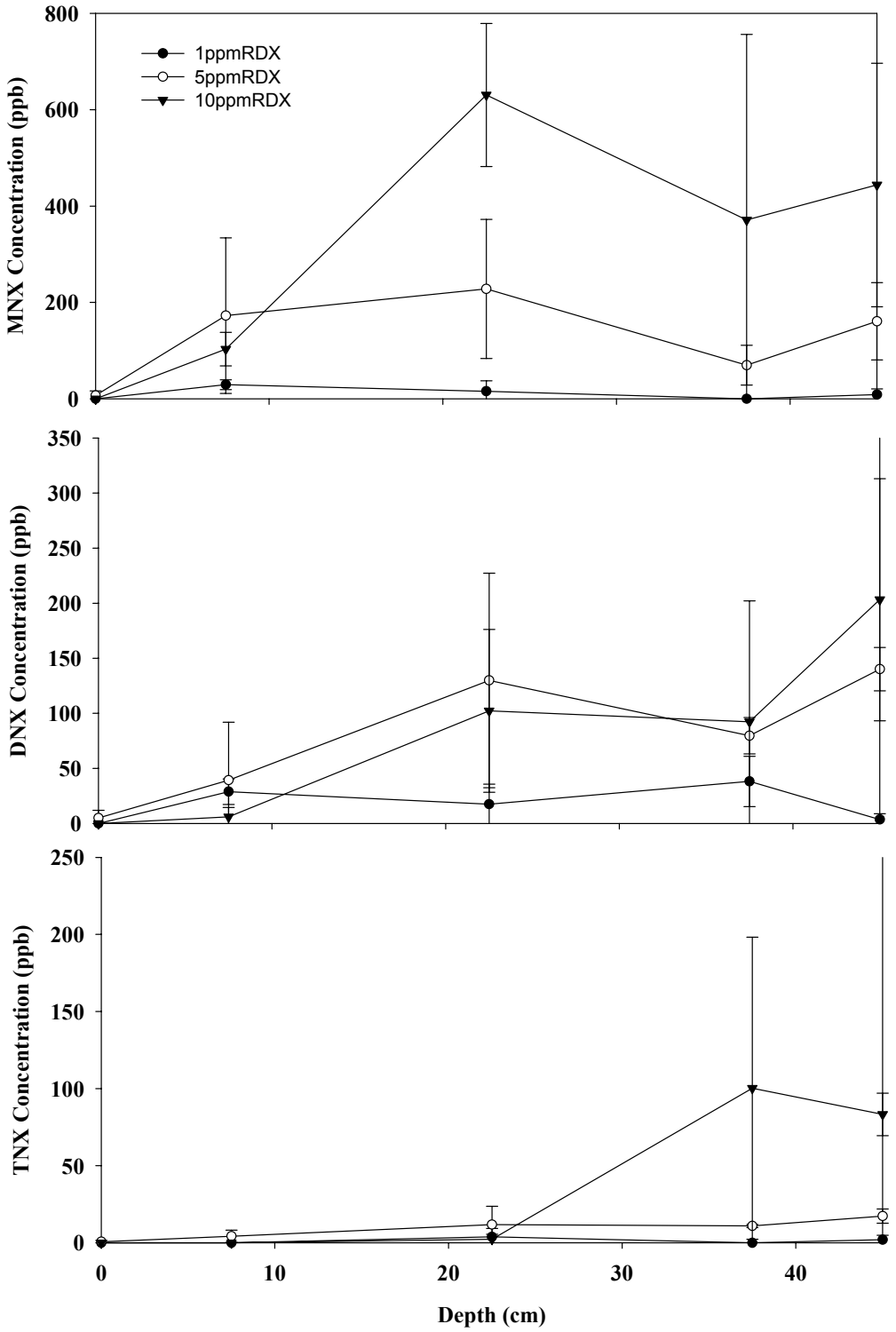

Figure 6. Distribution of MNX, TNX, and DNX in wetland Mesocosms for increasing RDX loading rates.

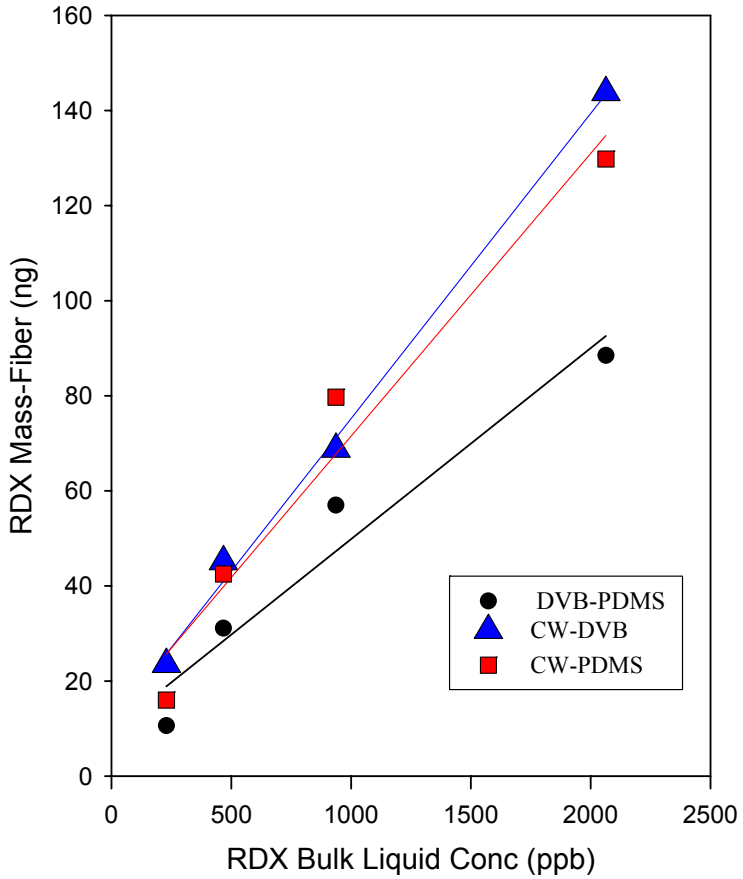

Figure 7. Residual RDX mass absorbed by the SPME fibers vs measured bulk liquid RDX concentrations

Table 1. Physical Characteristics of Mesocosms.

Media	Peat : Sand : Peat moss (2 : 2 : 1 by weight)
Bulk Density (g/cm3)	0.71
Media Porosity	0.79 ± 0.01
Hydraulic Conductivity (cm/day)	70.5 ± 11.9
Seepage Velocity (cm/s)	1.18 x 10-4 ± 0.02 x 10-4 cm
Hydraulic Retention Time (days)	5

Table 2 Loading rates and number of water and plant samples taken.

Date	RDX Conc.	NO3-N Conc.	Water Samples Taken	Plant Samples Taken
08/09/04-11/01/04	1ppm	5ppm	13	3
11/8/04-2/21/05	5ppm	5ppm	16	4
2/28/05-05/10/05	10ppm	5ppm	11	3
6/27/05-10/17/05	1ppm	1ppm	17	5
10/24/05-1/9/06	1ppm	0.1ppm	11	3

Table 3. First-order biodegradation of RDX and nitrate.

Treatment			Degradation Rates	
RDX (ppm)	Nitrate (ppm)	Planted	k_{RDX} (d^{-1})	k_{NO3} (d^{-1})
1	5	Yes	5.95	11.59
5	5	Yes	1.50	2.49
10	5	Yes	0.77	1.85
1	1	Yes	1.55	6.71
1	1	No	0.92	7.87
1	0.1	Yes	2.23	0.93
1	0.1	No	1.88	3.22

Table 4. RDX concentrations and the partition coefficients for RDX from bulk liquid phase for DVB-PDMS, CW-DVB, and CW-PDMS fibers.

Fiber Type	RDX Bulk Soln ppb	RDX mass-Fiber (ng)	Fiber Conc (ppm)	Kp	log Kp
DVB-PDMS	250	10.6	138	601	2.78
	500	31.1	404	865	2.94
	1000	57	741	791	2.90
	2200	88.5	1150	557	2.75
	500-Dupe	41	533	1140	3.06
CW-DVB	250	23.5	354	1547	3.19
	500	45.1	680	1455	3.16
	1000	68.8	1038	1107	3.04
	2200	143.8	2169	1051	3.02
	1000-Dupe	77.8	1173	1252	3.10
CW-PDMS	250	16	208	908	2.96
	500	42.5	552	1182	3.07
	1000	79.7	1036	1106	3.04
	2200	129.8	1687	817	2.91
	250-Dupe	24.2	315	1373	3.14

TITLE: **Evaluating Uptake of Incurred Explosives Residues**

STUDY NUMBER: **MRT-05-01**

SPONSOR: **Strategic Environmental and Research Development Program**
SERDP Program Office
901 North Stuart Street, Suite 303
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CONTRACT ADMINISTRATOR: **The Institute of Environmental and Human Health**
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TEST SITE:

RESEARCH INITIATION: **March 2004**

RESEARCH COMPLETION: **March 2005**

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

George P. Cobb
Co-Principal Investigator

Date

22.0 DESCRIPTIVE STUDY TITLE:
Evaluating Uptake of Incurred Explosives Residues

23.0 STUDY NUMBER:
MRT-05-01

24.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

25.0 TESTING FACILITY NAME AND ADDRESS:

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

26.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: March 2004
Termination Date: March 2005

27.0 KEY PERSONNEL:

Dr. George Cobb	Co-Principal Investigator
Mr. Nick Romero	Animal Care and Dosing
Mr. Jordan Smith	Animal Care and Euthanasia
Ms. Xiaoping Pan	Analyte Quantification
Dr. Kang Tian	Analyte Quantification
Dr. Ronald J. Kendall	Testing Facility Manager / Principal Investigator

28.0 STUDY OBJECTIVES / PURPOSE:

To determine the concentration of explosives and their metabolites in rodents following dosing of food containing incurred residues.

29.0 STUDY SUMMARY:

RDX and TNT are widely used explosives within the DOD. Many military installations have soil and/or groundwater contamination problems. These compounds are relatively tightly bound to organic matter in nature and are metabolized by soil microbes. We performed scoping studies wherein deer mice (*Peromyscus maniculatus*) are provided a

diet containing plant and earthworm material with incurred RDX, TNT and in all likelihood their primary metabolites.

30.0 TEST MATERIALS:

Test Chemical name: RDX
CAS number: 121-82-4
Characterization: Residues in plants cultivated in soils from Aberdeen Proving Grounds
Source: Waterways Experiment Station

Test Chemical name: TNT
CAS number: 38082-89-2
Characterization: Residues in plants cultivated in soils from Aberdeen Proving Grounds
Source: Waterways Experiment Station

31.0 JUSTIFICATION OF TEST SYSTEM:

The US Department of Defense desires knowledge regarding the trophic transport and disposition of explosives to assist risk assessors in their evaluation of ecological risks at military sites. For this reason a common species with wide distribution, the deer mouse (*Peromyscus maniculatus*) was selected. Dosing was conducted in a laboratory environment to control as many variables as possible.

32.0 TEST ANIMALS:

Species: *Peromyscus maniculatus*
Age: 45-90 day old
Number: 36
Source: University of South Carolina Peromyscus Stock Center
Sex: Male

33.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The study number was placed on the dosing room door and each cage contained information describing the study number, the mouse identification, sex and dose group. Each animal was also identified with a unique ear tag pattern.

34.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Effects and trophic transfer of TNT and RDX, respectively, on *Peromyscus maniculatus* (deer mouse) were determined by dosing mice with food contaminated with (a) TNT, and (2) RDX, incorporated into the tissues of *Lolium perenne* (perennial ryegrass) and *Eisenia fetida* (earthworm) in a study funded through ERDC-EL, Vicksburg, MS. Standard rodent chow was mixed with these powdered food stuffs. Dosing proceeded with as many animals as we calculated could be fed for 6 weeks with diets containing

decreasing percentages of each incurred residue type. We determined TNT, RDX, and selected metabolites in tissues and blood of the mice.

14.0 METHODS:

Animals: Sexually mature male deer mice were purchased from the Peromyscus Stock Center (Univ. of SC, Columbia, SC) and housed in an AALAC accredited facility at Texas Tech University. Mice were housed separately and maintained in environmentally controlled conditions of 18:6h light-dark cycle, 25° C, and relative humidity of 50%. Mice were acclimated for 1 week prior to dosing. Deer mice were randomly assigned to control and explosives contaminated food (see Table 1 for concentrations). Animals were weighed (±0.01g) before the initiation of the study. Access to food was provided on an *ad libitum* basis. Mice were dosed with powdered rodent chow (Purina Mills, St. Louis, MO, USA), amended with explosives residues from one of two sources, i.e., grass or earthworms

For each contaminant/food type combination, six animals were isolated for each dose group. The mice were housed as one per cage with aspen shavings as bedding. Environmental conditions were 12 h light: 12 h dark and temperatures of 21-24 ^{o}C. Each mouse received food and water *ad libitum*. All animals were exposed for 35 days to TNT, or 21 days to RDX. The weights of the animals and remaining food were also measured at regular intervals; i.e., at days -7, 0, 1, 7, 14, 21, 28, and 35 for TNT, and at days -7, 0, 1, 7, 14, and 21 for RDX. Every time the food stock was replenished in a cage, the weight of the food remaining before replenishment was recorded, and the food consumed calculated.

Preparation of food with incurred TNT or RDX: Plants and earthworms were cultivated in soils containing RDX or TNT by researchers at the Waterways Experiment Station. Each soil type was spiked with radiolabeled forms of RDX or TNT to allow better quantitation. Freeze-dried plant and earthworm materials were then shipped to TIEHH for testing.

Standard rodent chow was mixed with the plant and worm materials contaminated with TNT or RDX. Preparation was done by personnel from the food nutrition laboratory of the Animal Science Department at Texas Tech University, Lubbock, TX, USA. Each mixture was prepared by adding a preweighed amount of freeze-dried plant or earthworm material to an appropriate amount of powdered rodent chow (Table 1). Each food type was first hand-mixed with a stainless steel trowel for 5 minutes and subsequently mixed in a Hobart mixer (Hobart Corp; Troy, OH, USA) for 15 minutes. Doses were stored in air-tight plastic containers until use. Three trowels were available for hand-mixing to minimize reuse. Each trowel, mixing bowl and impeller was washed with water and acetone before and between uses. As much as 1.2 kg of food was available for each dose group. Each freeze-dried food amendment contained TNT or RDX as the primary contaminant. Radioactivity in the samples was used for dose confirmation (Table1), and the radioactivity increased linearly with dose.

General Observations: The general health of all animals was assessed daily by examination of the coat, activity, and food and water consumption. Body weights were recorded weekly and at necropsy. At day 30 animals were narcosed with CO_2. Animals were killed by cervical dislocation, and terminal blood samples were collected by cardiac puncture. Sera and liver were removed, weighed, and frozen (-20C) until chemical analysis. The accumulation of incurred TNT and RDX were evaluated after exposure.

Extraction of explosives and metabolites: Tissues were mixed with 8-10 g dried Na_2SO_4 using a mortar and pestle. A Dionex Accelerated Solvent Extractor (Model 200, Salt

Lake City, UT, USA) was used for all extractions as described in published methods developed in the Analytical Core of this research program (Pan et al., 2005, 2006) and are described here briefly. Each extraction cycle included 5-min preheating, and 5-min static extraction with 100% acetonitrile at 100°C and 1500 psi. Extracts were collected in glass vials and reduced to 1-2 mL using rotary evaporation in preparation for florisil and styrene-divinyl benzene cleanup (Pan et al., 2005). These steps removed large amounts of interfering compounds and pigments. The extract volumes were reduced to 1.5 mL under nitrogen using a N-EVAP^{TM} 111 (Organomation Associates, Inc. Berlin, MA, USA). The final volumes were adjusted to 2 mL with MilliQ water, filtered through a 0.20 µm membrane filter (Millipore, Bedford, MA, USA) into an autosampler vial, and stored until further analysis.

Blood samples were mixed with 7 mL of acetonitrile for every mL of blood. Mixtures were vortexed every 30 min for 2-3 h. Extracts were centrifuged at 3,500 rpm for 15 min. Supernatants were collected and purified using Florisil SPE cartridges. Extract volumes were reduced to 1-2 mL under nitrogen, and filtered through a 0.20 µm membrane filter prior to analysis.

Chemical Analyses: Chemical TNT and RDX concentrations were determined in blood using GC-ECD as developed in the analytical core for this research program (Zhang et al., 2005). An Agilent 6890 GC system (Palo Alto, CA) with automated 2 µl splitless injections was used with a 30m x 0.25 mm HP-5 column experiencing an oven temperature from 90 to 250 ^{o}C over a period of 13 min. The ECD was operated with an Ar/methane makeup of 40 mL min^{-1} and a constant current mode. Analyses were performed for TNT, RDX, MNX, DNX, and TNX. Detection limits of explosives in samples ranged from 0.2 to 2 ng mL^{-1} for the analytes used for calibration.

Explosives proved regularly to be below the detection limit of this method. Therefore, a more sensitive method using liquid chromatography-mass spectrometry (LC-MS) with selected ion monitoring (Pan et al., 2006) was also explored to measure the chemical levels. The latter method proved to be successful for the determination of TNT, RDX, and their known metabolites at the pg-level. Livers from the RDX portion of the study were evaluated by this technique.

7. RESULTS:

Given the low concentrations of toxicants incorporated into food, quantifying parent and metabolite concentrations was difficult. The chemical RDX-derived residues were determined in the blood of mice by GC-ECD. Concentrations were low with means ranging from 1.7 to 4.4 ng RDX ml^{-1} and 0.46 to 0.69 ng TNX ml^{-1} (Table 2). RDX derived residues in mouse liver were also determined by LC-MS (Table 3). Mean TNT, RDX, MNX, DNX and TNX concentrations in livers of mice receiving RDX treated food ranged from 0.95 to 20 ng/g but did not have concentrations that were different from

those found in control livers (P>0.44). Similarly, the TNT concentrations in muscle were low (Table 4) and treatment animals accumulated similar concentrations as did control animals.

Insignificant of TNT, RDX and RDX transformation products were accumulated during the study (Tables 2 through 4) indicating limited trophic transfer of TNT and RDX from food items to rodents. The possible reasons for this are the low doses used, rapid metabolism, or poor uptake via the rodent GI tract.

Table 1. Doses prepared by amendment of standard rodent chow with freeze-dried plant or worm material.

Explosive Dose	Food		
	% DW	[Explosive] (mg kg^{-1}) Nominal	Radiation Counts (millions)[a]
TNT			
Control	0	0	0
Plant, low	0.50	14.4	2.692
Plant, medium	1.49	42.9	7.262
Plant, high	4.04	116.3	14.199
Worm, low	0.42	4.1	3.287
Worm, high	1.32	12.9	7.680
RDX			
Control	0	0	0
Plant, low	0.46	15.0	7.130
Plant, medium	0.91	29.6	23.686
Plant, high	1.89	61.4	61.369
Worm, low	0.35	15.2	4.516
Worm, high	1.0	43.4	16.143

a- each food type was spiked with radiolabeled explosive before receipt at TIEHH to allow analytes to be detected more readily.

Table 2. TNT and RDX-residues in *P. maniculatus* blood following ingestion of contaminated food (N=6 unless specified in footnotes).

Exlosive/ Dose	Explosive concentration in blood (ng mL^{-1})[a]		
	TNT Dosing	RDX Dosing[b]	
	TNT	RDX	TNX
Control	0	0	0
Plant, low	6.87 (6.40-12.53)[c]	3.35 (1.02-11.02)	0.52 (0.33-0.80)

Plant, medium	9.08 (7.09-12.70)	4.37(0.60- 31.69)	0.53 (0.21-1.31)
Plant, high	31.11(23.23-53.3)[d]	2.64 (1.52- 4.58)-	0.57 (0.25-1.28)
Worm, low	9.93 (6.40-17.80)	4.28 (0.92-19.77)	0.46 (0.30-0.71)
Worm, high	8.05 (5.38-12.53)	1.74 (1.06-2.84)	0.69 (0.32-1.48)

[a]- corrected mean (95% confidence interval), t=2.571,
[b]- MNX and DNX were not detected
[c] N=4, t=3.182
[d] N=5, t=2.776

Table 3. Mean RDX and metabolite concentrations in *Peromuscus maniculatus* liver following ingestion of residues incurred in food. (N=6).

Explosive/ Dose	Explosive concentration in liver (ng g^{-1})[a]				
	TNT	RDX	MNX	DNX	TNX
Control	2.09 (1.22-3.72)	8.99 (3.21-27.69)	8.48 (3.31-23.91)	2.66 (0.49-16.00)	2.02 (0.59-7.69)
Plant, low	5.59 (1.40-18.95)	9.01 (2.38-37.54)	15.11 (7.14-35.24)	19.38 (9.91-41.73)	1.12 (0.43-3.11)
Plant, medium	2.37 (1.69-3.66)	7.30 (1.21-48.37)	6.04 (1.70-21.41)	6.95 (1.14- 46.79)	1.26 (0.44-3.99)
Plant, high	3.37 (1.50-7.43)	10.41 (3.28-36.43)	7.79 (3.51-17.25)	4.34 (0.80- 25.97)-	1.90 (0.49-7.98)
Worm, low	11.27 (2.37-48.07)	15.22 (5.97-42.74)	10.08 (5.78-19.38)	10.75 (2.22-57.15)	1.70 (0.36-8.97)
Worm, high	4.12 (2.35-7.63)	10.41 (2.36-50.56)	12.40 (6.08-27.92)	11.72 (2.21-68.39)	0.93 (0.31-3.06)

[a]- mean (95% confidence interval), t=2.571

Table 4. Chemical TNT residues in *P. maniculatus* muscle following ingestion of TNT contaminated food. (N=6).

Explosive/ Dose	Explosive concentration in liver (ng g^{-1})[a]
	TNT
Control	17.16[a] (8.63-36.7)
Plant, low	79.83 (28.34-244.34
Plant, medium	70.06 (28.37-188.40)
Plant, high	31.51 (12.41-85.81)

Worm, low	40.63 (19.26-93.20)	
Worm, high	15.49 (7.02-36.35)	

b- mean (95% confidence interval), t=2.57

DISCUSSION:

This study did not demonstrate significant uptake of TNT or RDX into mouse tissues. It is entirely possible that the dosing concentrations were too low for intake to exceed metabolic capacities. The maximum dose represented only a few percent of contaminated food in the diet. This dose should be increased to evaluate accumulation of these contaminants in the terrestrial environment.

8. REFERENCES:

Pan X, Zhang B, Cobb GP**.** 2005. Extraction and analysis of cyclonite (RDX) and its nitroso-metabolites in animal tissue using gas chromatography with electron capture detection (GC-ECD). *Talanta*. 813-824.

Pan X, Zhang B, TA Anderson, Cobb GP. 2006. Determination of N-nitroso metabolites of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in soils by pressurized liquid extraction (PLE) and liquid chromatography-electrospray ionization - mass spectrometry (LC-ESI-MS). *J. Chromatog. A.* 1007:2-9.

TITLE:	Phase VII Analytical Support
STUDY NUMBER:	AS-05-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
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
RESEARCH INITIATION:	July 2004
RESEARCH COMPLETION:	December 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

George P. Cobb
Co-Principal Investigator

Date

1.0 **DESCRIPTIVE STUDY TITLE**:
Phase VII Analytical Support

2.0 **STUDY NUMBER**:
MRT-05-01

3.0 **SPONSOR:**
Strategic Environmental and Research
Development Program
SERDP Program Office
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4.0 **TESTING FACILITY**:
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5.0 **PROPOSED EXPERIMENTAL START & TERMINATION DATES**:
Start Date: July 2004
Termination Date: December 2005

6.0 **KEY PERSONNEL**:
Dr. George P Cobb, Co-Principal Investigator / Study Director / Study Advisor
Dr Kang Tian, Instrument Manager
Ms. Xiaoping Pan, Research assistant
Mr. Jun Liu, Research Assistant
Dr. Ronald Kendall, Principal Investigator

7.0 **STUDY OBJECTIVES / PURPOSE:**
Our group was charged with quantification of energetic compound residues (Figure 1) in environmentally relevant samples.

This required method validation for several sample types and method implementation within ongoing research projects.

8.0 **TEST MATERIALS**:

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazacyclooctane	HMX	2691-41-0
2,4,6-trinitrotoluene	TNT	118-96-7
Hexahydro-1,3,5-trinitro-13,5-triazacyclohexane	RDX	21-82-4
Hexahydro-1,3,5-trinitroso-1,3,5-triazine	TNX	13980-04-6
Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine	MNX	5755-27-1
Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine	DNX	80251-29-2

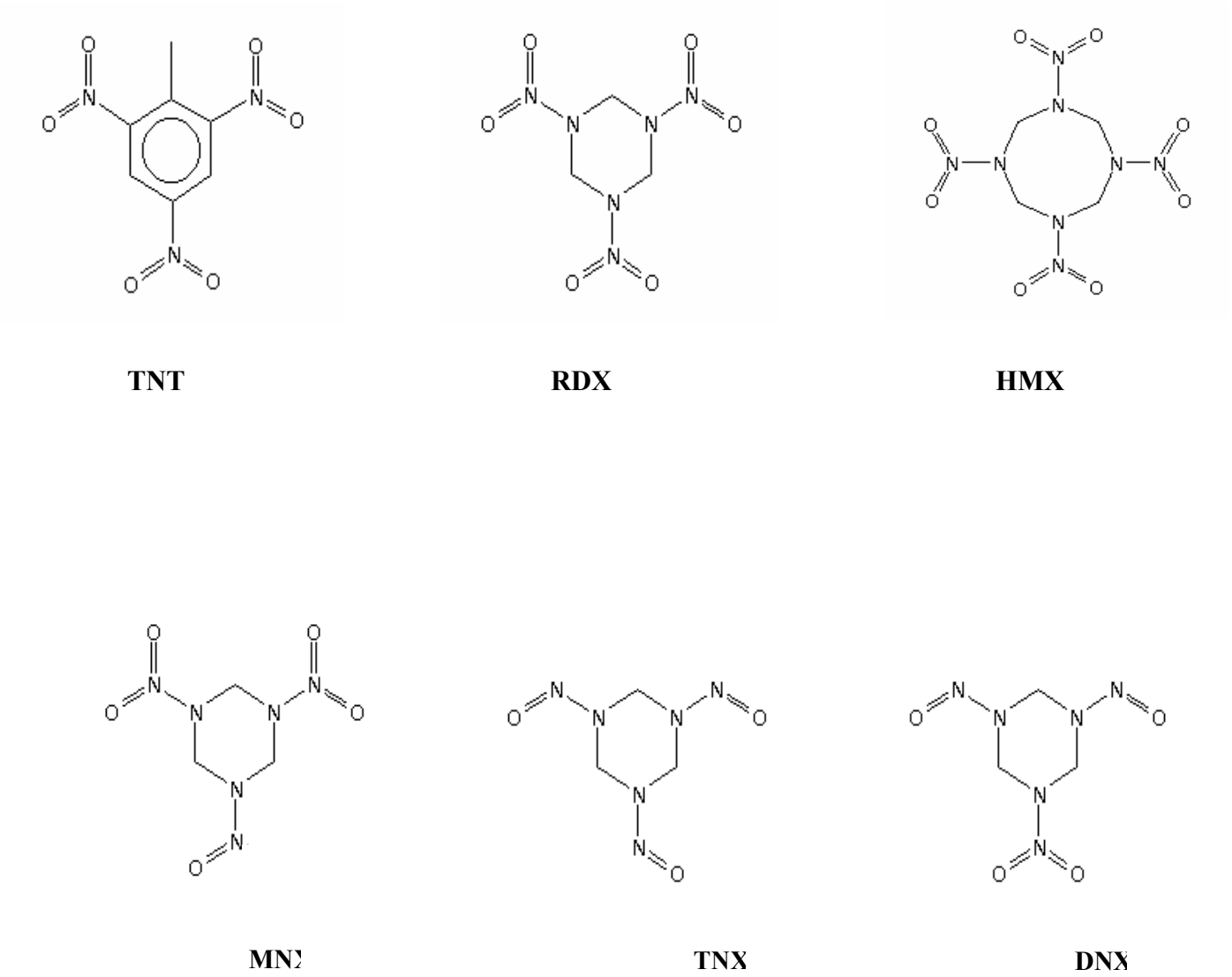


Figure 1. Structures of targeted energetic compounds and their transformation products

9.0 **EXPERIMENTAL DESIGN:**

All methods were validated on three separate days by two individuals. All limits of detection (LODs) were determined using analyses of at least 7 samples that contained analyte concentrations within 8 times the suspected LOD.

All analyses for toxicants in fate and effect studies used techniques previously verified within the Analytical Support section of this research program.

10.0 **METHODS**:

General: In previous years, we developed methods to determine explosives in water, soil, organ tissue, blood, plants, eggs, and animal food With the exception of water analysis each of these methods required Extraction with acetonitrile, and clean-up with florisil solid phase extraction (SPE) columns. In the case of liver and egg samples styrene-divinyl benzene SPE columns were also needed.

GC-ECD: Analyses were performed with a HP 6890 Series gas chromatograph (GC) equipped with an HP 6890 autosampler and an electron capture detector, all controlled by HP 6890 Series Chemstation from Hewlett-Packard (Agilent, Palo Alto, California, USA). Separation was performed on a capillary HP-5 column from Hewlett-Packard (Wilmington, DE, USA). Helium served as carrier gas at a constant flow-rate during the run at 80cm/sec. All gases were supplied by Texas Tech University (Lubbock, TX).

The oven temperature program began at 90°C, held for 2 min, increased to 130°C at a rate of 25°C /min, then made a 10°C /min ramp to 200°C, finally increased to 250°C at a rate of 25°C/min. The temperature of the injection port was 170°C, while that of the detector was 200°C. A 2 µL standard or sample was injected in splitless mode, and the ECD was operated in the constant current mode.

HPLC-UV: The HPLC apparatus was a Hewlett-Packard HP 1100 chromatographic system interfaced with the HP ChemStation software and equipped with a binary pump G1312A, an ultraviolet detector and an autoinjector with a 20 µL loop. The detector was operated at excitation and emission wavelengths of 254 nm. Separations were performed with a reverse-phase C18 column (Supelco, Bellefonate, PA).

For energetic compounds RDX, TNT, MNX, TNX and DNX, the mobile phase consisted of 50% acetonitrile and 50% ultra-pure water. For HMX, gradient mobile phase was employed from 50% acetonitrile and 50% ultra-pure water to 30:70 acetonitrile / water for 5 mins, followed by a gradient to 50:50 acetonitrile / water. The flow rate for all solvents was 1 mL/min, and the injection volume was 15 µL. Chromatography was performed at room temperature (about 25°C). Fresh mobile phase was prepared daily.

LC-MS: A Finnigan LCQ advantage HPLC system consisting of a Surveyor vacuum membrane degasser, a Surveyor gradient pump and a Surveyor autosampler, was coupled to the ion trap mass spectrometer. Chromatographic separation was achieved at room temperature using a Supelco RP C18 column (4.6 * 25 mm, 5-um packing). The isocratic effluent composed of a 50/10/40 (v/v/v), methanol/isopropanol /1.0mM acetic acid running at the flow rate of 0.5 mL/min. The injection volume for all samples was 25 µL and the temperature of the autosampler was kept at room temperature. MS analyses were conducted on a Thermo-Finnigan LCQ advantage ion trap mass spectrometer (San Jose, CA, USA) using ESI interface, in negative-ion mode. Helium was used as damping and collision gas for ion trap, while nitrogen served as sheath and auxiliary gas for ion source. The MS acquisition parameters were: heated capillary temperature 140 ^{o}C; ionization voltage 3.5 kV; sheath gas nitrogen flow rate 28 L/h; auxiliary gas nitrogen flow rate 7 L/h.

11.0 RESULTS

To aid in the evaluation of the potential toxicity nitramine explosives, simple and sensitive quantitative liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) methods were optimized for analysis of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Under negative ionization mode, nitramine explosives can form adduct ions with various organic acids and salts, including acetic acid, formic acid, propionic acid, ammonium nitrate, ammonium chloride, sodium nitrite, and sodium nitrate. Table 1 shows the relative HMX adduct ion abundance when using different additives. Acetic acid was chosen as additive and the ion $[M+CH_3COO]^-$ was used for selective ion monitoring (SIM) in this study (Table 2). Table 3 shows some important LC-ESI-MS operation parameters for HMX analysis, and Table 4 shows some ESI-MS operation parameters for RDX and its N-Nitroso derivatives analysis. Good sensitivity was achieved with low acetic acid concentration in mobile phase and relatively low heated capillary temperature. Figure 2 shows the relationships between target ion abundances with the acetic acid concentrations in the mobile phase across a wide range of concentrations. And Figure 3 shows the relationships between target ion abundances with the heated capillary temperatures. For RDX and its N-nitroso derivatives, the method detection limits (MDLs) were 1.46, 1.46, 1.69, and 1.93 µg/kg for RDX, MNX, DNX, and TNX in soil, respectively. And linearity spanned the range of 5 – 500 µg/L, with correlation coefficients > 0.998. For HMX, the MDL was 0.78 pg in standard solutions and linearity (R^2>0.9998) was obtained at low concentrations (0.5-50 µg/L). Figure 4 shows representative LC-ESI-MS chromatograms for analysis of RDX and its N-nitroso derivatives. And Figure 5 shows LC-ESI-MS chromatograms for HMX analysis.

A liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the analysis of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) was also developed. The electrospray ionization collision-induced dissociation (CID) pattern of HMX was observed and shown in Table 5. And the fragmentation pattern m/z: 355 → m/z: 147 and 174 was chosen for determination of HMX in samples. Important MS/MS operation conditions were also optimized and are shown in Table 6. Using this quantification technique, the method

detection limit was 1.57 µg/L and good linearity was achieved in the range of 5 – 500 µg/L. Figure 6 shows LC-ESI-MS/MS chromatograms for HMX analysis.

We also added to the capabilities for quantification of high explosives in biological tissues by validating an efficient analytical method for HMX in eggs. The method included solvent extraction with ultrasonication followed by cleanup using florisil and styrene-divinyl benzene (SDB) cartridges. Egg extracts were analyzed by high performance liquid chromatography- mass spectrometry (HPLC-MS). Matrix effects to LC-MS were studied and our cleanup procedure proved efficient. Good recoveries and accuracy were achieved. Overall recoveries from eggs containing 10, 50, 250 and 1000 ng/g of HMX were 84.0%, 88.0%, 90.6% and 87.4%. And a method detection limit (MDL) of 0.15 ng/g was achieved by the method.

Applications

These analyses have been applied to several research projects involving different high explosives in biotic (earthworm, lizards, lizard eggs, quail, quail eggs, mice) and abiotic (water, soil, sediment) media. This involved analysis of a significant number of samples (Table 7). Results of these analyses are reported and interpreted in the respective fate and/or effects research projects.

Table 1. Relative HMX adduct ions with different additives (1mM in mobile phase B).

Candidate Additives	Adduct Ion	m/z	Ion Relative Abundance %
Formic acid	$[M + HCOO]^-$	341	30
Acetic acid	$[M + CH_3COO]^-$	355	100
Propionic acid	$[M + CH_3CH_2OO]^-$	369	45
Ammonium nitrate	$[M + NO_3]^-$	358	80
Ammonium chloride	$[M + Cl]^-$	331,333	15
Sodium nitrite	$[M + NO_2]^-$	342	70
Sodium nitrate	$[M + NO_3]^-$	358	80

Table 2. Ions for Selective Ion Monitoring (SIM)

Analyte	CAS #	Molecular Weight	Ion monitored $[M+59]^-$
HMX	2691-41-0	296	355
RDX	121-82-4	222	281
MNX	5755-27-1	206	265
DNX	80251-29-2	190	249
TNX	13980-04-6	174	233

Table 3. Some important optimized LC-ESI-MS operation parameters for HMX analysis

LC conditions	MS conditions
Mobile phase A: Methanol	Mode: Negative
Mobile phase B: 0.5 mM	Ion spray voltage (KV): 3.5
aqueous acetic acid	Sheath gas flow rate (L/hr): 44.0
A: B = 60:40 (v/v)	Aux/Sweep gas flow rate (L/hr): 53.1
Flow rate: 0.5 ml/min	Capillary voltage (V): - 6.3
Injection: 25 µL	Capillary temp (°C): 140.0
	Multipole 1 offset (V): 1.7
	Lens Voltage (V): 25.6
	Multipole 2 offset (V): 7.0
	Multiple RF Amp (Vp-p, sp): 500.0

Table 4. Some important optimized ESI-MS operation parameters for analysis of RDX and its derivatives

ESI Source		**Ion Optics**	
Mode:	Negative	Multiple 1 offset (V):	2.0
Ion spray voltage (KV):	3.52	Lens Voltage (V):	23.6
Sheath gas flow rate(L/hr):	31.9	Multiple 2 offset (V) :	5.4
Aux/Sweep gas flow rate(L/hr):	56.3	Multiple RF Amp (Vp-p, sp) :	500
Capillary voltage (V):	-19.5	Coarse trap DC offset (V) :	10.0
Capillary temp (°C):	140.0	Fine trap DC offset (V):	10.0

Table 5. Collision induced daughter ions of HMX

Parent ion		**Daughter ions**		
m/z	**Structure**	**m/z**	**Relative abundance %**	**Proposed Structure**
355	$[M+CH_3COO]^-$	295	2	$[M-H]^-$
		221	11	$[M - H - CH_2NNO_2]^-$
		192	40	$[M - CH_2NNO_2 - NO]^-$
		174	36	$[M - 3O-CH_2NNO_2]^-$
		147	100	$[M – 2CH_2NNO_2]^-$
		117	12	$[M – 2CH_2NNO_2 - NO]^-$

Table 6. Some important optimized ESI-MS/MS operation conditions for HMX analysis

ESI Source		**Ion optics and ion trap**	
Mode:	Negative	Lens Voltage (V):	14.0
Ion spray voltage (KV):	4.0	Multiple 1 offset (V) :	1.25
Sheath gas flow rate(L/hr):	47.0	Multiple 2 offset (V) :	7.0
Aux/Sweep gas flow rate(L/hr):	27.0	Multiple RF Amp (Vp-p) :	600
Capillary voltage (V):	-12.0	Trap DC offset (V) :	10.0
Capillary temp (°C):	140.0	Normalized collision energy (%):	32

Table 7. Chemical analyses performed within the SERDP Analytical Core during Calendar 2005.

Analytical Activity	**Sample analyses**
Method development	500
Method validation	500
Fish dosing	676
Fish tissue	178
Amphibian dosing	136
Plant dosing	108
Plant	100
Rodent RDX and TNTdosing	12
Rodent RDX and TNTtissue	285
Quail food	58
Quail food stability	60
Quail Eggs	192
Lizard dosing	284
Lizard egg	150
Mesocosm Dosing	230
Mesocosm Output	890
Total Samples Analyzed	**4359**

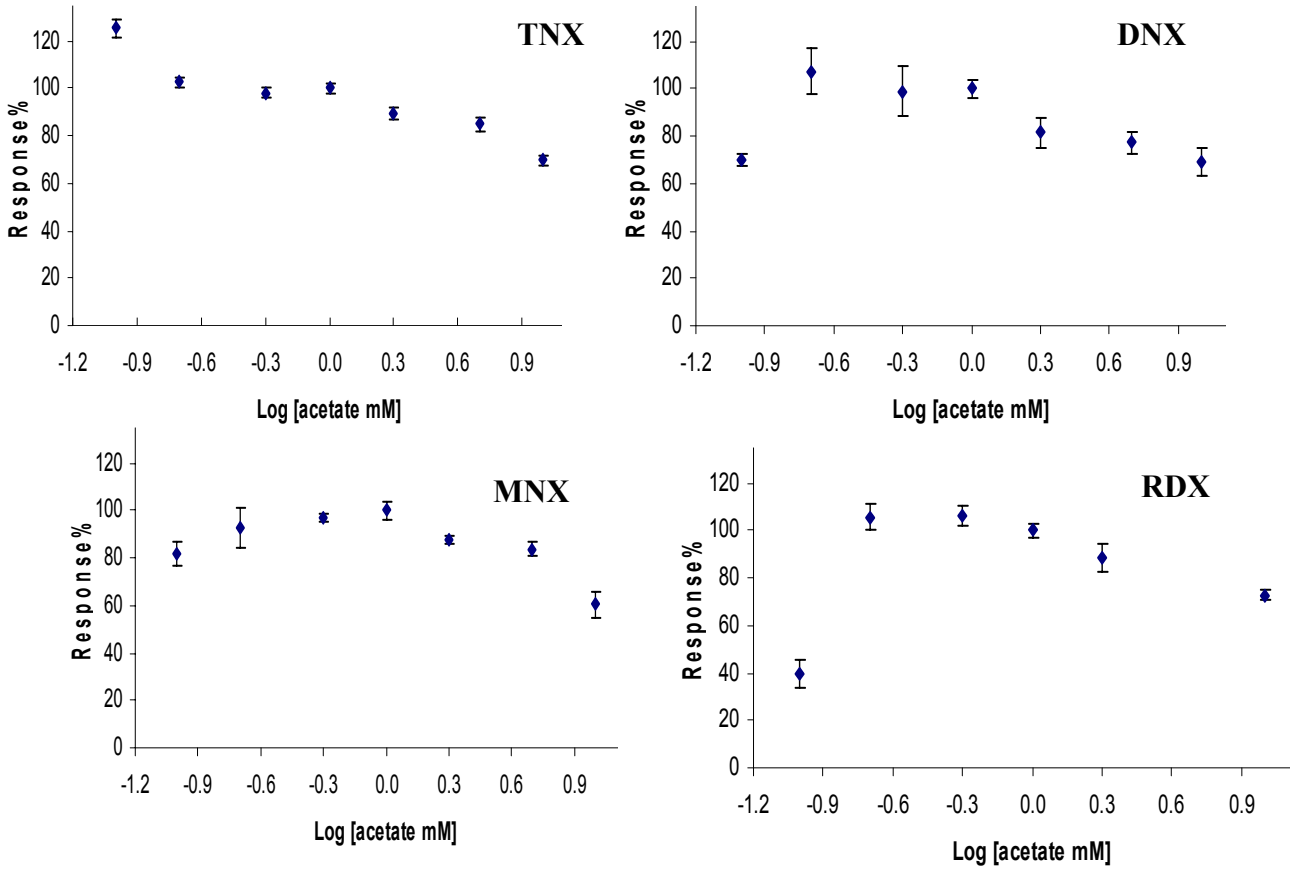


Figure 2. Percent response vs Log [acetate concentration (mM)] in mobile phase B when injecting 250 µg/L of analyte. Y values were calculated as the ratio of signal values at certain acetate concentrations to the signal value obtained at 1 mM acetate at mobile phase B for the same compound. Error bars indicate standard deviation for triplicate injections.

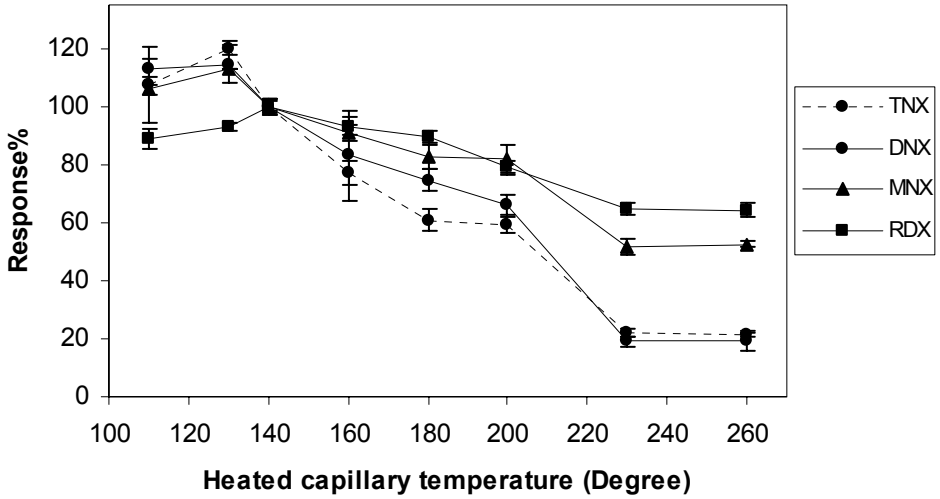


Figure 3. Percent response vs Heated capillary temperature when injecting 20 µg/L analytes. Y values were calculated as the ratio of signal values at certain temperature to the signal value obtained at 140 °C for the same compound. Error bars indicate standard deviation for triplicate injections.

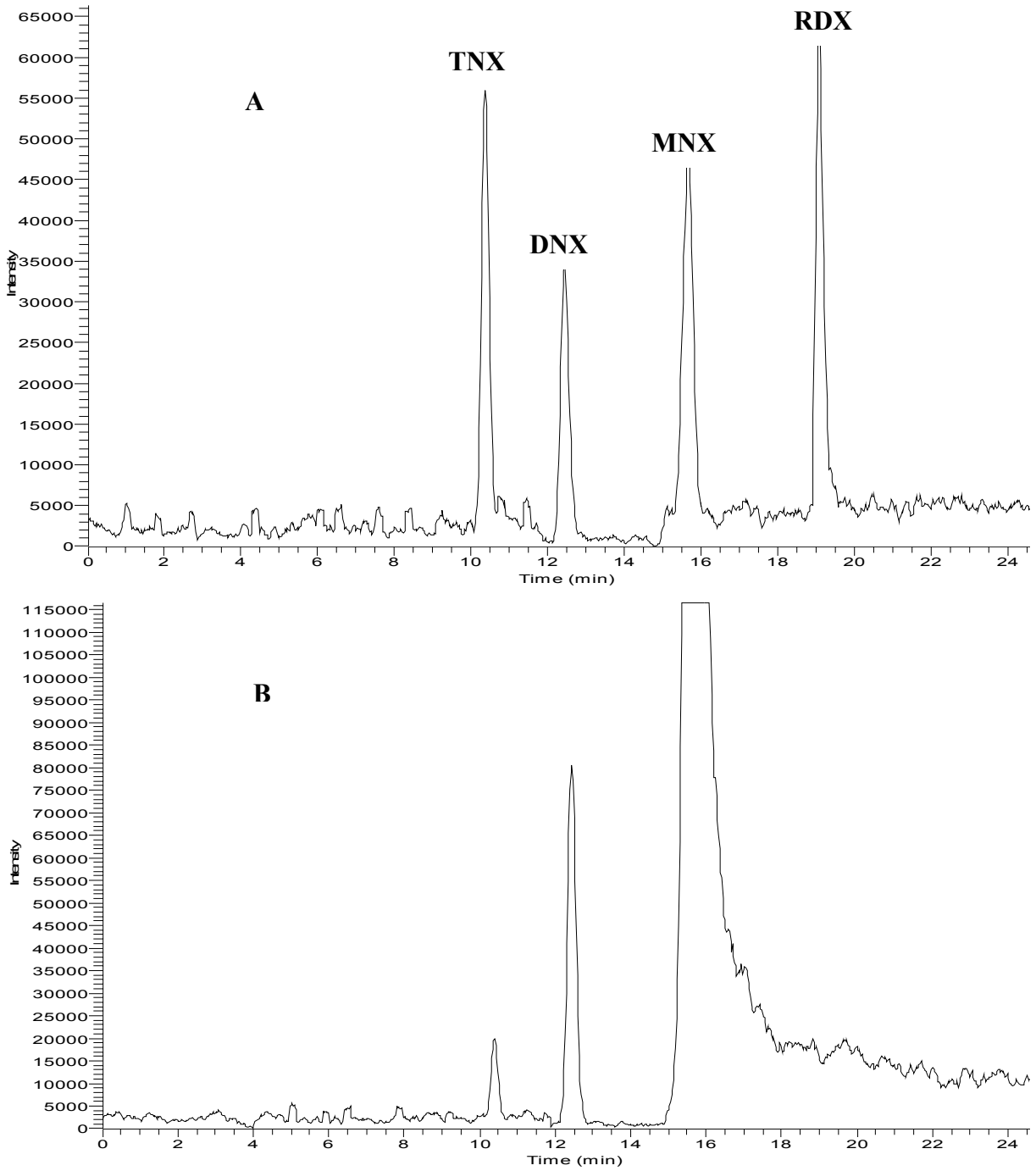
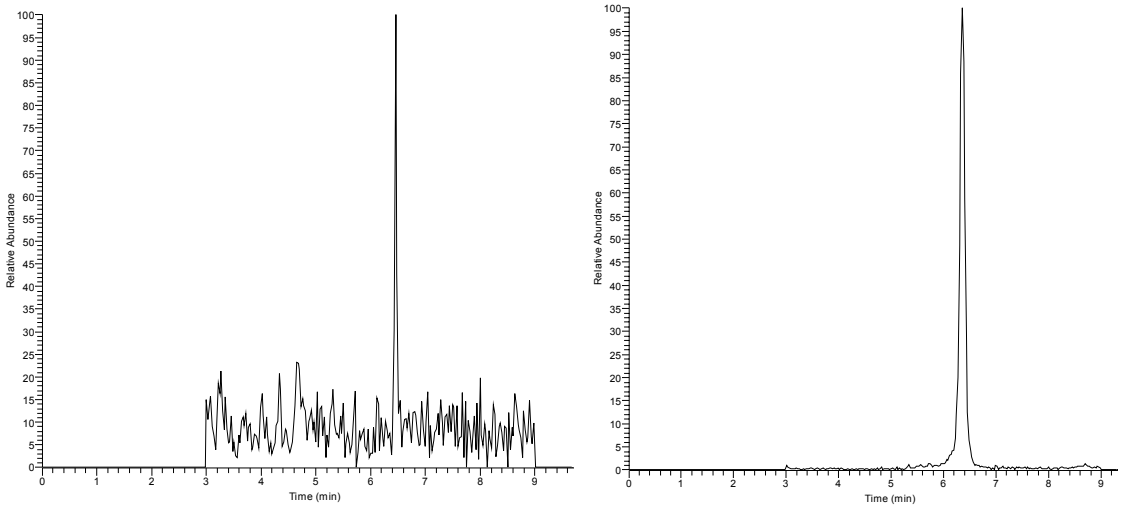
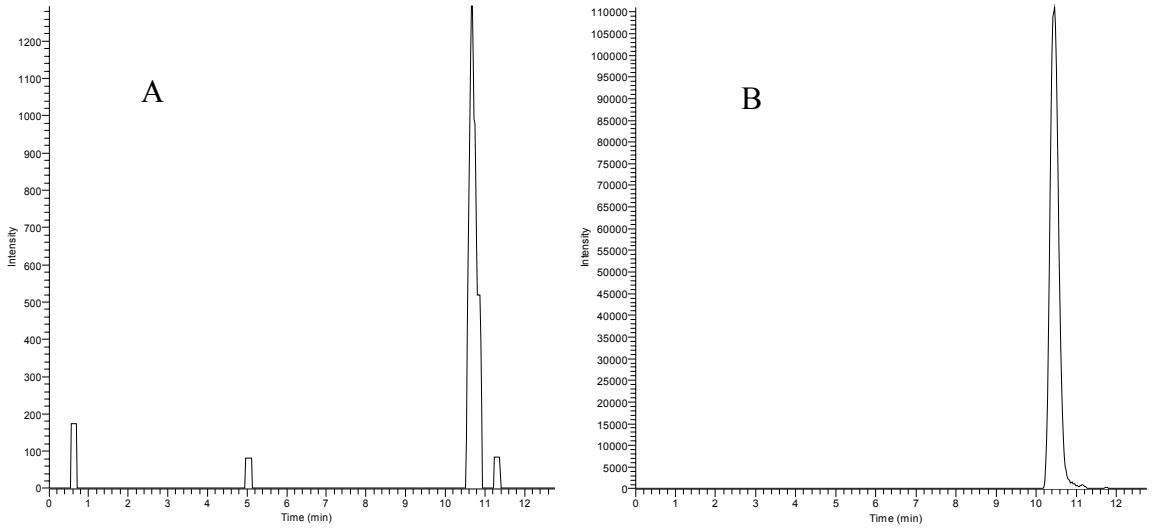


Figure 4. Representative chromatograms of A) a blank soil sample spiked with 20 µg /kg RDX, MNX, DNX, and TNX, and B) a real soil sample containing TNX (7.1 µg /kg), DNX (46.4 µg /kg), and MNX (1543.0) µg /kg (dilution factor = 5).



(A) (B)

Figure 5. LC-ESI-MS (SIM) chromatograms of (A) Mass chromatogram of 0.1 µg/L HMX standard; and (B) Occurred lizard egg sample with HMX 30.6 µg/L.



(A) (B)

Figure 6. Representative LC-ESI-MS/MS chromatograms of A) HMX 5 µg/L in solvent; and B) a real soil sample that contained HMX (210.6 µg /kg) (Diluted sample). SRM: 355>147, 174.

12.0 References:

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Zhang B, Pan X, Smith J, Anderson TA, Cobb GP. 2006. Determination of Trace Amounts of Energetic Compounds in Blood by Gas Chromatography with Electron Capture Detector (GC/ECD) and by High Performance Liquid Chromatography with Ultraviolet Detector (HPLC/UV). *Anal Chim Acta* (In Press)

Pan X, Tian K, Cobb GP. Analysis of Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by Liquid Chromatography / Electron Spray Ionization / Mass Spectrometry(LC-ESI-MS). Submitted to *Anal. Chim. Acta.*

TITLE: Invertebrate Developmental Toxicity of Explosive Metabolites in Soil

STUDY NUMBER: INVDEV-05-01

SPONSOR: Strategic Environmental and Research Development Program
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RESEARCH INITIATION: January 1, 2005

RESEARCH COMPLETION: December 31, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Todd A. Anderson Date
Co-Principal Investigator

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 17, 1989.

Submitted By:

_____ _____

Ryan Bounds
Quality Assurance Manager

Date:

1.0 DESCRIPTIVE STUDY TITLE:
Invertebrate Developmental Toxicity of Explosive Metabolites in Soil

2.0 STUDY NUMBER:
INVDEV-05-01

3.0 SPONSOR:
Strategic Environmental Research and Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

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: January 1, 2005
Termination: December 31, 2005

6.0 KEY PERSONNEL:
Dr. Todd Anderson, Co-Principal Investigator / Study Director / Study Advisor
Mr. Baohong Zhang, co-investigator
Ms. Christina Freitag, co-investigator
Mr. Ryan Bounds, Quality Assurance Manager
Dr. Ronald Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:
The proposed focus of this subproject was to address data gaps related to the potential environmental impact of two degradation metabolites of the explosive RDX (TNX and MNX). These experiments were follow-up studies to those conducted earlier on the biological availability and invertebrate toxicity of TNX and MNX in soil. The developmental toxicity of TNX and MNX to cricket (*Acheta domesticus*) eggs was evaluated. We assessed reproductive/developmental success (egg hatching) upon exposure to TNX and/or MNX.

8.0 STUDY SUMMARY:
The effect of two major hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) metabolites, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), on cricket (*Acheta domesticus*) survival and reproduction was studied. RDX metabolites did not have adverse effects on cricket survival, growth, and egg production. However, MNX and TNX did affect egg hatching. MNX and TNX were more toxic in spiked sand than in topical tests. TNX was more toxic to eggs than MNX. Developmental stage and exposure time affected hatching. After 30 days exposure to

MNX or TNX, the EC20, EC50, and EC95 were 47, 128, and 247 µg/g for TNX, and 65, 140, and 253 µg/g for MNX in topical tests. In sand, the EC20, EC50, and EC95 were 21, 52, and 99 µg/g for MNX, and 12, 48, and 97 µg/g for TNX. No gross abnormalities in cricket nymphs were observed in all experiments indicating that neither TNX or MNX is teratogenic in this assay.

As a side project, the effect of MNX and TNX on earthworm (*Eisenia fetida*) cocoon hatching was also studied. The results indicate that MNX and TNX inhibit cocoon hatching in both topical tests and soil tests. However, there was no significant affect of either chemical on the hatchling survival and growth.

9.0 TEST MATERIALS:

Test Material: laboratory sand
Source: Fisher Scientific

Test Material: silt loam soil
Source: Harlan County, NE

Test Chemical: MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine)
CAS Number: 5755-27-1
Characterization: Purity confirmed by source.
Source: SRI International

Test Chemical: TNX (hexahydro-1,3,5-trinitroso-1,3,5-triazine)
CAS Number: 13980-04-6
Characterization: Purity confirmed by source.
Source: SRI International

Reference Chemical: acetonitrile
CAS Number: 75-05-8
Characterization: ACS-Certified.
Source: Fisher Scientific

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:

Recently, much research effort has been focused on filling data gaps related to the fate/toxicity of explosive materials (EMs) in soil. With the exception of CL-20, the results of this effort have been a better characterization of the bioaccumulation/bioavailability, invertebrate toxicity, and plant toxicity and uptake of a variety of parent EMs and the development of Ecological Soil Screening Level (Eco-SSL) benchmarks for use in ecological risk assessments at explosives-contaminated sites.

While much invertebrate and plant toxicity information has been recently obtained through SERDP-sponsored research on the parent EMs, to our knowledge very little data exist on the potential environmental impact of the degradation metabolites of compounds such as HMX and RDX. The products of the biotic and abiotic degradation of these compounds may also pose toxicological risk to terrestrial and aquatic organisms.

11.0 TEST ANIMALS:

Invertebrate Eggs or Cocoons

Cricket (*Acheta domesticus*)

Earthworm (*Eisenia fetida*)

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

All test systems (crickets, cricket eggs, earthworm cocoons) were placed in bottles with labels containing the appropriate identification information for the test system. Collected samples were placed in individually labeled bags/containers and stored appropriately according to TIEHH SOP IN-3-02.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

A variety of controls were used throughout the course of the experiments to ensure the quality of the data generated. Solvent controls (sand or soil amended with acetonitrile only) and negative controls (sand or soil without test compound or solvent) were included in all trials. The solvent controls were prepared in the same manner as toxicant-spiked systems without the toxicant. All data analyses were conducted on measured metabolite concentrations rather than nominal concentrations. Data were processed using standard statistical software (SigmaPlot, Version 8.0, and SigmaStat, Version 2.03, SPSS, Chicago, Illinois, USA).

14.0 METHODS:

14.1. Test organisms

Crickets (*Acheta domesticus*) were purchased from Carolina Biological Supply Company (Burlington, NC). They were fed a diet consisting of dog food. Water was supplied daily. Crickets were maintained in aquaria on a 12 h light:12 h dark photoperiod at room temperature (~20°C). Each aquaria hosted about 15-20 pairs of adult crickets. Cricket eggs were collected as needed for use in experiments.

Earthworm (*Lumbricus rubellus*) cocoons were obtained from Advanced Biotechnology, Inc. (Elliott, IL).

14.2. Chemicals

MNX (purity > 99%) and TNX (purity > 99%) were obtained as solids from SRI International (Menlo Park, CA). Ultra-pure water (> 18 MΩ) was prepared by a Barnstead NANOpure infinity ultrapure water system (Dubuque, IA). Stock solutions (1000 µg/mL) of MNX and TNX were individually made in acetonitrile and stored at -20 °C until use. All concentrations of MNX and TNX in water, sand, and soil were confirmed using GC-ECD as described previously (Pan et al., 2005; Zhang et al., 2005).

14.3. Cricket egg production
A total of 150 g of fine colored sand (Activa Products Inc., Marshall, TX) was weighed and placed into a 200-mL beaker. Then, 20 mL ultra-pure water was used to wet the sand. Sand was evenly divided into 10 groups and placed into 10 individual 50-mm Petri dishes (VWR International, West Chester, PA). Finally, the sand-filled Petri dishes were put into a 500-mL Redi-Pak straight-sided jar (VWR International, West Chester, PA). Then, two adult female (presence of an ovipositor) crickets were put into the jar and placed in an incubator, overnight, at 28 °C in the dark. After about 12 h of incubation, eggs were harvested for topical tests or other experiments.

14.4. Effect of RDX metabolites on adult cricket survival and egg production
Acclimated adult crickets were fed MNX- or TNX-contaminated dog food for two weeks. Contaminated food was prepared according to the following procedure. Dog food was spiked with 1000 µg/mL MNX or TNX in acetonitrile to obtain final food concentrations of 10 and 100 µg/g. An identical volume of acetonitrile was also added to dog food for the control group (0 µg/g MNX or TNX). MNX and TNX were spiked individually. Spiked food samples were thoroughly mixed in order to distribute the contaminant evenly and allow the solvent to evaporate. Spiked food samples were further stored for 24 hours in the dark under a chemical hood to permit the complete evaporation of acetonitrile. Each treated group (40 adult crickets) was housed in one individual aquarium. Food and water were supplied daily. The behavior and survival of crickets was observed daily. After two weeks of treatment, the number of surviving crickets was recorded. Then, the crickets were allowed to lay eggs in clean colored sand following the same procedure described above.

All laid eggs were maintained (in clean sand) in an incubator at 28 °C in the dark. After 45 days, the number of hatched eggs and nymphs was determined.

14.5. Topical test: Effect of MNX and TNX on cricket eggs and earthworm cocoons
Two layers of filter paper were placed in 10-cm Petri dishes. Then, 30 cricket eggs harvested from the egg production experiments described earlier were placed on the filter paper. Milli-Q water with different concentrations (0, 10, or 100 µg/mL) of TNX or MNX was added to the exterior of each egg. Three replicates were constructed for each of the exposure concentrations. All treatments were incubated in the dark at 28 °C. Petri dishes were opened each day for observation. The earthworm cocoon experiments were performed in a similar manner.

14.6. Sand test: Effect of MNX and TNX on cricket egg hatching
Two healthy adult female crickets were put into a 500-mL glass jar. Each jar contained a 50-mm Petri dish in which 15 g of MNX- or TNX-contaminated sand was contained. Contaminated sand was prepared according to the following procedure. First, 75 g sand was weighed for each treatment. Then, the sand was spiked with 1000 µg/mL MNX or TNX in acetonitrile to final concentrations of 10 or 100 µg/g, individually. Spiked sand was thoroughly mixed in order to distribute the contaminant evenly and allow the solvent to evaporate. Spiked sand was further stored for 24 hours in the dark under a chemical

hood to permit the complete evaporation of acetonitrile. After 24 hours, the sand was wetted with 10 mL of ultra-pure water (> 18 MΩ). Finally, the spiked sand was evenly divided into five 50-mm Petri dishes (5 replicates). Another 75 g of sand, as control, was treated using the same procedure except that it was spiked only with acetonitrile.

Crickets were kept in the jars overnight (~12 hours), then removed to allow the laid eggs to incubate in the sand in the presence of MNX or TNX. After 45 days, the number of eggs and nymphs was recorded.

14.7. Soil test: Effect of MNX and TNX on cricket eggs and earthworm cocoons
Thirty-five grams of silt loam soil (Harlan County, NE) was placed into a 50-mL glass jar (VWR International, West Chester, PA). Silt loam soil contained 2.5% organic matter, 34% sand, 54% silt, and 12% clay (pH = 7.0). Soil was spiked with MNX or TNX in acetonitrile to final concentrations of 0, 10, 100, and 1000 mg/kg. The spiked soils were mixed thoroughly and placed in a chemical hood overnight to allow the acetonitrile to evaporate completely. Then, 5 mL of Milli-Q water was added to each jar to moisten the soil. Finally, 20 cricket eggs were put into each jar and covered by a thin layer of spiked soil. Each treatment group contained three replicates. After 40 days of exposure, the number of nymphs was recorded. The earthworm cocoon experiments were performed in a similar manner, except that the soil used was a sandy loam (Terry County, TX).

14.8. Statistical analysis
All obtained data were statistically analyzed using statistical software (SigmaPlot, Version 8.0, and SigmaStat, Version 2.03, SPSS, Chicago, Illinois, USA). Analysis of variance (ANOVA) was used to compare the data among different treatments. All percent data were arcsine transformed. If there was a significant difference among groups or times, LSD multiple comparisons were conducted to compare the mean of each treatment group or time for determining the bounded No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) values. Each measured parameter was fitted and analyzed using a linear regression model ($y = a + bx$). The 95% confidence intervals (C.I.) were calculated using R project (http://www.r-project.org/).

15.0 RESULTS:

15.1 Effect of MNX and TNX on adult cricket survival and egg production
After 14 days of feeding with MNX- or TNX-contaminated food, there was no difference in survival of adult crickets between treatment groups (10 or 100 µg/g MNX or TNX) and the control group.

Feeding MNX- or TNX-contaminated food did not affect egg production or the hatching success of produced eggs (**Figure 15.1** and **15.2**). About 40% of the eggs hatched (regardless of treatment), and no gross abnormalities in the nymphs were observed for any of the test groups.

15.2. Topical test: Effect of MNX and TNX on egg production and hatching
In the topical test, cricket egg hatching decreased as concentrations of MNX or TNX increased. A dose-response relationship was evident for the hatching of cricket eggs

topically exposed to MNX and TNX (**Figure 15.3**). Without MNX or TNX exposure, 50 ± 7.2% of eggs hatched. After 30 days of exposure to 10 µg/mL MNX or TNX, hatching was 51 ± 7.6% and 48 ± 6.0% for MNX and TNX exposure, respectively. Low concentration (10 µg/mL) exposure to MNX or TNX did not significantly affect cricket egg hatching (**Figure 15.3**). However, the high exposure concentration (100 µg/mL) did affect egg hatching ($p = 0.03149$). After 30 days of exposure to 100 µg/mL MNX or TNX, only 33 ± 12.9% and 30 ± 7.2% of eggs hatched, respectively. Compared with the control, the hatching rates decreased by 34% and 40% after 30 days of exposure to 100 µg/mL MNX or TNX. Based on 30 days of exposure to MNX or TNX, the EC20, EC50, and EC95 were for 47, 128, and 247 µg/mL for TNX, and 65, 140, and 253 µg/mL for MNX (**Table 15.1**).

Developmental stage and exposure time affected cricket hatching (**Figure 15.4**). Short-term exposure to MNX or TNX at a late embryonic developmental stage did not cause a significant decrease in cricket egg hatching. **Figure 15.4** clearly indicates that there was no significant difference among hatching rates for the control group and treatment groups after only 10 days of exposure to MNX or TNX for a total incubation time of 45 days. However, if newly laid eggs were immediately treated with MNX or TNX for at least 30 days, 100 µg/mL MNX or TNX did cause a significant decrease in egg hatching ($p = 0.0315$) (**Figure 15.4**).

Nymph crickets from these experiments (observed under a microscope) showed no morphological abnormalities.

15.3. Sand test: Effect of MNX and TNX on egg production and hatching
Crickets laid eggs in both TNX- and MNX-contaminated sand and uncontaminated (control) sand. Each cricket laid approximately 120 eggs during an overnight period; there was no significant difference in the numbers of eggs laid in contaminated sand or control sand (**Figure 15.5**).

Although TNX or MNX in sand did not affect egg production, both contaminants did affect the hatching of eggs, and this effect was concentration-dependent (**Figure 15.6**). Cricket eggs began hatching after 30-35 days of incubation in sand. For the control group, 43 ± 5.1% of eggs hatched after 45 days of incubation in sand without MNX or TNX. Adding 10 µg/g TNX significantly affected egg hatching ($p = 0.00134$); only 31 ± 2.7% of eggs hatched, a decrease of 29% compared with control. However, the same amount of MNX in sand did not statistically reduce cricket egg hatching. It is possible that this lack of an effect was obfuscated by the large variation among the MNX replicates at this concentration (41 ± 28.1% of eggs hatched in sand with 10 µg/g MNX). At the high concentration (100 µg/g), both MNX and TNX significantly inhibited cricket egg hatching ($p < 0.001$). Only 3.4 ± 3.4% and 1.6 ± 3.6% of eggs hatched in TNX- or MNX-contaminated sand (100 µg/g) after 45 days of incubation, respectively. This suggested that TNX inhibited cricket egg hatching more than MNX at the low concentration (10 µg/g); both MNX and TNX inhibited cricket egg hatching at the higher concentration (100 µg/g). The EC20, EC50, and EC95 were 21, 52, and 99 µg/g for MNX and 12, 48, and 97 µg/g for TNX (**Table 15.1**).

15.4. Soil test: Effect of MNX and TNX on egg production and hatching
In the non-contaminated silt loam soil, 40 ± 5.4% of incubated eggs hatched. Adding 10 or 100 mg/kg MNX or TNX in silt loam soil did not significantly affect cricket egg hatching. However, 1000 mg/kg MNX or TNX did significantly affect egg hatching. TNX at 1000 mg/kg in soil completely inhibited hatching, whereas only 3.3 ± 4.7% of eggs hatched in 1000 mg/kg MNX-spiked silt loam soil (**Figure 15.7**).

15.5. Topical test: Effect of MNX and TNX on earthworm cocoon hatching
MNX and TNX significantly affected cocoon hatching in the topical test (**Figure 15.8** and **15.9**). After 9 weeks of incubation, about 50% of the cocoons hatched in the control group (without MNX or TNX). One µg/mL MNX or TNX did not significantly affect the cocoon hatching. However, 10 µg/mL MNX and TNX significantly decreased the hatching of cocoons. Adding 100 µg/mL MNX or TNX almost completely inhibited earthworm cocoon hatching.

15.6. Soil test: Effect of MNX and TNX on earthworm cocoon hatching
The effect of MNX and TNX on earthworm cocoon hatching was also investigated in sandy loam soil. The results indicated that TNX and MNX affect cocoon hatching in a concentration-dependent manner (**Figure 15.10**). However, it appears that there was no effect on hatchling survival and growth (**Figure 15.11** and **15.12**).

16.0 DISCUSSION

In these experiments, three different approaches were evaluated to determine the effect of two major RDX metabolites (MNX and TNX) on cricket reproductive success. All of the tests indicated that MNX and TNX have somewhat adverse effects on cricket reproductive success, as evidenced by egg hatching. In all experiments, TNX inhibited egg hatching more than MNX. This suggests that TNX was more toxic to cricket eggs than MNX, although the difference is not large. These toxicity results are also similar to our results on earthworms. In our previous RDX metabolite toxicity study with earthworms, it was found that the EC50s were 526 mg/kg for MNX, and 364 mg/kg for TNX in the same silt loam soil (Zhang et al., 2006a). Both the topical test and sand test were good approaches as screening tests to determine the toxicity of RDX metabolites to cricket eggs. These methods are simple, easy to control, and are relevant as examples for maximum contaminant bioavailability. Both are suitable for testing other compounds using crickets or other organisms such as earthworms.

MNX and TNX appeared to display greater toxicity in sand than in the topical test or natural soil test, as evidenced by MNX and TNX having much lower Lowest Observed Effect Concentrations (LOEC) or ECx in sand. The reason for this is possibly due to the cricket eggs being exposed to MNX or TNX isotropically in sand as opposed to the topical tests. Exposure from multiple directions may cause the eggs to absorb more contaminant. Some caution is warranted in this interpretation given the difficulty in comparing the exposure for these two somewhat different toxicity tests. For the sand and soil tests, it is important to note that the silt loam soil contained 2.5% organic matter. Organic matter sorbs MNX or TNX and may make them less biologically available

(Zhang et al., 2006b). Thus, MNX and TNX were less toxic in silt loam soil than in laboratory sand.

Embryos of the cricket (*Acheta domesticus*) were highly sensitive to chemicals (benz[g]isoquinoline-5,10-dione, benzo[h]quinoline-5,6-dione) and developed gross morphological abnormalities after exposure to a number of complex organic mixtures (Walton, 1981; Walton et al., 1983). In addition, crickets display a critical period of teratogen sensitivity and an ability to metabolize xenobiotics during development. In this experiment, although MNX and TNX inhibited cricket egg hatching under certain conditions, no gross abnormalities in cricket nymphs were observed. This indicates that MNX and TNX are not mutagens or teratogens in this assay.

Developmental stage and exposure time affected cricket hatching. In this experiment, we found that only eggs exposed to MNX or TNX for at least 30 days produced low hatching rates. There was no significant effect between the control group and the treatment groups that were exposed to MNX or TNX only for 0-10 days. This indicates that early development of cricket embryos may be more sensitive to MNX or TNX, or MNX and TNX only produce a biological affect after they accumulate to high concentrations in eggs with time.

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:

Pan, X., B. Zhang, and G.P. Cobb. 2005. Extraction and analysis of trace amounts of cyclonite (RDX) and its nitroso-metabolites in animal liver tissue using gas chromatography with electron capture detection (GC–ECD). Talanta 67:816-823.

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APPENDIX

Table 15.1 Toxicity comparison of RDX metabolites (MNX and TNX) to cricket eggs based on sand test and topical test (30 days of exposure).

RDX Metabolite	EC	Sand test * µg/g	Topical test * µg/mL
MNX	20	29 (3-55)	65 (44-86)
	50	52 (33-71)	140 (110-170)
	95	99 (89-109)	253 (210-296)
TNX	20	12 (4-20)	47 (40-54)
	50	48 (41-55)	128 (114-142)
	95	97 (91-103)	247 (133-261)

Data were processed using standard SPSS software (SigmaPlot, Version 8.0.) Each measured parameter was fitted and analyzed using a linear regression model ($y = a + bx$).
* Values in parentheses represent lower and upper 95% confidence limits.

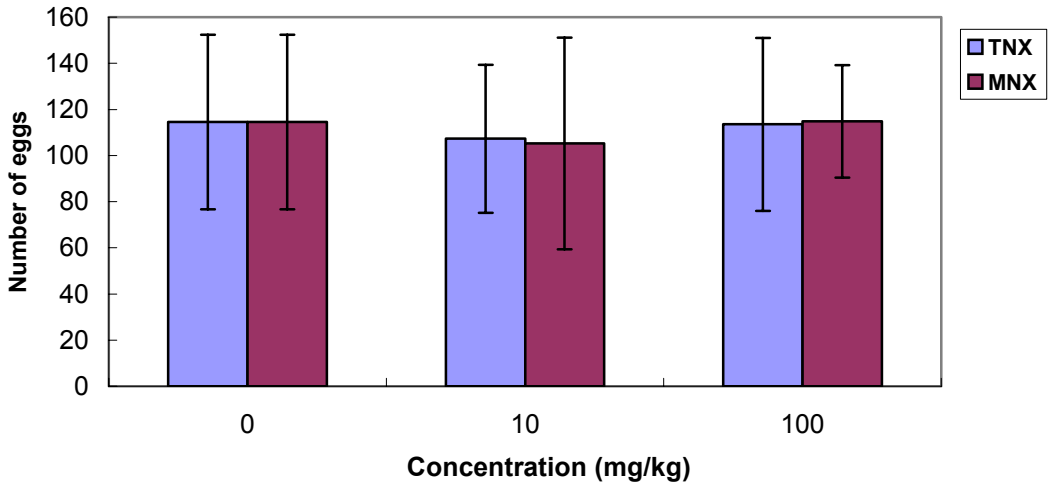

Figure 15.1 Effect of TNX- or MNX-contaminated feed on cricket egg production. Error bars indicate one standard deviation (n = 3).

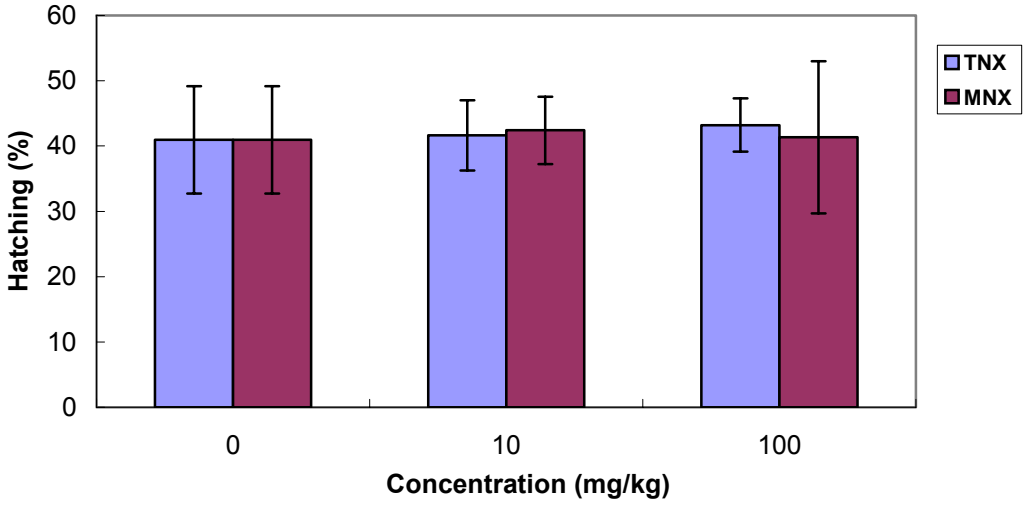

Figure 15.2 Effect of TNX- or MNX-fed crickets on egg hatching. Error bars indicate one standard deviation (n = 3).

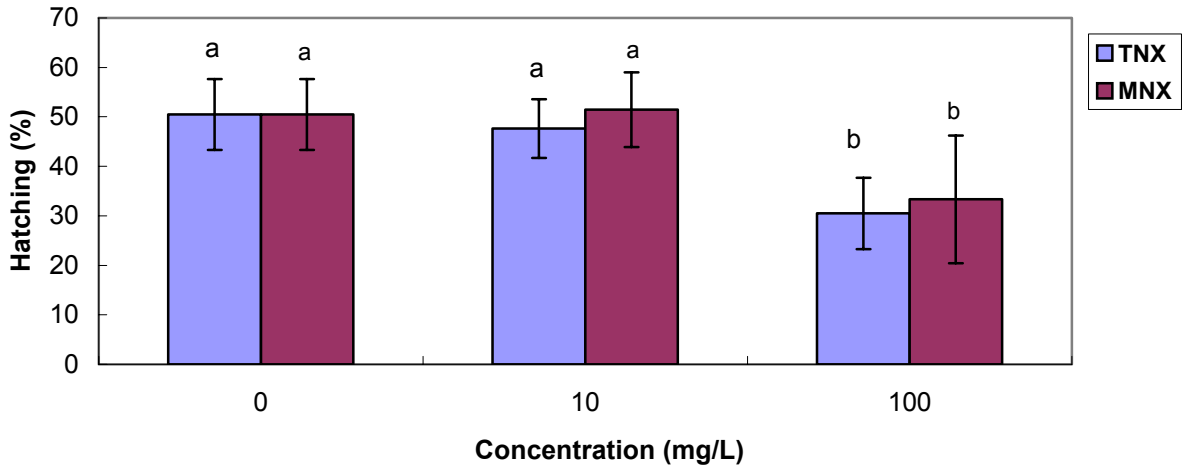

Figure 15.3 Effect of TNX and MNX on egg hatching after 30-d exposure in a topical test. Error bars indicate one standard deviation (n = 3).

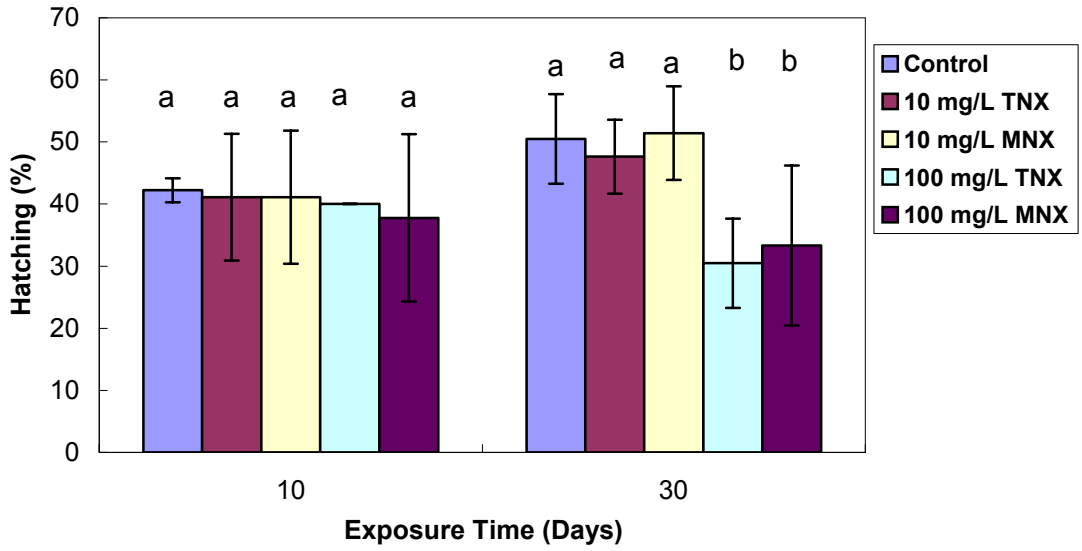

Figure 15.4 Effect of exposure time on cricket egg hatching in a topical test. Error bars indicate one standard deviation (n = 3).

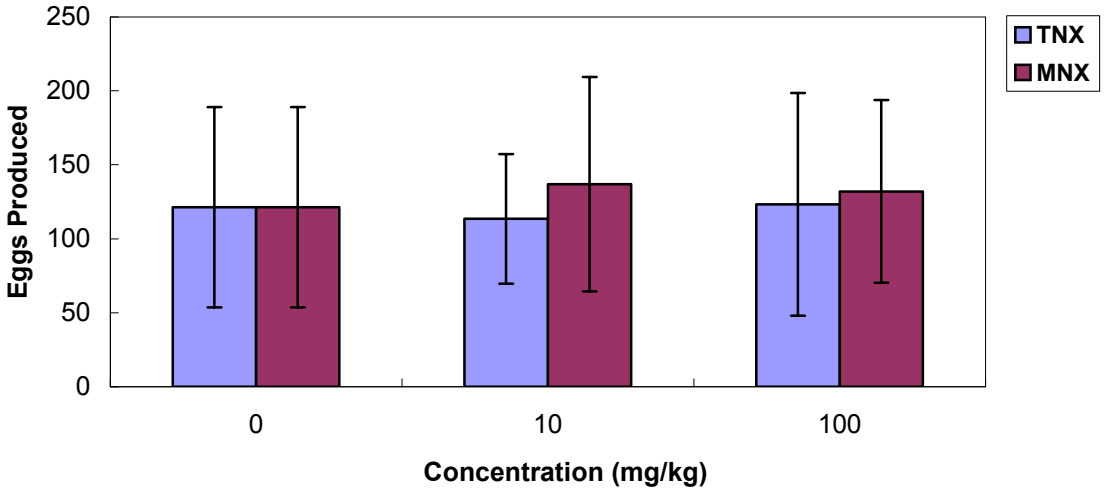

Figure 15.5 Eggs laid on TNX- or MNX-contaminated sand. Error bars indicate one standard deviation (n = 3).

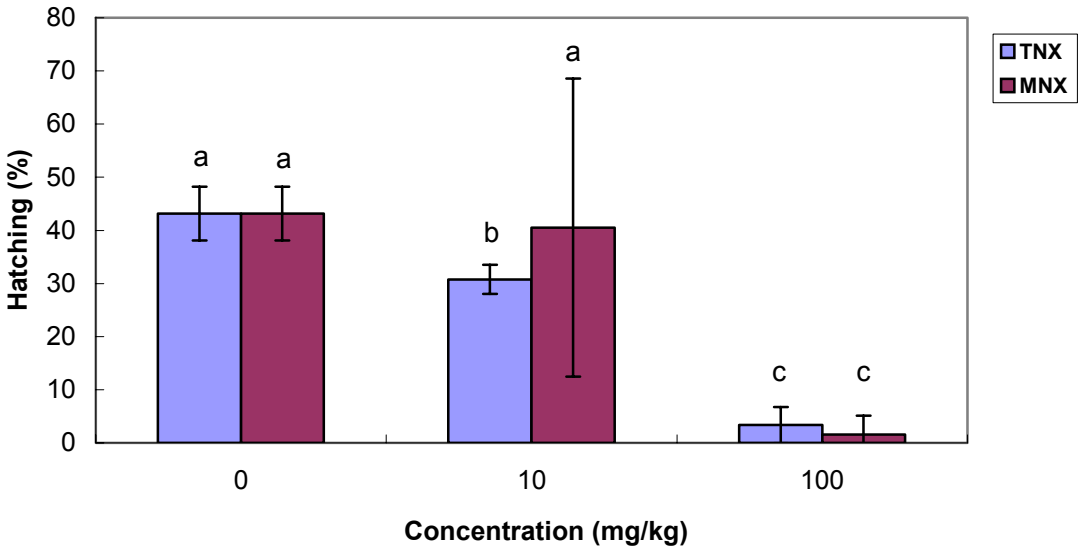

Figure 15.6 Effect of MNX or TNX on cricket egg hatching in contaminated sand. Error bars indicate one standard deviation (n = 5).

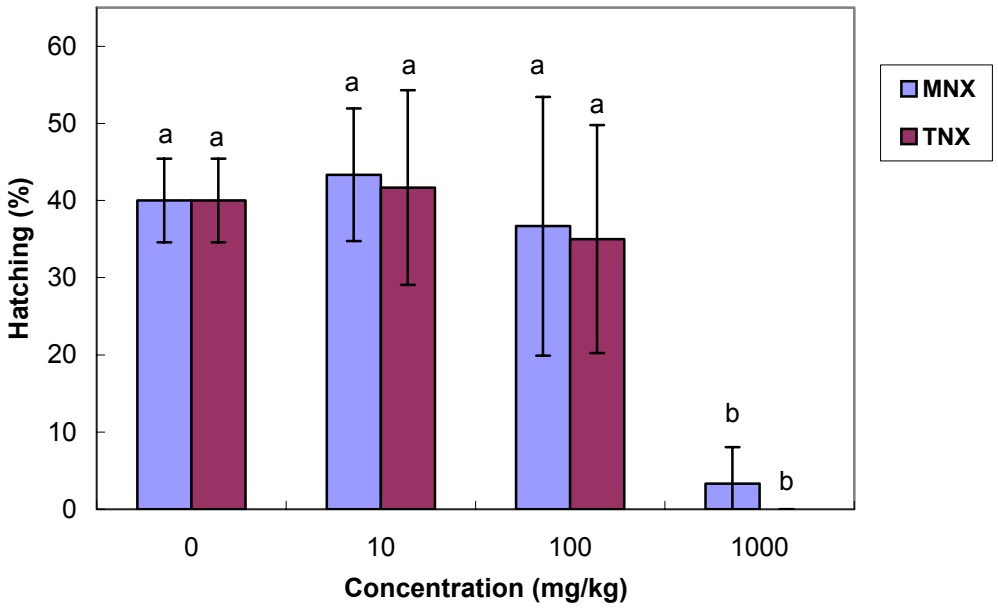

Figure 15.7 Effect of MNX and TNX on cricket egg hatching in silt loam soil. Error bars indicate one standard deviation (n = 3). 1000 mg/kg of TNX completely inhibited egg hatching.

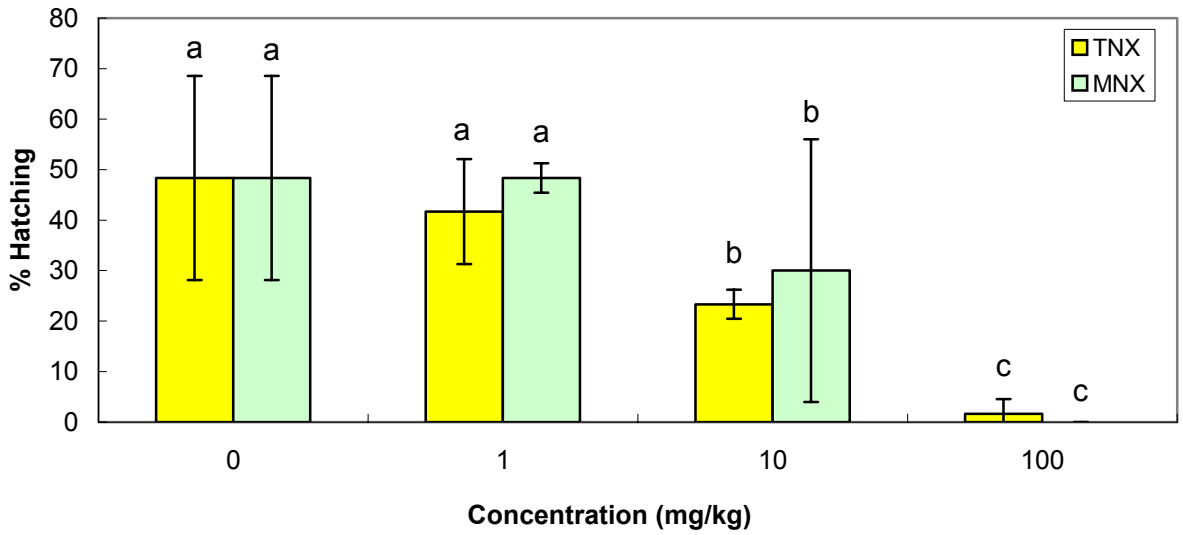

Figure 15.8 Effect of MNX and TNX on cocoon hatching in a topical test (63 day exposure).

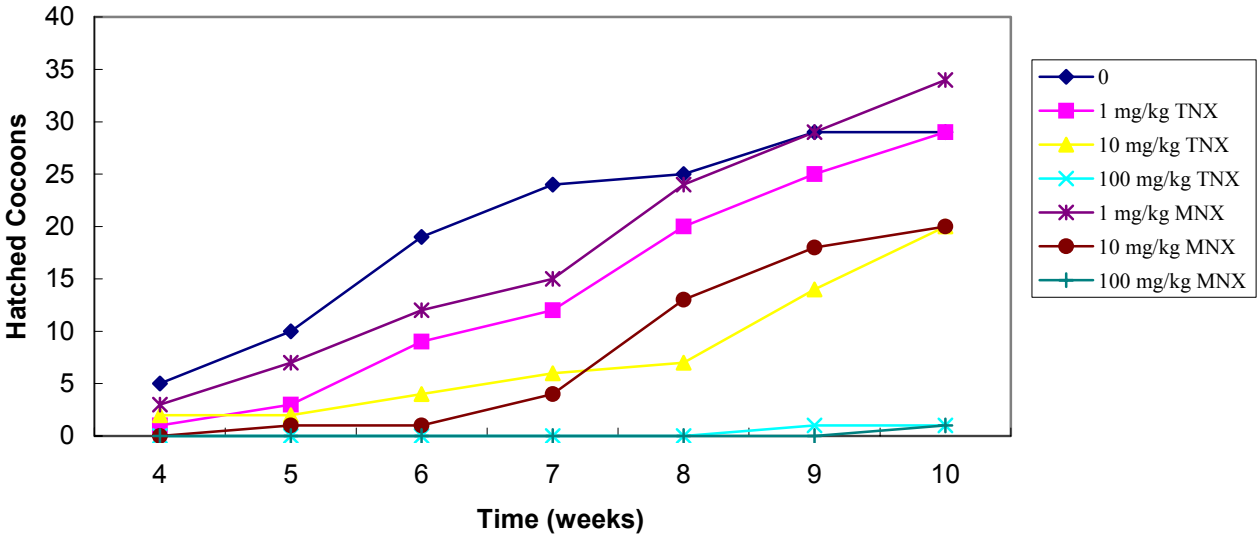

Figure 15.9 Time course of the effect of MNX and TNX on cocoon hatching in a topical test.

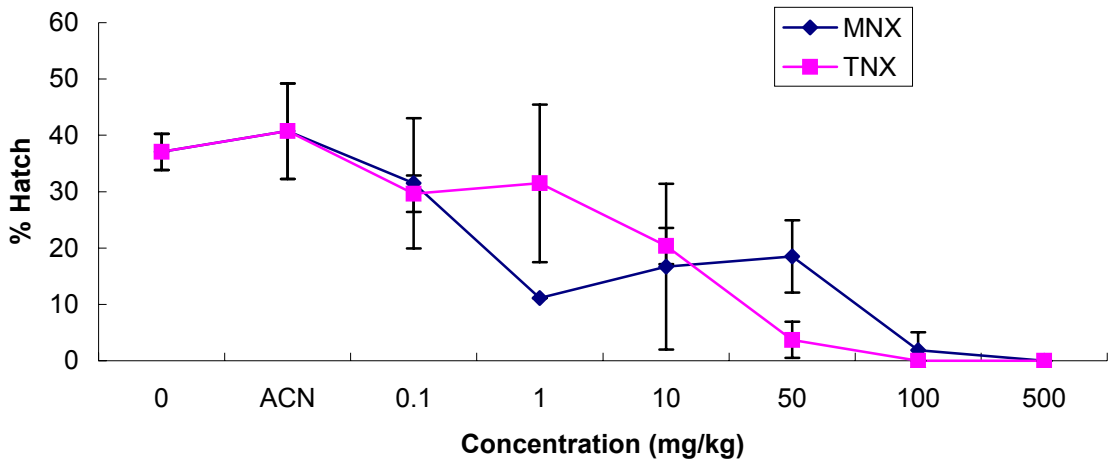

Figure 15.10 Effect of TNX and MNX on cocoon hatching in sandy loam soil (90-d exposure).

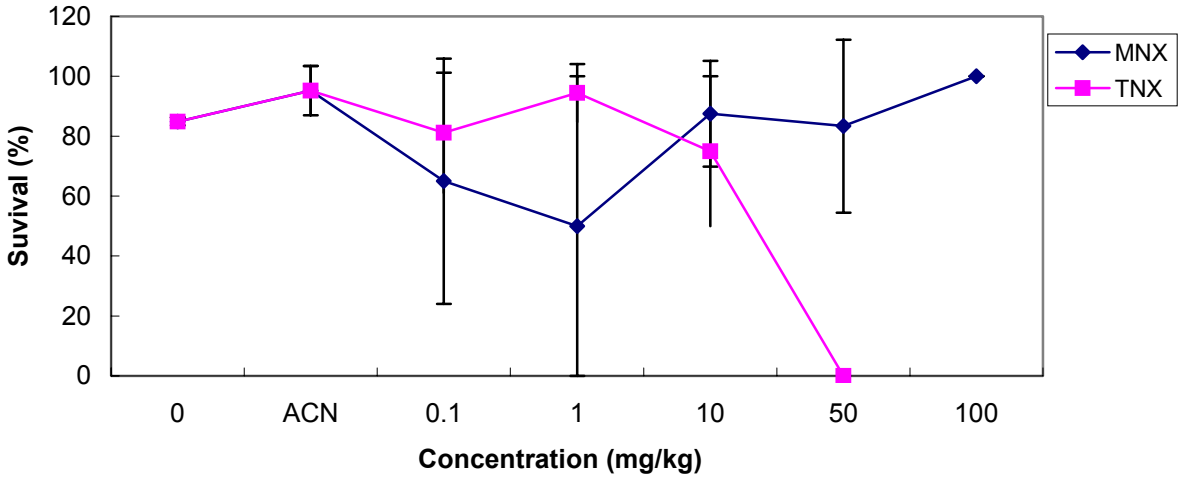

Figure 15.11 Effect of TNX and MNX on earthworm hatchling survival in sandy loam soil (90-d exposure).

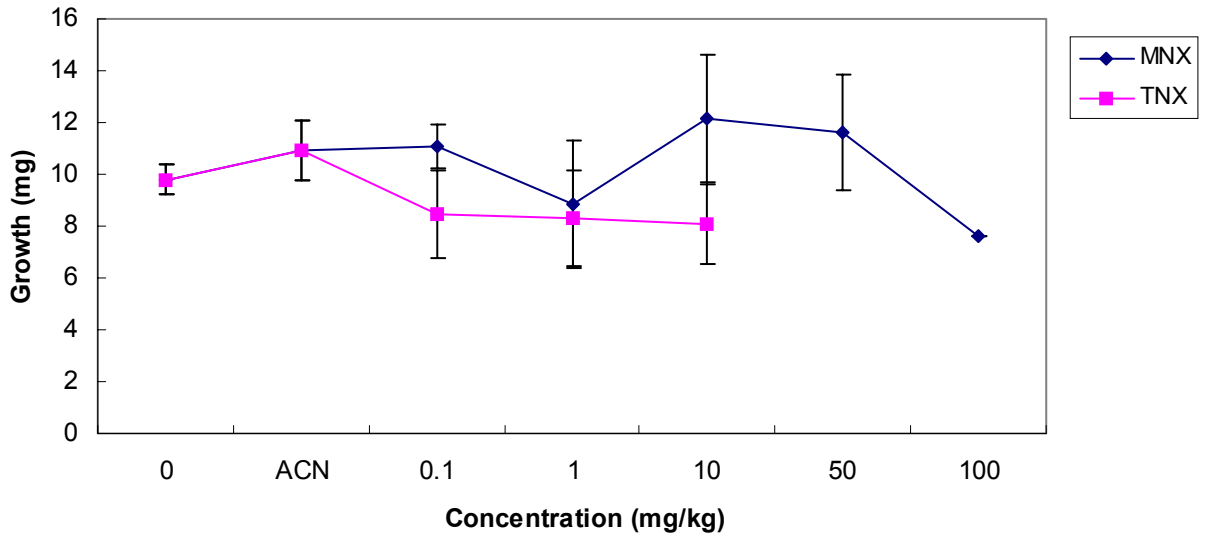

Figure 15.12 Effect of TNX and MNX on earthworm hatchling growth in sandy loam soil (90-d exposure).

Development of Polyclonal Antibody for Biomarkers of Effects following Exposure to RDX Metabolites

STUDY NUMBER:	PABE-05-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
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:	November, 2004
RESEARCH COMPLETION:	December, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in compliance with Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Ernest Smith, PhD 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 17, 1989.
Submitted By:

_____ _____
Quality Assurance Manager Date

1.0 DESCRIPTIVE STUDY TITLE:
Development of Polyclonal Antibody for Biomarkers of Effects following Exposure to RDX Metabolites

2.0 STUDY NUMBER: PABE-05-01

3.0 SPONSOR:

Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME & ADDRESS:
Animal Facility
Human Sciences Building
Texas Tech University

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: November 2004
Termination Date: December 2005

6.0 KEY PERSONNEL:
Ernest Smith, Project Manager / Co-Principal Investigator
Angella Gentles, Co-Principal Investigator
Bharath Ramachandran, Study Director
Ryan Bounds, Quality Assurance
Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:
The purpose of this sub-project is to develop polyclonal antibodies specifically for the deer mice.

8.0 STUDY SUMMARY:
In this study, deer mice were euthanized and liver quickly removed and placed in liquid nitrogen. They were then processed to isolate total RNA, which was used in reverse transcription polymerase chain reaction (PCR) to generate the cDNA for aryl hyrdrocarbon receptor (AhR)gene. A parial deer mouse specific cDNA sequence was obtained. The cDNA was used to generate a deer mouse specific probe and a set of reverse and forward primers that were utilized in real time PCR to quantitate the relative AhR gene expression in various deer mice tissue. The results show the AhR is differentially expressed in deer mouse tissue. The partial sequence was subsequently transcribed to the representative protein sequence. The protien sequence was used to generate polyclonal antibodies antisera against the deer mouse AhR protein.

9.0 TEST MATERIALS:

Peptide sequence (C)SKRHRDRLNTELDR-cooh

10.0 JUSTIFICATION OF TEST SYSTEM

Deer mice were used in this project because they are ubiquitous, opportunistic and are sentinel for wildlife. They are also easily handled, permit large sample sizes, and are more economically feasible in a study of this nature. Live animals are necessary because culture and computer models cannot simulate changes in general homeostasis. In addition, culture and computer models would not provide pertinent scientific data for future use in risk assessment.

11.0 TEST ANIMALS:

Species:	Deer Mice
Strain:	Wild type
Age:	adults
Sex:	Males
Number:	Deer mice = 10 of adults
Source:	In house breeding colony

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each cage was labeled as indicated in TIEHH SOP **IN-3-06;** label included 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 of pups, 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:

Adult animals were selected for tissue collection and isolation of mRNA. Male were selected for this procedure as we do not expect that the AhR would be different between males and females.

14.0 METHODS

14.1 TEST SYSTEM ACQUISITION, QUARANTINE, AND ACCLIMATION

Adult deer mice were obtained from our breeding colony at Texas Tech University. They were maintained in standard cages lined with sani-chip bedding and kept on a 16L: 8D light regimen.

14.2 ANIMAL EUTHANASIA AND SAMPLE COLLECTIONS

Adult animals were euthanized using carbon dioxide asphyxiation and selected tissues were quickly extirpated, wrapped in pre-labeled foil, and placed in liquid nitrogen.

RNA Isolation

Total RNA was extracted, according to manufacturer's procedure, from kidneys using Trizol Reagent (BRL, Gaithersburg, MD). The tissues were homogenized in Trizol Reagent (0.5 mg tissue/ml Trizol) and incubated at room temperature (RT) for 5 min. Chloroform (0.2 ml/ml Trizol) was then added to each tube. After this, the tubes were then shaken and incubated at room temperature (rt) for 3 min. They were then centrifuged at 12,000-x g for 15 min at 4 C. The aqueous phase was transferred to a new tube and 0.5 ml isopropyl alcohol (per ml supernatant) and incubated for 10 min at RT. These were then centrifuged at 12,000 x g for 10 min at 4 C. The supernatant was removed and the pellet washed with 75% ethanol. The pellets were air dried for 5-10 min and dissolved in 50µl nuclease-free water. The RNA concentration was then determined spectrophotometrically at 260nm.

cDNA Cloning, Sequencing and RT-PCR analysis of the deer mice mRNA

An aliquot of total mRNA (2µg) of Deer mouse mRNA was reverse transcribed, using an oligodT-primed first-strand kit (Ambion, TX) to generate deer mice cDNA for amplification and gene isolation.

For PCR amplification, reverse and forward oligonucleotide primers were designed according to the sequence for AhR in *Mus musculus*. PCR was carried out using Failsafe kit (Epicentre, WI). PCR was conducted for 40 cycles of denaturation (92 C, 30 sec), annealing (53 C, 30 sec.) and extension (72 C, 45 sec.), with a 5 min final extension. The deer mouse-AhR PCR product was sequenced for identification and verification using ABI (Perkin Elmer) DNA sequencer by Texas Tech Biotechnolgy Center.

Subsequently, the deer mice specific cDNA sequence was submitted to ABI primer design software for the development of Taqman specific probes and primers for Real Time PCR quantification of mRNA equivalents (Smith et al., 2002).

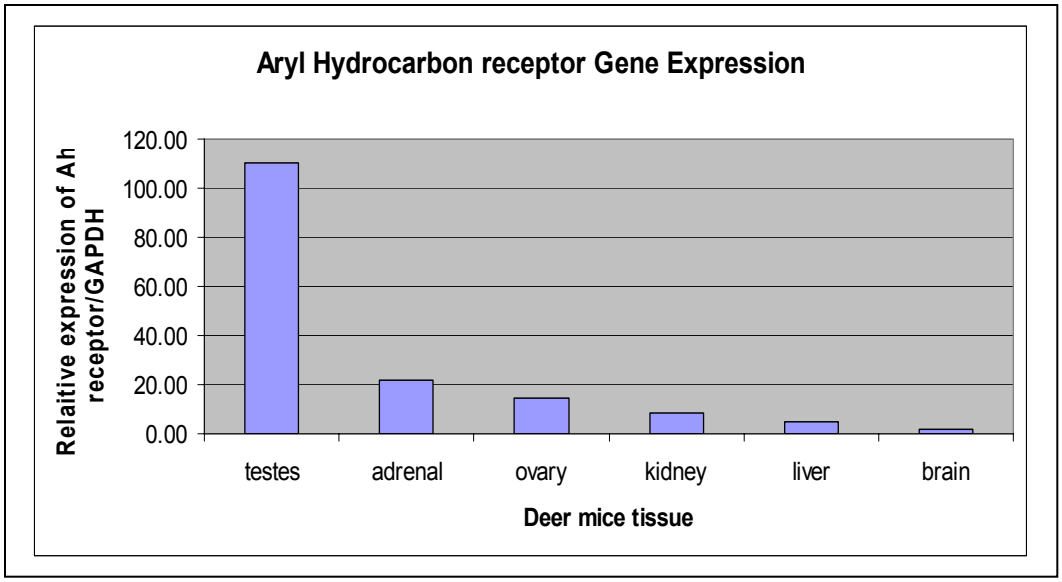
Sense primer sequence – CGC ACA TGG TAC CCA CCT GTA
anti-sense primer – GCC GGT CCC CCT CAA G
Taqman probe – 6fam-CAT GGC CGT CTC TCA CAT GAA GT - tamara

Antibody Development
Expressed deer mouse cDNA gene sequence was used to synthesize deer mouse specific protein at Zymed laboratories. The protein product was using HPLC. The purified peptide was combined with an immunogen for the development of AhR polyclonal antisera over a 90 day period of time. Polyclonal antibody titer was monitored using an ELISA protocol reading at wavelength of 405/490nm.

15.0 RESULTS

Deer mouse mRNA was isolated and successfully transcribed to cDNA. A partial cDNA sequence for AhR was generated by PCR and using deer mouse specific primers. The PCR product was sequence and compared to sequence provided by Dickerson and Frame (2004, personal communication), as outline below. Deer mouse specific real time primer set and probe were subsequently used to determine the relative expression of AhR in different tissues of the deer mouse.

Figure 1. Relative expression of AhR in deer mouse tissues.



Anti-peptide Sequence

A standard 15 residue amino acid peptide sequence (C)SKRHRDRLNTELDR-cooh *(NH-serine-lysine-arginine-histidine-arginine-asparic acid-arginine-leucine-asparagine-threonine-glutamic acid-leucine-aspartic acid-arginine-cooh*) was used for the generation of this polyclonal antibody. This peptide is equivalent to the deer mouse AhR amino acid number 35 to 48.

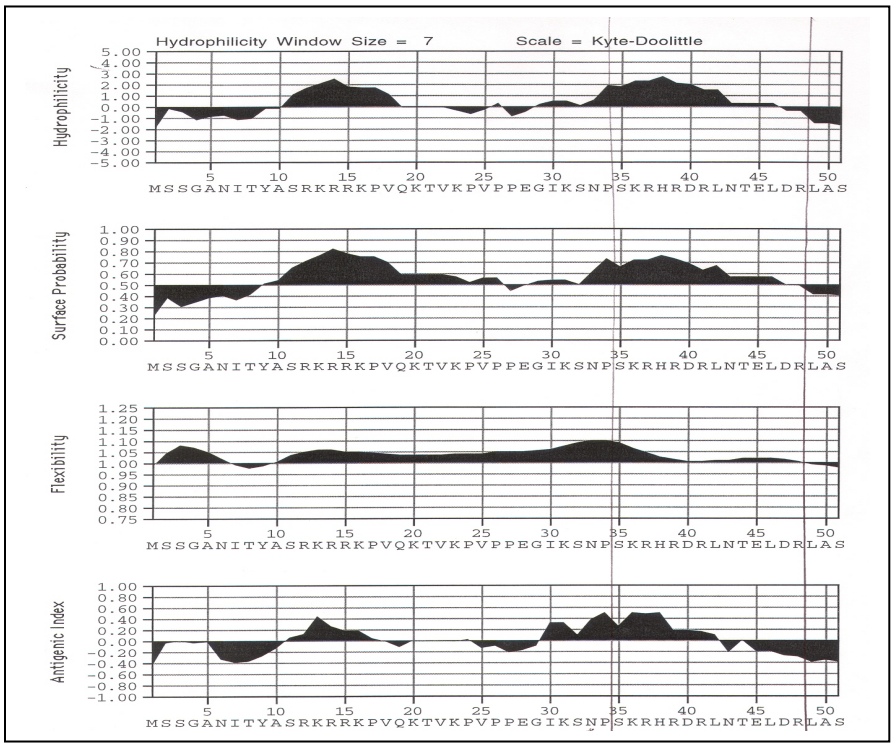


Figure 2. Protein tool box plot of selected the AhR amino acid sequence identifying the levels of antigenic index, flexibility, surface probability and hydrophilicity.

16.0 DISCUSSION

We have successfully isolated expressed aryl hydrocarbon receptor gene from the deer mouse and reversed transcribed a partial sequence to cDNA. The cDNA was used in real time quantification to determine relative expression of AhR in several deer mice tissue. Subsequently, computational analysis protein toolbox plots were used to characterize various segment of the isolated sequence for the selection of a 15 amino acid peptide. Based on hydrophilicity, surface probability, flexibility and antigenic index the following seqeuence - *(NH-serine-lysine-arginine-histidine-arginine-asparic acid-arginine-leucine-asparagine-threonine-glutamic acid-leucine-aspartic acid-arginine-cooh* - was selected as the best of three sequences that were identified for the generation of *Peromyscus maniculatus* arylhydrocarbon receptor antisera.

A specific anti-peptide antibody that is capable of distinguishing a speicifc protein from similar or related proteins is a very powerful technique for application in basic research and diagnostic applications (Yen, 2004). These antipeptides can be developed as new biomarkers of effects. We currently have the AhR antisera at the Institute of Environmental and Human Health. This resource will be shared with other members of the SERDP research team and researchers that are funded by SERDP upon request. Due to the limited quantity of the antisera for the deer mice AhR, sharing will be on a limited basis for external requests.

17.0 REFERENCE LIST

1. Dickerson R. L. and Frame, L (2004) Personal communication

2. Yen S. E. (2004). Anti-peptide Antibodies. Zymed Laboratories California.

APPENDIX

AhR sequence (Dickerson Personal communication 2004)

atggcggcgactacagctaacccagaaatgacatcagatgtaccatcgctaggttccaccattgcttctggaaaccctggacctg
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TITLE:	Reproductive toxicity of RDX in zebrafish
STUDY NUMBER:	ZEB-05-02
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
TEST SITE:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
ANIMAL TEST SITE:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
RESEARCH INITIATION:	August 2004
RESEARCH COMPLETION:	December 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Reynaldo Patiño
Co-Principal Investigator

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 17, 1989.

Submitted By:

_____ _____

Ryan Bounds
Quality Assurance Manager

Date

1. Descriptive Study Title:
Reproductive toxicity of RDX in zebrafish

2. Study Number:
ZEB-05-02

3. Sponsor:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. Testing Facility Name and Address:
Texas Cooperative Fish and Wildlife Research Unit
Texas Tech University
Box 42120, 218 Agricultural Science Building
Lubbock, Texas 79409-2120

5. Proposed Experiment Start and Termination Dates:

Start date:	August 2004
Termination Dates:	December 2005

6. Key Personnel:
Reynaldo Patiño, Co-Principal Investigator
Sandeep Mukhi, Study Director
George Cobb, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principal Investigator

7. Study Objectives/Purpose:
The goal of this proposed research is to generate a database for ecologically relevant, lethal and sublethal effects of RDX and its metabolites in an important group of aquatic vertebrates, the fishes. In our previous studies, we characterized the lethal (LC50) and sublethal effect of RDX on somatic growth and the bioaccumulation pattern of RDX in whole fish. In this study, we examined the sublethal effect of RDX on reproductive performance.

8. Study Summary
The objective of this study was to examine the effect of RDX on reproductive performance of zebrafish. Adult males and females were exposed to control, water, or two environmentally relevant concentrations of RDX, 0.5 and 3.2 ppm, for a period of 6 weeks. Male and female fish were exposed separately. Female fish tanks containing 8 females were the unit of replication for this study. Every two weeks, they were paired with 4 similarly-treated males and packed egg volume, egg fertilization rate and embryo hatching rate were determined. Mean packed-egg volume seemed to be increased in the 0.5-ppm group at 2 weeks of exposure but not at 4 or 6 weeks. No significant effects of

RDX on packed egg volume were noted at 3.2 ppm. Egg fertilization and embryo hatching rates were not affected at any RDX concentration during the exposure period. Overall, the results of the present study do not suggest deleterious effects on zebrafish reproductive performance when exposed to RDX at environmentally relevant concentrations. However, this study did not include measures of larval or juvenile health.

9. Test Materials:

Test Chemical name: Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)
CAS number: 121-82-4
Characterization: white powder
Purity: 99.9% pure as indicated by supplier
Stability: The chemical was found to be stable at least for 1 week in our test system
Source: Accurate Energetics (McEwen, TN, USA)

Reference Chemical name: Calcium Chloride
CAS number 10035-04-8
Characterization: coarse white powder or mixture with medium size granules.
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Magnesium Sulfate
CAS number: 100-34-99-8
Characterization: colorless crystals
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Potassium Chloride
CAS number: 7447-40-7
Characterization: white crystalline granules
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Sea Salts
CAS number: Not applicable
Characterization: an artificial salt mixture closely resembling the composition of the dissolved salts of ocean water.
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Aquarium Systems, Inc.

Reference Chemical name: Sodium Bicarbonate
CAS number: 144-55-8
Characterization: white crystalline powder

Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Sodium Chloride
CAS number: 7647-14-5
Characterization: white crystalline granules
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Ultrapure water with added salts needed by fish will be used as reference solution to which negative reference material or test material will be added for treatments.

CAS Number: Not applicable
Characterization: water quality will be tested by chemical analysis and pH will be monitored regularly.
Purity: ultrapure
Stability: stable
Source: Steam plant reverse-osmosis (RO) water that has been run through a carbon filter and a de-ionizer to convert it to ultrapure water was used in this study. To this RO water ROrite® (25 g /100 liter of RO water) was added to make suitable for zebrafish.

10. Justification of Test System:

The cyclic nitramine, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), is widely used as an explosive in commercial and military operations. It is believed that the large-scale manufacture, use, and improper disposal of RDX have led to contamination of soil and ground water by this compound and its metabolites (Sunahara et al. 1999). RDX in natural soil environments is metabolized to other compounds (Sheremata et al. 2001). RDX metabolites may include hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). RDX and its metabolites do not strongly adhere to soil particles, and there is a high potential for leaching from contaminated soils into surface waters. Among fishes, the number of species for which the acute (lethal) toxicity of RDX has been tested is small (Bentley et al. 1977).

In previous studies (DoD-SERDP, Phase VI), we examined the acute (lethal) toxicity of RDX (Mukhi et al., 2005b) as well as its sublethal toxicity on the growth and bioaccumulation in zebrafish (Mukhi et al., in review). The purpose of the present study (DoD-SERDP, Phase VII) is to examine the sublethal effects of RDX on the reproductive health of adult fishes. The model organism for the present study continues to be the zebrafish (*Danio rerio*). Zebrafish are frequently used in biomedical research and have the advantage of a wealth of information concerning its genetics and developmental biology. Also, the zebrafish genome sequencing project and the availability of commercial cDNA microarrays allow for the convenient application of current tools in molecular biology to this species. Further, zebrafish are easy and economical to maintain in the laboratory, and we have prior expertise and experience using this species for toxicological research.

11. Test Animals:

Species: *Danio rerio*, Zebrafish
Strain: Wildtype
Age: Adult zebrafish
Number: 72 females and 36 males (2:1 ratio of female to male)
Source: Purchased from local vendor

12. Procedure for Identifying the Test System:

The experimental units (aquarium) were labeled with the study protocol number, ACUC number, chemical and its concentration and the contact person name on it. There were three replicates for each treatment and each treatment was color coded for easy visual identification. Due to anticipated difficulty in identifying the males from the females after spawning, males were tagged with Visible Implant Elastomer (VIE) at the base of dorsal fin according to the manufacturer's protocol (Northwest Marine Technology, Shaw Island, WA, USA).

13. Experimental Design Including Bias Control:

Adult male and female zebrafish were exposed to RDX using a static renewal system according to contaminant exposure procedures previously developed in our laboratory (Mukhi et al. in review). The basic experimental design included control (no RDX), low (0.5 ppm) and high (5 ppm) nominal RDX concentrations. Each exposure was conducted in triplicates aquaria for females and single aquarium for males. The exposure period was 6 weeks. Once in 2 weeks during the exposure, spawning containers were placed in the female tanks and the 8 female fish in each tank will be combined with 4 males from the male exposure tank. Reproductive success was assessed by measuring packed-egg volume, fertilization rate and hatching rate. Cumulative packed-egg volumes were analyzed as previously described (Patiño et al., 2003).

Number of female fish required
8 females/replicate x 3 replicates x 3 treatments x 1 chemical = 72

Number of male fish required
12 male/replicate x 1 replicate x 3 treatment = 36

Total number of adult zebrafish required = 108

14. Methods

14.1. Chemical, Safety Procedures and chemical analysis

Hexahydro-1,3,5-trinitro-1,3,5-triazine (CAS Reg. No. 121-82-4) was obtained from Accurate Energetics (McEwen, TN, USA). The chemical was 99% pure and supplied in desensitized form, containing 20% water by volume. Standard solutions for RDX were obtained from Supelco (Bellefonate, PA, USA). RDX was stored in a specially designed bunker on campus and handled with utmost care to prevent spark or shock which could trigger explosions. RDX was transported from the bunker to the study facility in the desensitized form. Water samples from the treatment tanks were analyzed for actual RDX concentrations by high performance liquid chromatography (HPLC) according to Mukhi et al. (2005a).

14.2. Experimental animal and standard rearing condition

The use of animals in this study was reviewed and approved by the Texas Tech University Animal Care And Use Committee (Lubbock, TX, USA). Four-month-old, adult wildtype zebrafish (*Danio rerio*) were obtained from local vendor (Lubbock, TX, USA) and allowed to acclimatize to our laboratory condition for a period of three weeks before the spawning trials began. As the objective of this study was to evaluate the reproductive toxicity of RDX, male and female zebrafish were reared separately. Eight females or 15 males were randomly distributed into each of 9 or 3 10-gallon aquarium respectively. Animal husbandry procedures for this study were as described by Mukhi et al. (2005a). Briefly, each aquarium was filled with 30 L of system-water (25 g of ROrite/100 liter of reverse-osmosis supply water) and fitted with two hand-made internal biofilters. Each aquarium was marked at 15-L and 30-L volume level to facilitate 50% or 100% water exchange during experimentation. A water current through the filter was maintained by airflow via glass pipette. The tanks were added with stresszyme in weekly interval to maintain a biofilm in the biofilter. Water quality parameters were maintained at recommended levels for zebrafish (pH 6.5-8.0, 26-28.5 ºC, 12/12 light/dark cycle). If the pH in the treatment tanks fell below 6.5, appropriate volume of 5-M NaOH solution was added to aquarium to bring within the optimal range. Fish were fed either with adult frozen *Artemia* or Tetramin® flakes (Tetra Sales, Blacksburg, VA, USA) twice daily to satiation. Every evening, leftover food and fecal material were removed by siphoning. Temperature and pH was measured daily and dissolved oxygen, specific conductivity, salinity, unionized ammonia and nitrate was measured at least once weekly. Half of the water volume (15 L) was removed and replaced with clean system-water twice weekly.

Due to anticipated difficulty in identifying the males from the females after spawning, males were tagged with Visible Implant Elastomer (VIE) at the base of dorsal fin according to the manufacturer's protocol (Northwest Marine Technology, Shaw Island, WA, USA). Males were allowed to recover for at least 2 weeks after tagging before the start of spawning.

14.3. RDX exposure

Two environmentally relevant, nominal concentrations of RDX (0.5 and 5 ppm) were chosen for the experiment in addition to a control group. Female tanks were considered the unit of replication, and each treatment was conducted in triplicate for females (8 fish per tank). Males for each treatment were maintained together in a single tank (12 fish per tank). Therefore, the experiment consisted of 9 female-tanks and 3 male-tanks. A static renewal exposure procedure was followed as described earlier (Mukhi and Patiño, 2005). Briefly, every week, 50 percent of treatment water in each aquarium was renewed twice with preheated-aerated-fresh treatment water from the overhead tanks. Water samples (2-3 ml) were collected from each tank once a week before the second water exchange for verification of actual RDX concentrations in treatment tanks. Fish feeding and swimming behaviors were observed daily and any sign of abnormal behavior was recorded.

14.4 Measurement of Somatic Growth

Fish wet-weight was measured by placing the fish (all 8 females per tank or all 12 males per tank) in a pre-zeroed, 1-L beaker with water. The weight was taken one day prior to placement in the spawning chambers in order to minimize stress, which could affect spawning performance. Also, the weight was measured before the evening meal. As male tanks were not replicated, their weight was not used for statistical analysis.

14.5. Effect of RDX on packed-egg volume

The spawning procedure was as described as Patino et al. (2003). Briefly, plastic shoe boxes (30 cm long X 15 cm wide X 5 cm deep) coated with silicon were used as spawning chambers. The spawning unit consisted of an upper chamber for holding the fish and a lower chamber for collecting the eggs. Holes were drilled on the side walls of the upper chamber to allow air exchange to occur, and its bottom was replaced with a silicon-coated plastic mesh to allow the eggs to fall into the lower chamber. The day before spawning, fish were fed only the morning meal and a single spawning container was placed in each female fish tank. The 8 females from that aquarium and four males from the same RDX-treatment tank were placed into the spawning chamber. The following morning, fish were removed from the spawning container and returned to their original tanks approximately 2.5 hours after lights on. Fecal matter and other debris were removed from the egg slurry using pipettes and by rinsing 4 times with fresh zebrafish water. Packed-egg volume was determined by volume displacement in a graduated 5-ml glass cylinder.

The first spawning trial was conducted one week before the initiation of RDX treatment. The purpose of this spawning was to confirm spawning readiness and to synchronize reproductive cycles. The fish were then spawned once every 2 weeks after the initiation of RDX exposure. Therefore the packed-egg volume was measured at 1 week prior to exposure and 2, 4, and 6 weeks after initiation of exposure. One female was lost from one of the 5-ppm tanks following the first spawning; therefore, this replicate was eliminated from the study.

Rates of fertilization and hatching rate were also calculated. For this purpose, approximately 100 eggs from each treatment replicate were incubated in 500-ml beakers containing 300 ml of zebrafish water at 28 °C. After 6-8 hours of fertilization, unfertilized eggs became white (opaque) and were removed from the incubation beaker and counted. The fertilized, translucent eggs were counted once and kept in the same beaker until hatching. Fifty percent of the water in the beakers was replaced with fresh-preheated (28°C) system water. Three days after spawning, the number of unhatched eggs was determined in each beaker. Percent of hatching was calculated from the total fertilized eggs present in the beaker at the beginning.

14.6. Data analysis

The weight of female fish was measured as group-weight (8 fish), and the packed-egg volume was measured in each tank replicate. Thus, in all cases, sample size per treatment for statistical analyses is the number of tank replicates (n = 3). Unless otherwise noted, the effects of waterborne RDX (concentration and exposure time) on somatic condition, egg volume, fertilization rate and hatching rate were initially

assessed by two-way analysis of variance (ANOVA). Fertilization rate and hatching rate were subjected to arcsine transformation before analysis. The effects of RDX exposure on these endpoints at each exposure period were then analyzed with one-way ANOVA followed by Duncan's multiple range test (Statistica®, StatSoft, Tulsa, OK, USA). These analyses were performed at the level of significance of $\alpha = 0.05$. The average value for each parameter in the text and figure has been mentioned as the average of three replicates ± Standard Error. Behavioral observations were qualitatively documented and assessed.

15. Results

15.1. RDX in experimental tank

The average measured concentrations of RDX in the experimental tanks were 0±0, 0.5±0.1 and 3.2 ± 0.2 ppm in control, 0.5 and 5 ppm nominal concentration group, respectively.

15.2. Effect of RDX on weight

Two-way ANOVA (treatment X time of exposure) indicated that only treatment had an effect on weight of females ($p<0.05$); whereas time and interaction between treatment and time had significant effect on growth. Mean weight of females did not differ one week prior to RDX exposure ($p>0.05$) and RDX did not affect their weight at 2 and 6 weeks after the onset of exposure ($p>0.05$, 1-way ANOVA); however, mean weights differed at 4 weeks of exposure ($p<0.05$, 1-way ANOVA; Figure 1). The weight of females in the 0.5-ppm treatment group was significantly higher ($p<0.05$) than the 3.2-ppm group.

15.3. Effect of RDX on feeding and behavior

Fish aggregation behavior during feeding was recorded qualitatively. In the control and 0.5-ppm treatment groups, feeding activity was not affected at any point during the experiment. However, feeding activity was reduced in the 3.2-ppm female treatment group within one day of starting the exposure, and in the single male tank suppressed feeding activity was evident after one week. The effect on feeding behavior was temporary and only lasted until the end of the second week of exposure. However, males and females from the 3.2-ppm treatment group also showed aggressive (fighting and chasing) beginning after the second day of exposure, and this behavior persisted throughout the exposure (6 weeks).

15.4. Effect of RDX on packed-egg volume

Two-way ANOVA (treatment X length of exposure) indicated that treatment ($p< 0.05$) and interaction between the treatment and length of exposure ($p<0.05$), but not length of exposure ($p>0.05$) had significant effects on packed-egg volume. One-way ANOVA for treatments at each spawning period indicated that the treatment effect was significant ($p<0.05$) only at 2 weeks of exposure. RDX stimulated egg production ($p<0.05$, Duncan's multiple range test) in the 0.5-ppm treatment group at 2 weeks of exposure compared to the control and 3.2-ppm exposure group (Figure 2). Egg production seemed to decrease in the 3.2-ppm group relative to

controls, but this decrease was not statistically significant. Packed-egg volume in the 0.5-ppm treatment group subsequently decreased from its peak value at 2 weeks, and was not different from the control at 4 and 6 weeks of exposure.

Egg volumes for each treatment replicate (excluding the packed-egg volume before RDX exposure) were added to calculate the cumulative packed-egg volume in each treatment. Results of one-way ANOVA indicated that treatment had an effect on the cumulative packed-egg volume ($p<0.05$); RDX at 0.5 ppm caused a higher cumulative egg volume relative to RDX at 3.2 ppm, with a trend to be also higher than control values (Figure 3).

15.5. Effect of RDX on fertilization and hatching rate

Values for percentage fertilization and percent hatching were subjected to an arcsine transformation to achieve homogeneity of variances. Two-way ANOVA (treatment X length of exposure) showed no treatment or interaction effects ($p>0.05$) either on fertilization rate or hatching rate, but length of exposure had an effect on fertilization rate with a slight length-dependent increase being apparent in all treatment groups ($p<0.05$, Figure 4).

16. Discussion

Contamination of the environment with military waste, including RDX, has been reported in various parts of the world (Small and Rosenblatt 1974, Aller 1985, Walsh and Jenkins 1992). In water bodies, the concentration of RDX varies widely and may go up to 109 ppm (Ryon et al. 1984). These high concentrations of RDX may be causing toxic effects and adversely impacting the aquatic biota. To our knowledge, this is the first report of the effect of RDX on reproductive performance in fish. The concentrations chosen for this study (1 and 3.2 ppm verified concentration) are well within environmentally relevant concentrations.

Exposure to RDX has been shown to affect behavior and body weight in different species including zebrafish (Mukhi et al. in review). In an earlier study, we observed that RDX at 9.6 ppm affected feeding behavior temporarily. Similar results were obtained in the present study, where feeding behavior was temporarily affected by exposure of zebrafish to 3.2 ppm RDX. However, a novel finding of the recent study was the appearance of aggressive starting about 2 weeks after initiation of exposure to RDX; this behavior lasted until the end of the experiment (6 weeks). In an earlier study, we observed that the RDX at 1 ppm and 9.6 ppm reversibly reduced the weight of zebrafish after 4 weeks of exposure, with full recovery from this effect after 12 weeks of exposure. In the present study, we did not observe loss of weight in the 3.2-ppm treatment females compared to control at any point of exposure period. It is possible that the alteration in feeding behavior is not significant enough to cause a reduction in feed intake in the females. Information on the effect of RDX on reproductive performance is limited to few species.

RDX seems to be toxic to reproductive success in earthworm (*Eisenia Andrei*; Robidoux et al., 2002), enchytraeid worms (*Enchtraeus albidus*, *E. crypticus*; Dodard et al., 2005) and northern bobwhite (*Colinus virginianus*; Gogal et al., 2003). However, RDX showed no adverse reproductive effects in rats exposed to concentrations as high as 50 ppm

RDX/kg in the feed. In the present study, no deleterious effects of RDX on spawn volume were found; in fact, the volume of egg produced in the 0.5-ppm treatment group seems to have increased at 2 weeks of exposure. This observation suggests that RDX at low concentration (0.5 ppm in this experiment) has some stimulatory effect on egg production early during the period of the exposure. Moreover, the stimulatory effect of egg production was short lived. The biological relevance of this observation is uncertain at this time. Stimulatory effects of certain chemical contaminants on the reproductive performance of fishes have been previously reported; this effect has been described as "hormesis" (Calabrese and Blain, 2005).

Maternal exposure to RDX did not affect egg fertilization and embryo hatching rates. At the present time, we are unaware of information available for other species concerning effects of parental exposure to RDX on embryo development. There is also no information available to assess whether RDX can be transferred the mother to the embryo.

Overall, the results of the present study do not suggest deleterious effects on zebrafish reproductive performance of exposure to RDX at environmentally relevant concentrations. However, this study did not include measures of larval or juvenile health.

17. 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 the completion date of the study.

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19.0 Figures

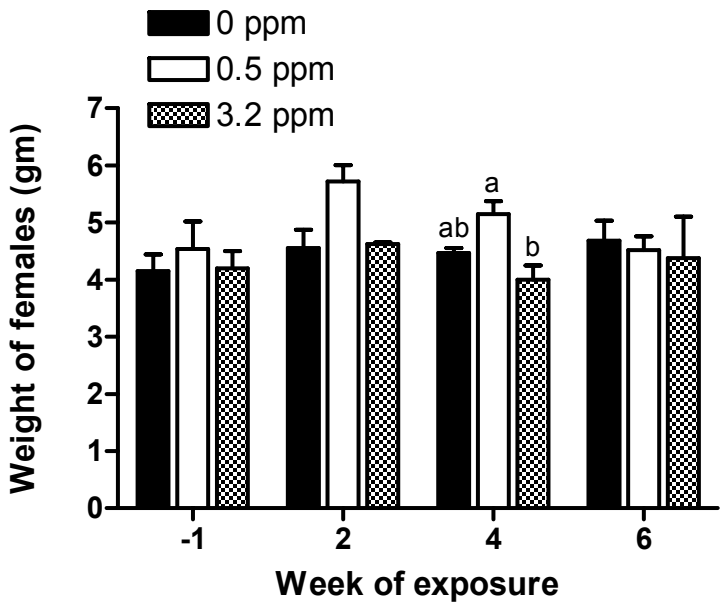

Figure 1: Effect of RDX on weight of female fish. Treatment with RDX, but not length of exposure, affected female weight (2-wat ANOVA). Bars associated with common letters are not significantly different (Duncan's multiple range test).

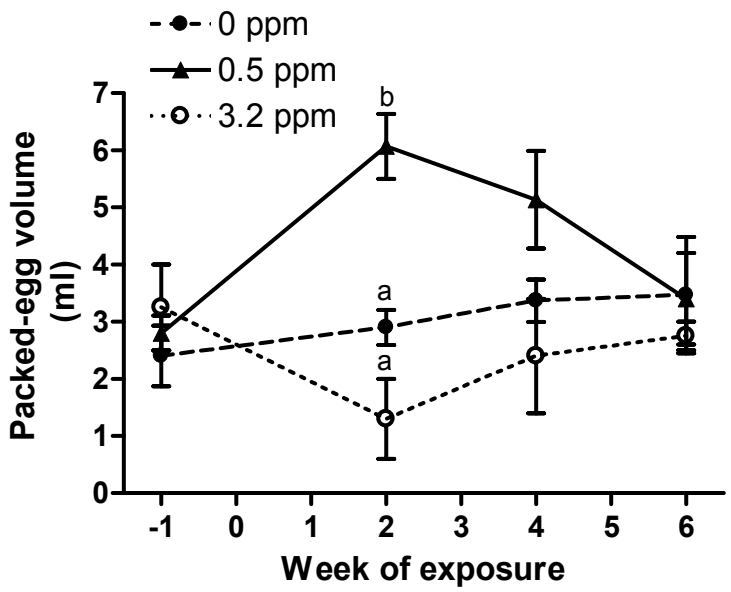

Figure 2: Effect of RDX on packed-egg volume. RDX stimulated egg production in the 0.5-ppm treatment group at 2 weeks of exposure (one-way ANOVA and Duncan's multiple range test; $p<0.05$). Although RDX reduced the egg production (almost by half) in 3.2 ppm group at 2 weeks, the effect was not significant.

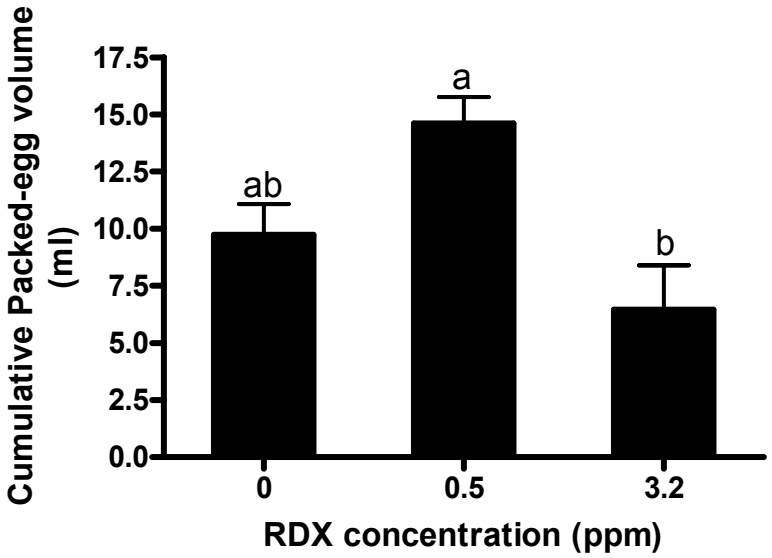

Figure 3: Cumulative effect of RDX on packed-egg volume. Cumulative packed-egg volume in the 0.5-treatment was significantly higher than 3.2 ppm group, whereas the cumulative packed-egg volume in the two RDX treatment group was not different from control (one-way ANOVA and Duncan's multiple range test; $p<0.05$).

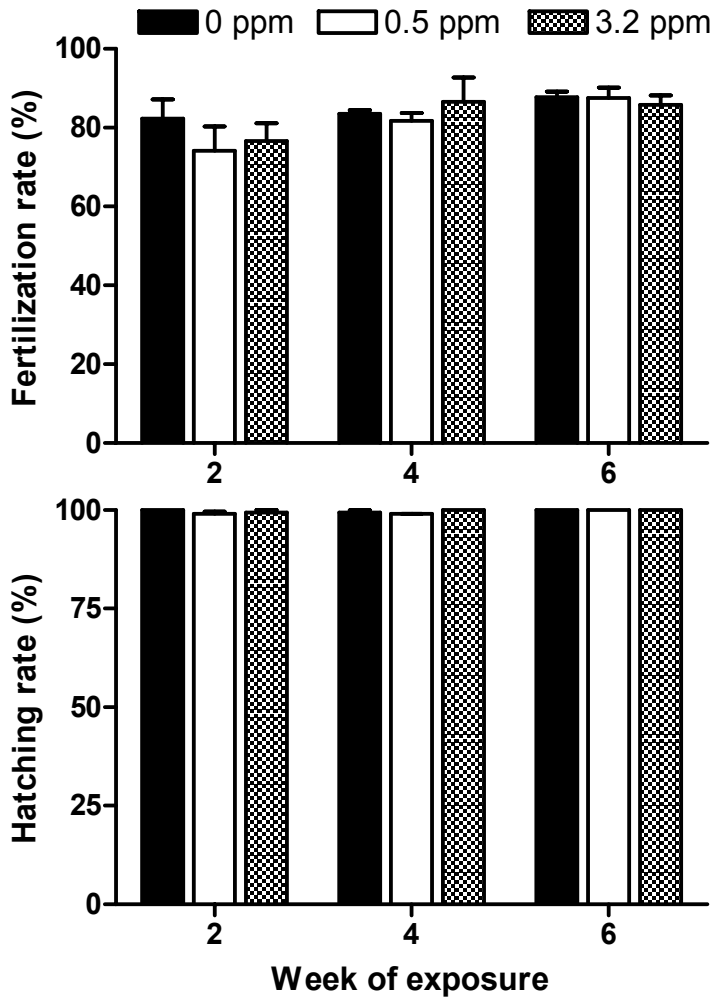

Figure 4: Effect of RDX on egg fertilization and embryo hatching rates. Parental exposure to RDX had no significant effect on these endpoints ($p>0.05$).

TITLE: Effects of explosive mixtures on Fathead Minnows and Larvae of *Xenopus laevis*

STUDY NUMBER: EXP-05-01

SPONSOR: Strategic Environmental and Research Development Program

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RESEARCH INITIATION: January 1, 2005

RESEARCH COMPLETION: December 31, 2005

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Figure 1. Mortality of Xenopus exposed to TNX +HMX. 11

GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Ernest Smith
Co-Principal Investigator

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 17, 1989.

Submitted by:

_____ _____

Ryan Bounds
Quality Assurance Manager

Date

1.0 DESCRIPTIVE STUDY TITLE:
Effects of explosive mixtures on Fathead Minnows and Larvae of *Xenopus laevis*

2.0 STUDY NUMBER:
EXP-05-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, Texas 79409

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: January 01, 2005
Termination Date: December 31, 2005

6.0 KEY PERSONNEL:
Mike Wages, Study Director
Ronald Kendall, Testing Facility Management/Principal Investigator
Ernest Smith, Co-Principal Investigator
Ryan Bounds, Quality Assurance Manager

7.0 STUDY OBJECTIVES / PURPOSE:
To determine the acute toxicity and effects of the mixtures of HMX, RDX, TNX, and TNT on growth and development of *Xenopus laevis* and fathead minnows (*Pimephales promelas*) larvae.

8.0 STUDY SUMMARY:
Xenopus laevis larvae were exposed to mixtures of RDX + HMX, RDX + TNX, and HMX + TNX in separate experiments. RDX and HMX were tested at saturated concentrations. Six concentrations of TNX were used in combination with RDX or HMX. *Xenopus* larvae were exposed to these contaminants starting at Nieuwkoop-Faber (NF) stages 8-10, and exposure was terminated at 96 hours. During the exposure and at termination, the number of dead and malformed embryos was counted. There were no effects of the combination of RDX + HMX on survival or development in this study. However, the combination of TNX +RDX and TNX+HMX resulted mortality, only at concentrations greater than 1000 ppm TNX plus saturated HMX. Similar results were observed for 1000 ppm TNX in combination with saturated RDX solution. No mortality was observed below 100 ppm TNX in combination with RDX or HMX. Based on the

lack of toxicity for the mixture of HMX and RDX (saturated solutions) and the information from the SAP at the summer 2005 meeting the evaluation of mixtures of these chemicals was not pursued any further.

Fathead minnow larvae were exposed to 7 concentrations (0.01, 0.1, 1, 10, 50, 100 and 1000 ppm) of TNX. At 10 days post-hatch, the larvae were exposed to TNX for 96 hours. During the exposure and at termination, the number of dead and malformed embryos was counted. TNX resulted in 100% mortality at 100 ppm and above. In comparison, 50 ppm resulted in 10% mortality. The significance of the mortality at 50 ppm was negated by a 20% mortality in the untreated controls. There was no mortality observed following exposure to saturated solutions of mixtures of RDX and HMX. Based on the lack toxicity at the point of saturation for HMX and RDX and the suggestion from the SAP at the summer 2005 meeting the evaluation of mixtures of these chemicals was not pursued any further for fathead minnow larvae.

9.0 TEST MATERIALS:

Test Chemical name: cyclotetramethylene-tetranitramine (HMX)
CAS number: 2691-41-0
Characterization: Determination of concentration in water samples.
Source: Aldrich Chemical Company

Test Chemical name: cyclotrimethylenetrinitramine (RDX)
CAS number: 121-82-4
Characterization: Determination of concentration in water samples.
Source: Aldrich Chemical Company

Test Chemical name: hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)
CAS number: 13980-04-6
Characterization: Determination of concentration in water samples.
Source: SRI International

Test Chemical name: trinitrotoluene (TNT)
CAS number: 118-96-7
Characterization: Determination of concentration in water samples.
Source: Aldrich Chemical Company

Reference Chemical name: FETAX medium will be prepared using de-ionized, carbon filtered water and reagent grade salts (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).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: Reverse osmosis and de-ionizer treated City tap water was used to prepare FETAX solution.

10.0 JUSTIFICATION OF TEST SYSTEM:

In natural systems, aquatic organisms are exposed to complex mixtures of contaminants. However, the toxicity of mixtures of explosive compounds has not been thoroughly examined in aquatic species. This is significant for DoD sites contaminated with explosives, because such contamination often consists of mixtures of RDX and HMX, and, when RDX breaks down, it may coexist with its metabolites (Hovatter et al. 1997, Sheremata et al.2001, Sunahara et al.1999). However, the toxicity of mixtures of explosive compounds and their metabolites has not been thoroughly examined in aquatic species.

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

Species: African clawed frog (*Xenopus laevis*) and fathead minnows (*Pimephales promelas*).

Strain: Outbred

Age: Larvae

Number: Approximately 2400 (*Xenopus*) and 200 (Fathead minnows)

Source: Xenopus were bred from captive stocks currently maintained in our laboratory and fathead minnow were obtained from a commercial vendor.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Glass Petri dishes were labeled, species name, animal use protocol number, project number, test system, and date of hatch.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Glass Petri dishes were labeled with treatment, species name, animal use protocol number, project number, test system, and date of hatch. Randomization was used to minimize bias.

14.0 METHODS:

14.1 Animal Selection and Receipt

Animals used were selected from in-house breeding colonies. Animals were selected for breeding if they were not previously bred within the last 60 days. Fathead minnows were obtained from a commercial vendor.

14.2 Assignment of Animals to Study Group and Identification

Larvae were placed into Petri dishes labeled with test chemical and concentration and identified by test group since identification of individual animals is not possible at this stage of development.

14.3 Acclimation

Xenpous larvae did not require acclimation. Fathead minnows were acclimatized for approximately 5 days.

14.4 Animal Husbandry

Animals were kept in water supplemented with 60 mg/L commercially available aquarium sea salts for adults (Goleman et al. 2001). Larvae were kept in FETAX solution, specifically formulated for the Xenopus larvae at this stage of development, according to ASTM (1998). One third of the FETAX solution was changed at least every other day. Aquaria were covered with plastic mesh to prevent escape. To breed frogs for egg production, adult males were injected with 300 and adult females were injected with 750 units of human chorionic gonadotropin dissolved in sterile water into the dorsal anterior lymphatic sac, and returned to tanks. After injection, one male and one female were placed in a 5.5 gallon aquarium with a false bottom (plastic-coated hardware cloth) to allow eggs to sink to the bottom without being eaten. Frogs were kept at a water temperature of 23º C (the preferred temperature of this tropical species) at a 12:12 h light;dark cycle. Water chemistry (pH, dissolved oxygen, ammonia, nitrate, nitrite, temperature) were monitored every other day, using a water quality meter (YSI, Inc.) and a spectrophotometic-based water quality kit (HACH, Inc.). Fathead minnows were kept in sea salt treated water without further modification.

14.5 Test Material Application

Test solutions consisted of RDX + TNX, HMX + TNX, and RDX + HMX. RDX, HMX, and RDX + HMX were dissolved in control medium (FETAX), and applied from stock solutions. Stock solutions consisted of saturated solutions of HMX or RDX. Eggs/embryo (Nieuwkoop -Faber [NF] stages 8-10, Nieuwkoop and Faber 1967) were placed into pre-cleaned Petri dishes and the hatched larvae were allowed to develop for 96 hours while being exposed to toxicants. Dishes were cleaned by washing according to SOP AQ-1-23 “Cleaning Glassware for Use with Xenopus laevis”, and all Petri dishes were baked at 250° C for 4 hours before use. For exposures, dishes were located in a Rubbermaid plastic tub in a controlled-temperature room. The arrangement of the dishes within the tub was randomized in order to avoid effects due to gradients in light and temperature in the laboratory, etc.

The overall experimental design consisted of range finding tests, in which the larvae were exposed to RDX + HMX, RDX + TNX, and HMX + TNX according to the following scheme:

TNX:

<u>Range finding tests</u>: 10 larvae/replicate x 2 replicates per treatment x 8 treatments

Treatment groups consisted of non-treated controls (FETAX), RDX-alone or HMX-alone (saturated solutions) and 6 concentrations of TNX combined with either RDX or HMX saturated solutions.

RDX + HMX
Range finding tests: 10 larvae/replicate x 2 replicate per treatment x 8 treatments
Treatment groups consisted of non-treated controls (FETAX), RDX-alone or HMX-alone (saturated solutions) and the saturated solution of RDX combined with HMX.

14.6 Food and Water Trace Contamination

Not determined.

14.7 Daily Observations

Each day, all Petri dishes were examined for dead and malformed embryos.

14.8 Euthanasia

At the end of the exposure, all animals were euthanized by immersion in 0.5 g/L MS222

14.9 Sample Collection

Tadpoles were collected at the end of exposure

14.10 Sample Analysis

TNX 1000 ppm was measured at 952.19 ppm.

RDX at water saturation was measured at 38.7 ppm.

HMX at water saturation was measured at 6.4 ppm.

15.0 PROTOCOL CHANGES / REVISIONS:

No protocol changes were made in this study

16.0 RESULTS:

Exposure to TNX at 0.01, 0.1, 1.0, 10, 100 and 1000 ppm in combination with either RDX or HMX resulted in toxic effects at concentrations above 100 ppm TNX. Similar results were observed for the combination of TNX above 100 ppm with HMX. The solutions of RDX and HMX tested in combination with TNX in this study were saturated solutions of RDX and HMX. Treated animals displayed stunted growth, edema, poor hatchability, severe malformation and mortality. In most cases the embryos did survive beyond 24 hours of hatching. Unlike the TNX, combination with either RDX or HMX, no toxic effects (neither mortality nor deformities) were observed when the animals were treated with the combination of RDX + HMX, even at the limit of RDX and HMX

saturated solutions. RDX and HMX were tested at 38.7 ppm and 6.4 ppm, respectively. This was determined to be at 100% water saturation at room temperature. It is not clear what toxic effect would be induced following longer duration of exposure to mixtures of these environmental contaminants.

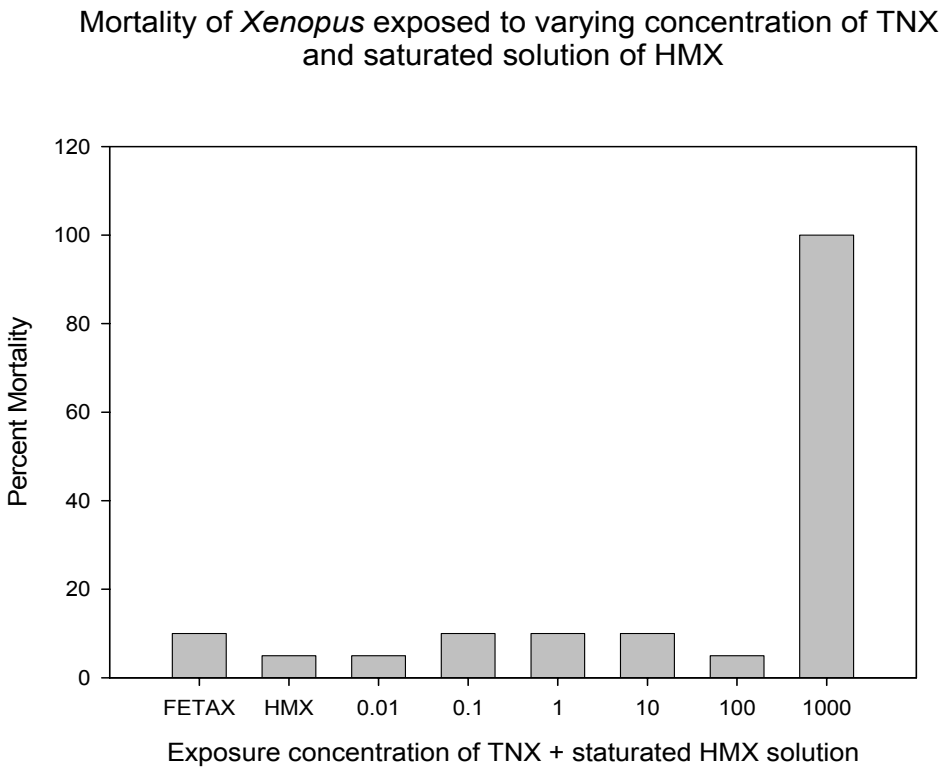

Figure 1. Mortality of Xenopus exposed to TNX +HMX.

Fathead minnow larvae were exposed to 7 concentrations (0.01, 0.1, 1, 10, 50, 100 and 1000 ppm) of TNX. The larvae were exposed to this contaminant starting at 10 days post-hatch and exposure was terminated at 96 hours. During the exposure and at termination, the number of dead and malformed embryos was counted. TNX resulted in 100% mortality at 100 ppm and above, while 50 ppm resulted in 10% mortality. The significance of the mortality at 50 ppm was negated by a 20% mortality in the untreated controls. There was no mortality observed following exposure to saturated solutions of mixtures of RDX and HMX. Based on the lack of toxicity at the point of saturation for HMX in combination with RDX and the suggestion from the SAP at the summer 2005 meeting the evaluation of mixtures of these chemicals was not pursued any further for fathead minnow larvae.

17.0 DISCUSSION:

The results presented above indicate that there is minimal hazard for RDX and HMX at environmentally relevant concentrations, at least for fathead minnows and *Xenopus*. TNX did show some acute toxicity, at levels above 100 ppm in combination with RDX or HMX. The teratogenic effects that we observed are more than likely associated with TNX since the exposure to RDX in combination with HMX did not affect the exposed animals even at the point of saturation. The "teratogenicity index" (TI = LC50/EC50), greater than 1.5 indicates a teratogenic hazard (ASTM 1998). The teratogenic hazard of TNX is 1.08, indicating no teratogenic hazard for *Xenopus* (Theodorakis, 2004). However, the concentrations of TNX in combination with RDX and MNX at which effects are seen are relatively high. Furthermore, TNX is typically found in the field in anaerobic environments. It would not be highly likely that *Xenopus* in the field would experience such concentrations. Thus, there does not appear to be an elevated hazard for this chemical for *Xenopus*. However, the question of the effects of these contaminants on native amphibian species that would be found at DoD contaminated sites requires characterization and evaluation. In addition, there are several sources of uncertainty for this hazard assessment, and these are as follows: 1) *Acute-chronic extrapolation*: It is currently unknown if there would be greater effects for longer exposures, beginning at the embryonic stage. It is also unknown if chronic exposures would lead to sublethal effects (e.g., effects on immunity, behavior, etc.), that would affect survival in the wild. 2) *Interspecific extrapolation*: The relative sensitivity of *Xenopus* compared to native frog species is currently unknown. 3) *Effects of biotransformation*: The test chemicals were exposed to young animals in this study, but older native species in the field are likely to metabolize these chemicals. It is not clear if the biotransformation effects would lead to less of the toxic compound in the animals.

18.0 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.

19.0 REFERENCES:

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Theodorakis, C. 2004. Final Report on Effects of RDX and HMX on Larvae of *Xenopus laevis* – RDX-03-01. SERDP.

TITLE: ENVIRONMENTAL MODELING

STUDY NUMBER: MOD-05-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

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

RESEARCH INITIATION: October 1, 2004

RESEARCH COMPLETION: December 31, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in compliance with Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Ken Dixon
Co-Principal Investigator

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 17, 1989.
Submitted By:

_____ _____
Ryan Bounds Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE:
ENVIRONMENTAL MODELING

2.0 STUDY NUMBER:
MOD-05-01

3.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, TX 79409-1163

5.0 EXPERIMENT START AND TERMINATION DATES:
Start Date: October 1, 2004
Termination Date: December 31, 2005

6.0 KEY PERSONNEL
Kenneth R. Dixon, Co-Principal Investigator / Study Director
Eric P. Albers, Research Assistant
Min Lian, Research Assistant
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principal Investigator

7.0 STUDY OBJECTIVES/PURPOSE
Modeling efforts utilized previously developed models to simulate the movement and effects of explosives. A suite of models has been developed to simulate the transport, uptake, and effects of explosives in terrestrial ecosystems (Figure 1). The emphasis in model development to date has been on uptake and distribution of explosives in mammal, bird, reptile, and plant species. Little information has been available on the effects of explosives on reptile species. The lab and field studies in this continuation provides data that can enhance the effects aspects of the models.

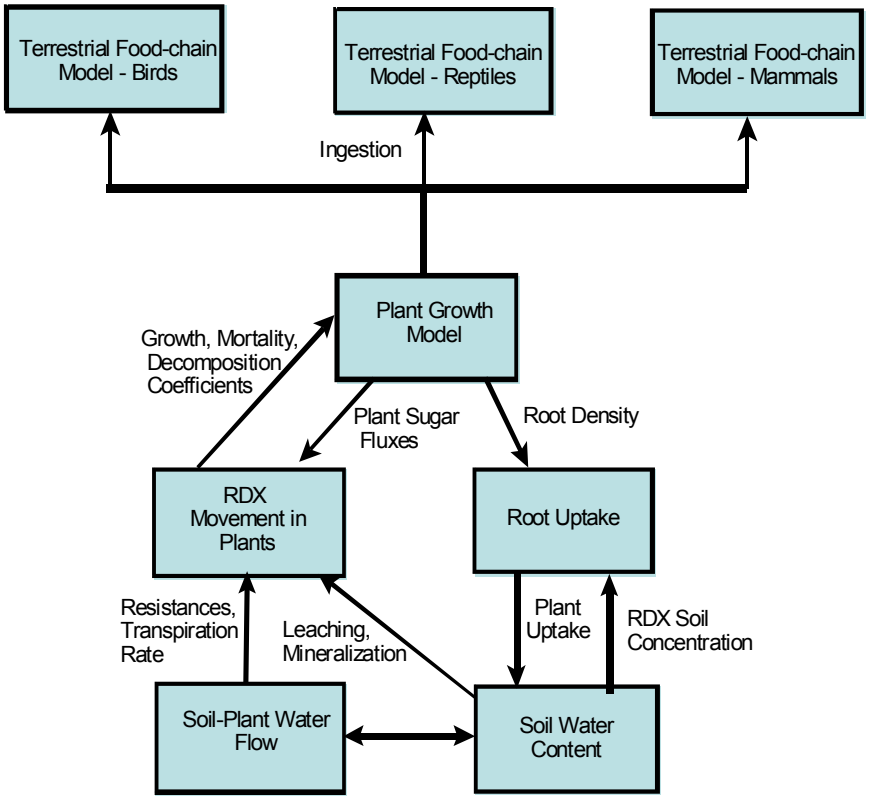

Figure 1. Flow diagram of explosives modeling suite

We also completed the implementation of the suite of models to provide for large-scale simulations, including estimates of risk for a risk assessment of explosives. This integrated suite of models will be used to assess explosives effects at different contaminated sites.

Small Mammal Model. The small mammal model was adapted from a similar model developed to predict the effects of perchlorate on thyroid activity (Dixon, et al. 2005). Mortality was added as a state variable in the model.

Bird Model. The bird model was adapted from a similar model developed to predict the effects of perchlorate on thyroid activity (Apodaca, et al. 2005). Food items for bird species and the explosives concentration in those items will be added to the model as those data become available from lab and field studies.

Plant Model. Little is known about explosives transport mechanisms in plants. Lab and field studies on explosives exposure in plants will provide data to incorporate more mechanistic transport processes in the plant models. Measured explosives concentrations in different plant

tissues will provide data for model calibration and validation.

8.0 METHODS

Small Mammal Model.

A physiologically based toxicokinetic (PBTK) model was developed to simulate the movement of explosives within small mammals. Contaminant movement was governed by a series of mass-balanced difference equations programmed in Matlab®. Model compartments and blood flow can be seen in Figure 2. Compartments in the small mammal model include blood, heart, brain, fat, kidneys, liver, gut wall, and gut contents (Figure 2). The primary environmental exposure pathway in small mammals is ingestion of explosive-contaminated food and water.

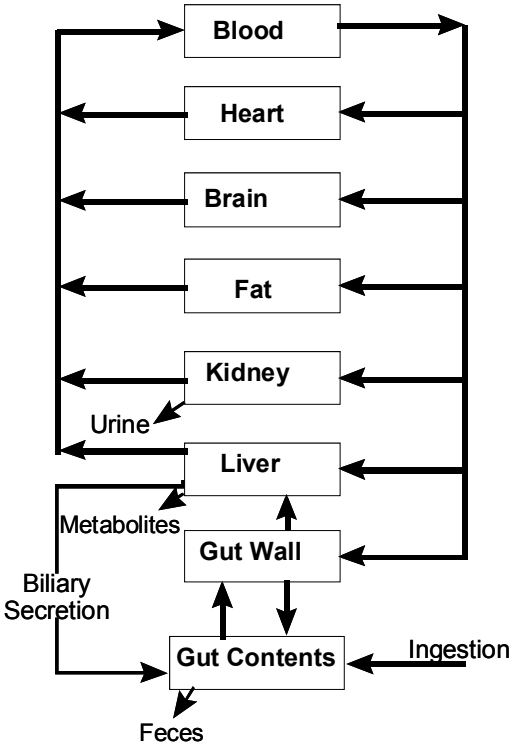

Figure 2. Flow diagram of small mammal PBPK model.

The amount of explosives distribution between venous blood and tissue concentration, $A_{i,t+1}$, is:

$$A_{i,t+1} = A_{i,t} + F_i\left(C_{vb} - \frac{C_i}{P_i}\right)$$

where,
$A_{i,t}$ is the amount of explosives in compartment i at time t, mg,
C_{vb} is the venous blood explosives concentration at time t, mg•L^{-1},
F_i is the blood flow rate into the compartment i, L•h^{-1},
P_i is the explosives partitioning coefficient for compartment i,

Explosives concentration in a compartment is the amount of explosives in the compartment divided by the mass of the compartment:

$$C_i = \frac{A_i}{m_i}$$

where m_i is the mass of compartment, g.

The amount of explosives in the gut, $A_{g,t+1}$, results from the rates of ingestion and elimination of explosives in the interval which can be described by the difference equation:

$$A_{g,t+1} = A_{g,t} + If_t + Iw_t + abs \cdot C_l - aks \cdot C_{gw} - akf \cdot C_g$$

where
C_l = explosives concentration in the liver, µg·g^{-1}
C_{gw} = explosives concentration in the gut wall, µg·g^{-1}
C_g = explosives concentration in the gut, µg·g^{-1}
$If_{i,t}$ = ingestion rate of explosives in food item i at time t, µg·g^{-1} h^{-1}
Iw_t = ingestion rate of explosives in drinking water at time t, µg·g^{-1} h^{-1}
abs = liver absorption rate constant, h^{-1}
aks = gut wall absorption rate constant, h^{-1}
akf = elimination rate constant, h^{-1}

The weight-specific mass ingestion rate of explosives in food, If_t, (µg·h^{-1}) may be written as

$$If_t = \sum_{i=1}^{m} p_i \times Cf_i \times \upsilon_i$$

where
p_i = proportion of total diet contributed by item i at time t
Cf_i = consumption rate of food item i, g·h^{-1}

v_i = explosives concentration in food item i , $\mu g \cdot g^{-1}$

Consumption rate is a function of body weight (USEPA 1993):

$$Cf = 0.398 \cdot W^{0.850}$$

where W = consumer body weight, g

Similarly, for ingestion of explosives in water, Iw_t ($\mu g \cdot h^{-1}$) is:

$$Iw_t = Cw \cdot \upsilon$$

where
Cw = consumption rate of water, $L \cdot h^{-1}$
v_i = explosives concentration in water, $\mu g \cdot L^{-1}$

Consumer body weight was modeled as simple exponential growth:

$$W = W_0 \, e^{b \cdot t}$$

where
W_0 = initial body weight, g
t = time, h
b = growth rate constant

Parameter estimation

Flow rates and tissue volumes were obtained from Brown, et al. (1997). Partitioning coefficients were calculated from data in Schneider, et al. (1978). Growth rate constants were estimated from lab data on growth of F1C deer mice dosed with varying concentrations of TNX in drinking water.

Bird Model.

A physiologically based toxicokinetic (PBTK) model was developed to simulate the movement of explosives within birds. Contaminant movement was governed by a series of mass-balanced difference equations programmed in Matlab®, similar to those in the mammal model. Model compartments and blood flow can be seen in Figure 3. Distribution was assumed to be flow-limited, i.e. chemical equilibrium existed between the tissues and blood leaving the compartment. An ingestion term was included to allow for the incorporation of multiple food sources of varying levels of toxicity.

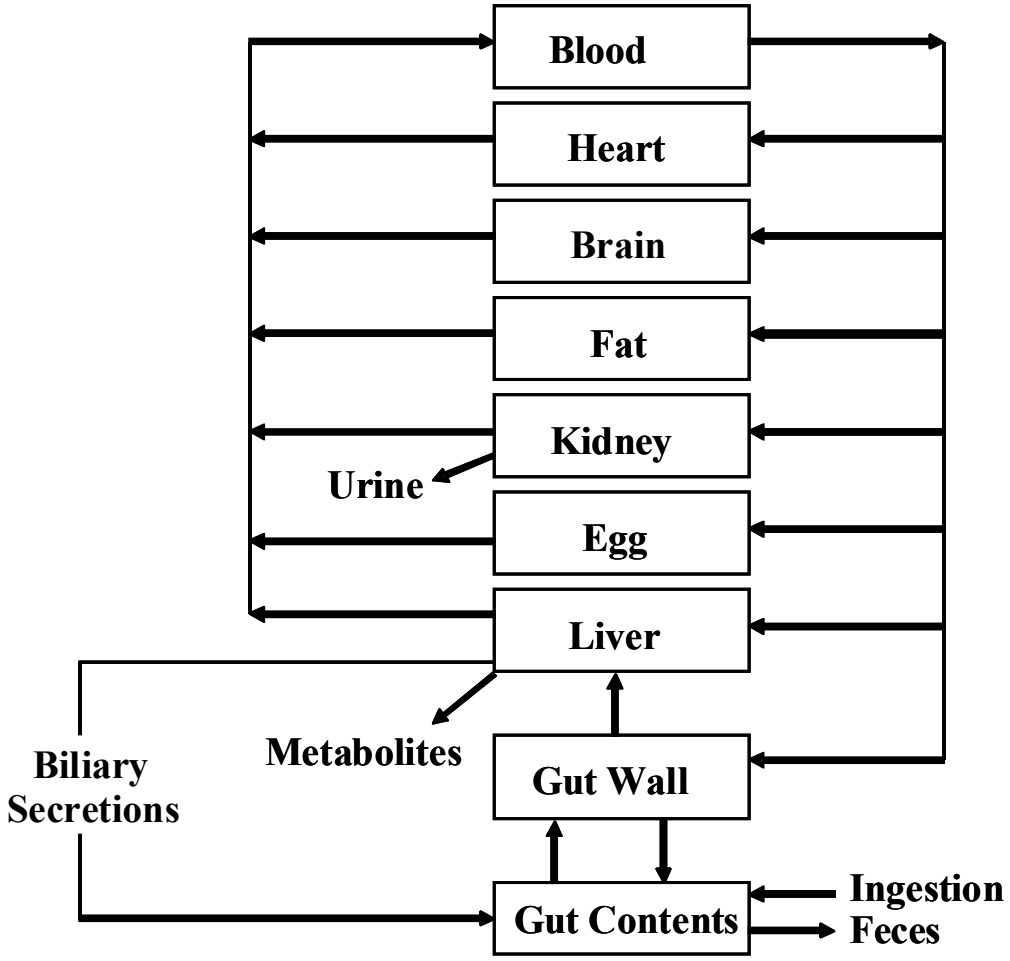

Figure 3. Flow Diagram of the PBTK model for explosives uptake in birds.

The previous model of RDX assumed that food consumption was a function of body weight (US EPA 1993). In our experiments with HMX, however, it appeared that bobwhites showed aversion to ingestion of food contaminated with RDX. Therefore, we modeled ingestion, *w*, as a

function of HMX concentration in food and body weight, *wt*, as a function of the mass of food ingested:

$$w = w_{\max}\left(1 - \frac{\nu(t - t_{\exp})}{560 \cdot \nu_{\max}}\right)$$

where
w_{max} = maximum ingestion rate
ν = HMX concentration in food, $mg \cdot kg^{-1}$
ν_{max} = maximum HMX concentration in food, $mg \cdot kg^{-1}$
t = time, h
t_{exp} = time of exposure

and

$$wt = 10^{(1.1765\log(w)+0.470)}$$

Reptile model.

A physiologically based toxicokinetic (PBTK) model was developed to simulate the movement of explosives within reptiles. Contaminant movement was governed by a series of mass-balanced difference equations programmed in Matlab®, similar to those in the bird model.

Ingestion in the reptile model differs from that in the bird model because of the less frequent feeding behaviour of reptiles. Consumption of insects, Nc_t, was assumed to be a Bernoulli random variable. The number of insects ingested was considered a Bernoulli trial in which an insect was ingested with probability p:

$$p(x) = \begin{pmatrix} 1-p & if\, x = 0 \\ p & if\, x = 1 \\ 0 & otherwise \end{pmatrix}$$

The assumption of a Bernoulli process was applied at an hourly time step in the model.

Plant Model.

The model simulates explosives uptake in terrestrial macrophytes and was programmed in Matlab using difference equations. To simulate and predict the uptake and transport of explosives in various terrestrial and aquatic plants, we developed new uptake and distribution components that are specific to explosives and modified the CERES model (Dixon, et al. 1978) by incorporating these new components (Figure 4). Additionally, an internal hydrological component was added to simulate environmental soil and water conditions.

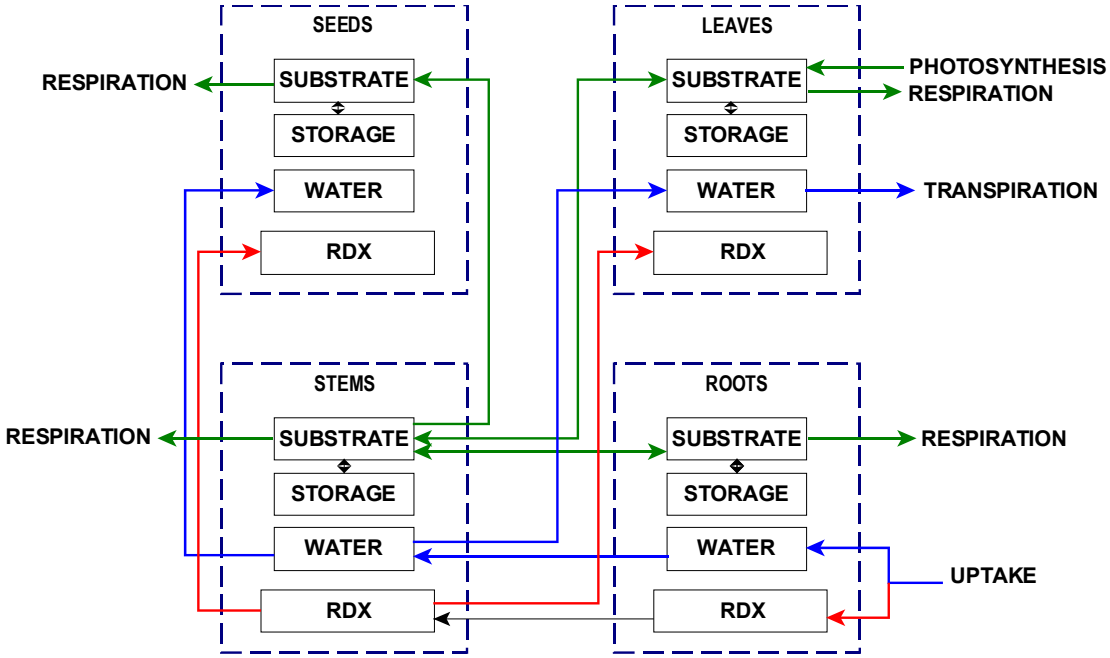

Figure 4. Flow diagram of plant uptake model

For the macrophyte model plant growth governing equations, we used those described by Dixon, et al. (1978).

The plant's water uptake is the product of the plant's ability to take up water, its leaf area, and the mass of water available to the plant in its growing soil horizon:

$$U_t^i = f \cdot L_t^{'} \cdot SM_A$$

where,

U_t^i = incremental water uptake at time t
f = water flow constant (h^{-1})
L'_t = Leaf Area Index
SM_a = Mass of water in Soil Horizon A ($g \bullet m^{-2}$ land area/hour)

Distribution of water and explosives between compartments is defined by the difference in water and explosives between compartments:

$$F_{ab} = \begin{cases} (S_a - S_b)/r_{ab} & t_1 < t \le t_4 \\ 0 & otherwise \end{cases}$$

where,
F_{ab} = flux from compartment a to compartment b ($g \bullet m^{-2}$ land area/hour)
S = amount of water in a given compartment ($g \cdot m^{-2}$ land area)
r_{ab} = water flux constant
t_1 = starting day of the growing season
t_4 = ending day of the growing season

The amount of explosives in individual compartments is defined by:

$$M_a = F_{ab} \cdot C_{\mathrm{RDX}}$$

where

M_a = mass of explosives in compartment a at time t ($\mu g \bullet m^{-2}$ land area)
F_{ab} = flux of water between the two involved compartments
C_{RDX} = RDX concentration in the incoming water

The ratio of the amount of explosives in the compartment to the biomass (wet weight) of the compartment determines the explosives concentration:

$$Q_{a,t} = \frac{M_{a,t}}{B_{a,t} + W_{a,t}}$$

where,
$Q_{a,t}$ = concentration of explosives in compartment *a* at time *t* (µg•g^{-1})
$M_{a,t}$ = amount of explosives in compartment *a* at time *t* (µg•m^{-2})
$B_{a,t}$ = biomass in compartment *a* at time t (g•m^2)
$W_{a,t}$ = mass of water in compartment a at time *t* (g•m^2)

Plant biomass is calculated by summing the soluble and insoluble photosynthate fractions (Dixon, et al. 1978):

$$B_a = S_a + ST_a$$

where,
$B_{a,t}$ = biomass of compartment *a* at time *t* (g•m^2)
$S_{a,t}$ = sugar substrate in compartment *a* at time *t* (g•m^2)
$ST_{a,t}$ = plant storage tissue in compartment *a* at time *t* (g•m^2)

For cattails, we added a term for uptake of explosives from standing water into plant stems:

$$M_{stem} = c \cdot (C_{\mathrm{RDX}} - Q_{stem}) \cdot B_{stem}$$

Model Assumptions:

- transport between leaves and stems occurs from the time of bud formation to the time of abscission.
- transport between stems and fruits occurs from the time of net photosynthesis to the time of abscission.
- transport between the stems and roots is assumed to occur throughout the year.

9.0 RESULTS

Small Mammal Model.

The earlier small mammal model was calibrated using data on the RDX distribution in different organs in the rat (Schneider, et al. 1978). The current model used laboratory data on chronic ingestion of TNX saturated drinking water at 1, 10 and 100 µg/L *ad libitum* by deer mice. The F1C generation showed a decreased rate of growth with increased TNX concentrations. The model predicted similar effects on growth (Figure 5). The lab data also showed accumulation of TNX in liver tissues in F1C deer mice exposed to 10 and 100 µg/L (Figure 6). Model predictions were within 95% C.I. of the mean (Figure 6).

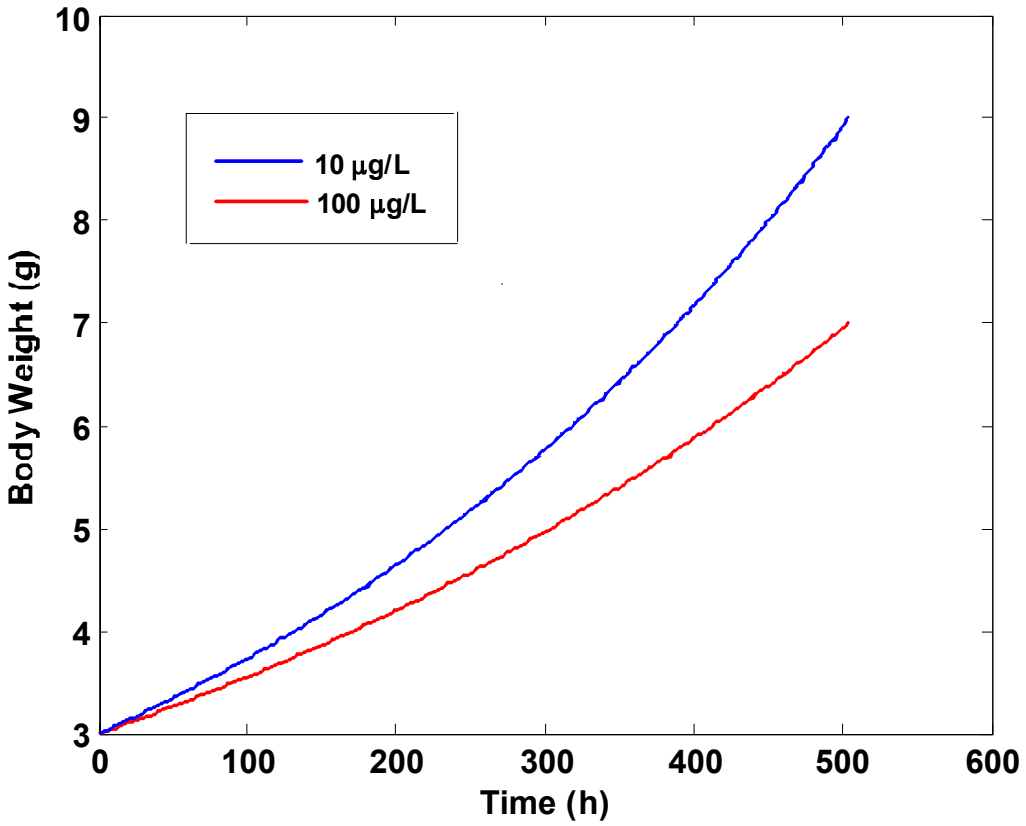

Figure 5. Predicted mean deer mouse body weights following exposure to TNX concentrations of 10 µg/L (blue) and 100 µg/L (red) in drinking water.

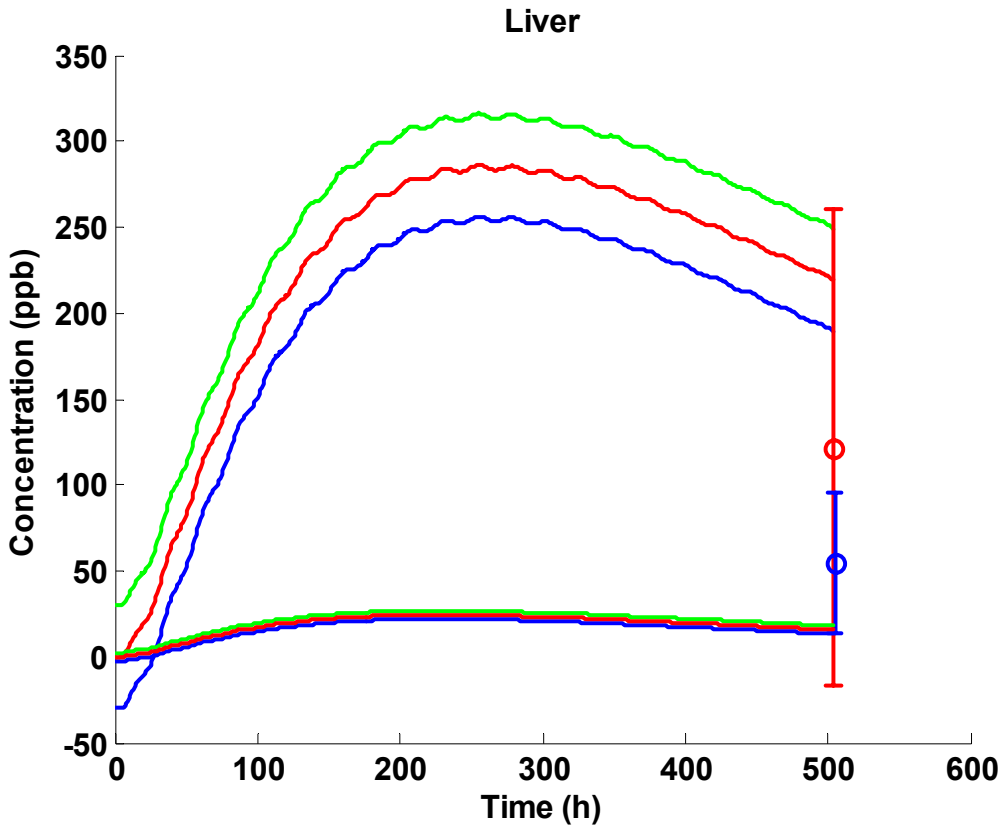

Figure 6. Observed and predicted liver TNX concentrations in deer mice. Predicted red lines are means and green and blue lines are upper and lower 95% confidence limits respectively. Red and blue circles with error bars are observed means ±S.E.

Note that the TNX concentrations increase over time with ingestion of contaminated drinking water and then decrease as body weight (and liver weight) increase.

Bird Model.

The previous model was calibrated using data from Gogal, Jr., et al. (2003). The model was calibrated further using data collected in the laboratory. In this lab experiment, quail were exposed to feeding mixtures of HMX ranging from 0 to 250 mg/kg. No birds died over the course of the feeding trial and no birds died in the simulation. Quail eggs had mean and standard error HMX concentrations of 1.48±0.30, 6.63±1.54, and 13.95±4.52 µg/g following ingestion of food at nominal concentrations of 25, 125, and 250 mg/kg, respectively. Predicted egg concentrations showed similar concentrations (Figure 7). Quail decreased food consumption and lost weight with increasing HMX exposure. At the 250 mg/kg exposure level, the average weight loss was approximately 40g. In the simulation at 250 mg/kg, predicted weight loss was of 40g occurred after 7 days of exposure starting at day 7 (hour 168) (Figure 8). Food consumption followed a similar pattern because body weight is a function of food consumption (page 12) (Figure 9).

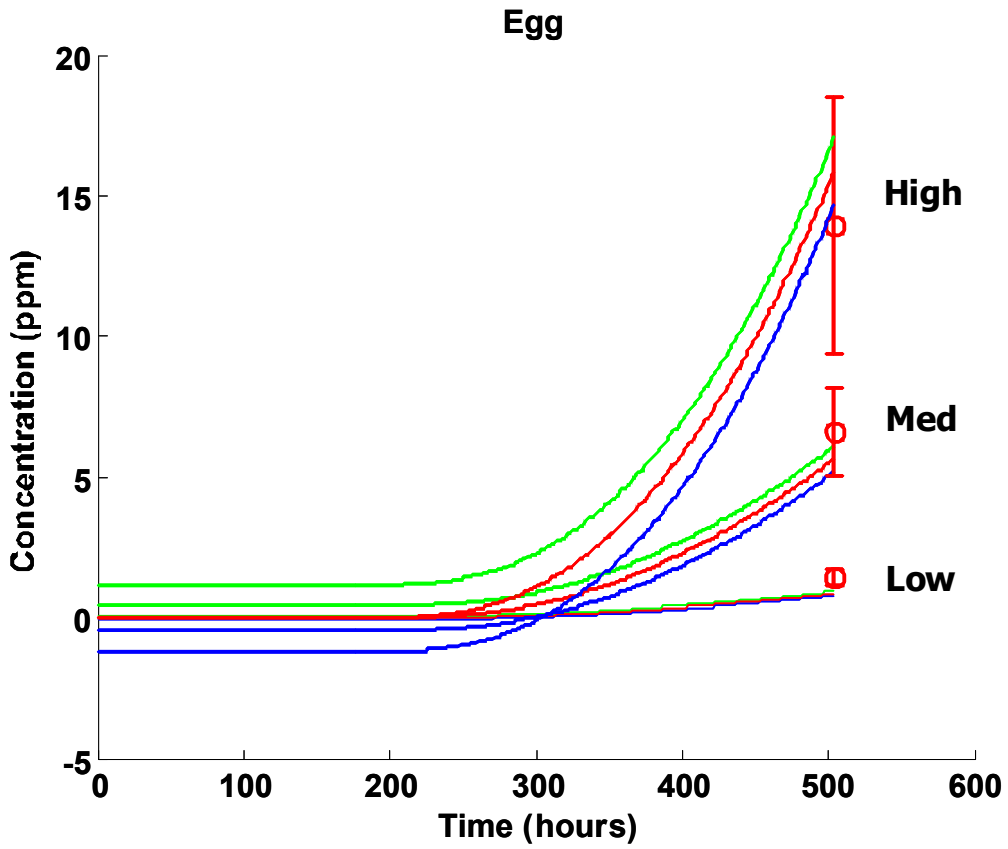

Figure 7. Observed and predicted HMX concentrations in bobwhite quail eggs resulting from ingestion of food with HMX concentrations of 25 (low), 125 (med), and 250 (high) mg/kg. Predicted red lines are means and green and blue lines are ± S.E.

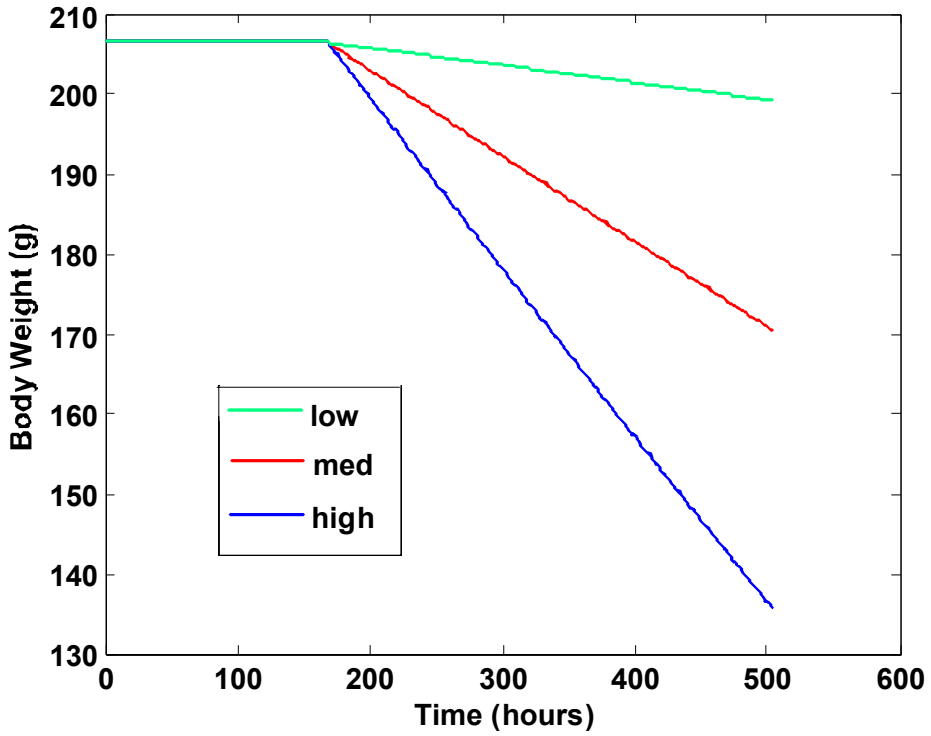

Figure 8. Predicted body weight (g) in a bobwhite with initial weight of 210 g following exposure to nominal HMX concentrations of 25 (low), 125 (med), and 250 (high) mg/kg in food starting at day 7 (hour 168).

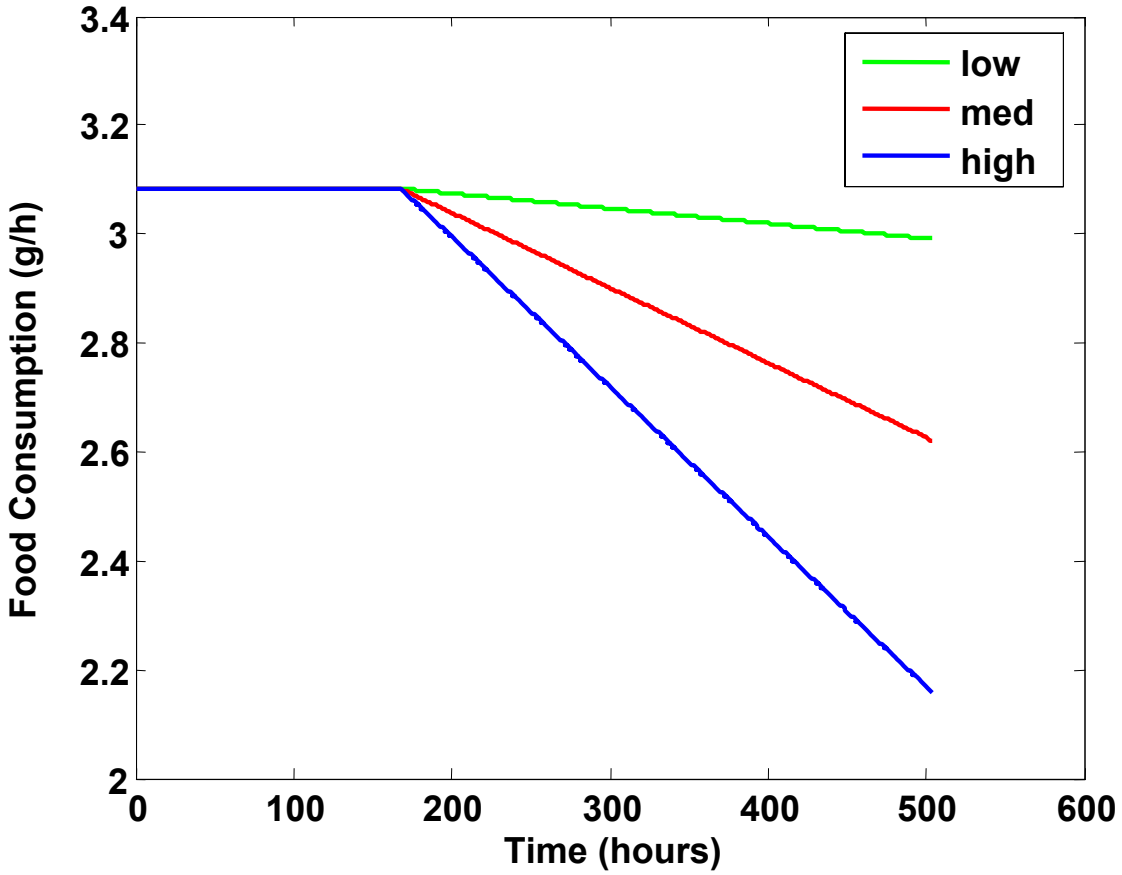

Figure 9. Predicted food consumption (g/h) in a bobwhite with initial body weight of 210 g following exposure to RMX concentrations of 25 (low), 125 (med), and 250 (high) mg/kg in food starting at day 7 (hour 168).

Reptile Model.

The model was calibrated using data collected in the laboratory. In this lab experiment, anoles were exposed to dosed crickets at a rate of 3 crickets per week. Crickets were dosed at 0, 500, and 1000 mg/kg. Using an estimate of an LD50 of >2000 mg/kg, no birds died in the simulation. Anole eggs had mean and standard error HMX concentrations of 0.637±0.048, 1.954±0.356, and 5.077±4.105 µg/g following ingestion of crickets by the adults at nominal concentrations of 0, 500, and 1000 mg/kg, respectively. Predicted egg concentrations showed similar concentrations (Figure 10).

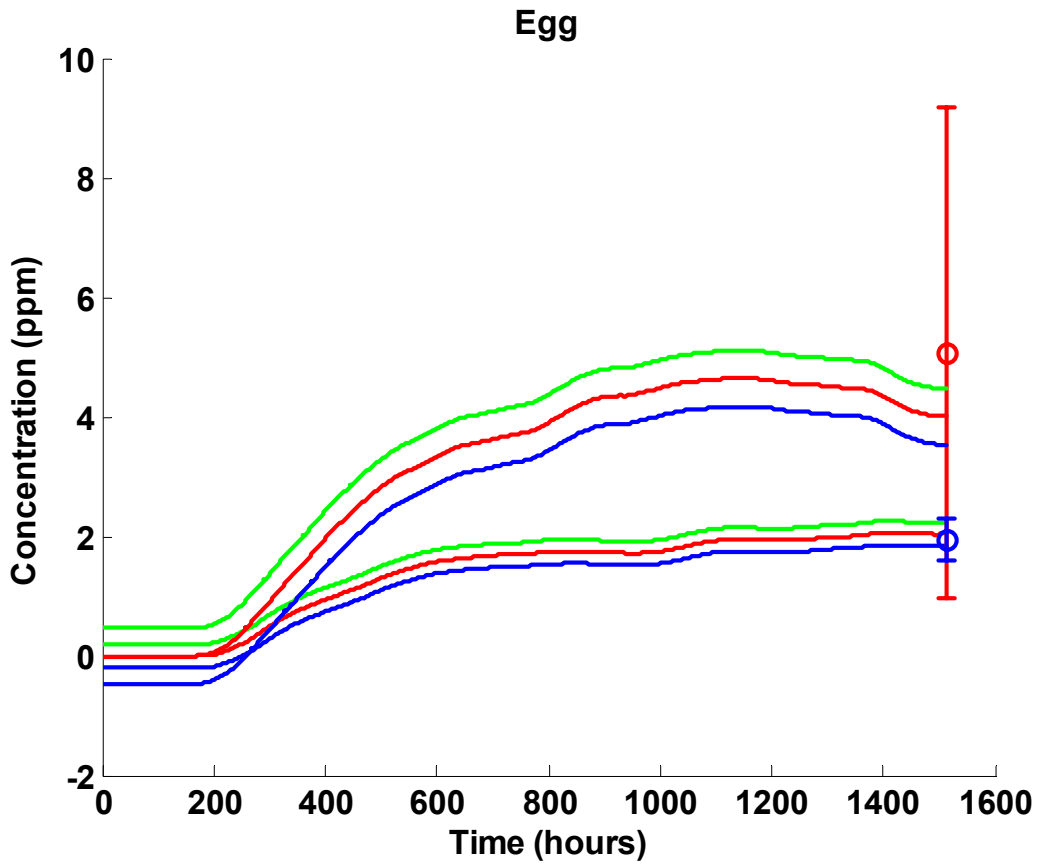

Figure 10. Predicted HMX concentration in anole eggs following exposure to HMX dosed crickets at concentrations of 500 and 1000 mg/kg starting at day 7 (hour 168). Predicted red lines are means and green and blue lines are ± S.E.

Plant Model.

The original plant model was calibrated using data on RDX uptake by tomato plants (Price et al. 2002). The current model used data on uptake of RDX in cattails in mesocosms following added influent at 1, 5, or 10 ppm RDX. Measured and predicted stem concentrations following 5 ppm influent showed similar values (Figure 11). The predicted explosives concentration in stems was adjusted to fit the observed data by reducing uptake from the water and reducing the flow into leaves and fruits. At the 10 ppm influent concentration, however, the model predicts a lower concentration than the reported mesocosm values (Figure 12).

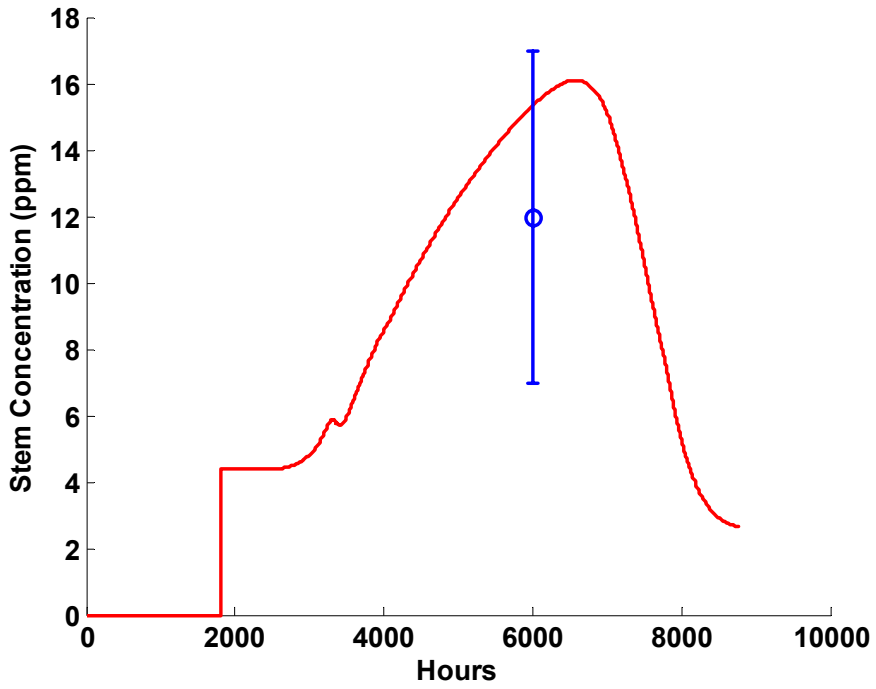

Figure 11. Observed and predicted RDX concentration in cattail stems (in red) following influent loading rate of 5 ppm. Observed concentration shown as mean ± standard error (in blue).

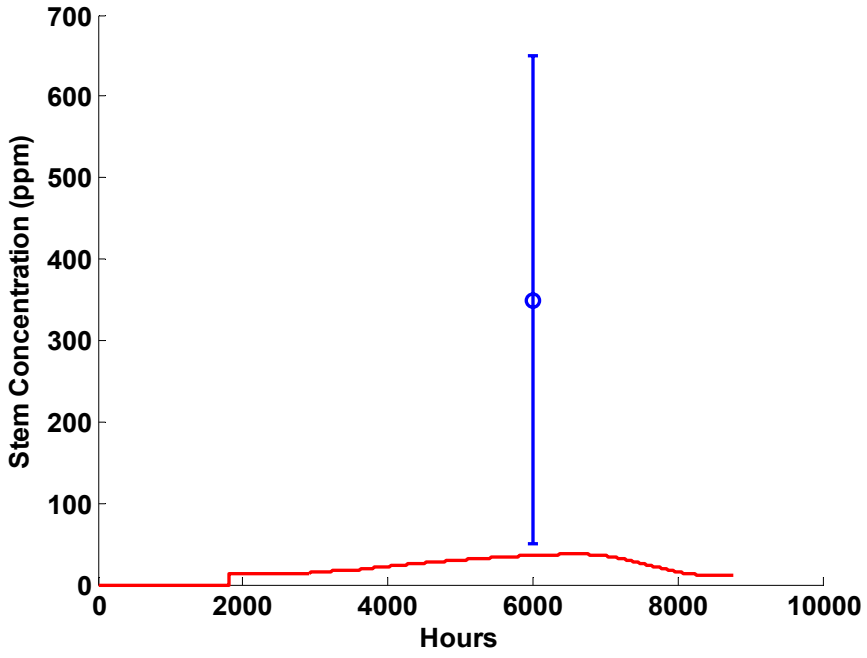

Figure 12. Observed and predicted RDX concentration in cattail stems (in red) following influent loading rate of 10 ppm. Observed concentration shown as mean ± standard error (in blue)

10.0 DISCUSSION

Small Mammal Model.

The predicted deer mouse liver concentrations of TNX fall within the 95% C.I. of the observed values. The variability of the observed data, however, is quite high. The C.I. of the higher dose rate (100 µg/L) completely contains that for the lower dose rate (10 µg/L). Further calibration of the model should await additional lab data.

Bird Model.

Calibration of the initial bird model was based upon RDX data from Gogal, Jr., et al. (2003). The current model was calibrated further using lab data on HMX with good agreement between observed and predicted values. Appropriate data on egg partitioning coefficients, excretion rates, and feeding avoidance are needed.

Reptile Model.

There was good agreement between observed and predicted concentrations of HMX in anole eggs. There was high variability associated with the high dose levels (1000 mg/kg) with the mean ± SE completely containing that of the lower dose level (500 mg/kg). This high variability demonstrates the need for larger sample sizes for future calibration efforts.

Plant Model.

The vascular plant model was developed under the assumption that water is the driving force behind the uptake and distribution of explosives in plants. Because the model under predicts tissue concentrations at the high influent loading rate (10 ppm), and the variability is quite high, additional data are needed for further calibration. The observed data suggest a nonlinear dose response of plant concentration to influent loading rate. The mechanism for this response needs to be identified before it can be incorporated into the model. Although parameter estimates were based on calibration with lab data, direct parameter estimation may improve the accuracy of the model predictions.

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TITLE: Developmental Response of Larval *Xenopus laevis* to TNX

STUDY NUMBER: TNX-05-01

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RESEARCH INITIATION: April 28, 2005

RESEARCH COMPLETION: December 31, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in compliance with Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Angella Gentles
Co-Principal Investigator

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 17, 1989.

Submitted By:

_____ _____

Ryan Bounds Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE:
Developmental Response of *Xenopus laevis* to TNX

2.0 STUDY NUMBER:
TNX-05-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite
Arlington, VA 22203

4.0 TESTING FACILITY NAME & ADDRESS:
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Texas Tech University
Texas Tech University Health Sciences Center
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Lubbock, TX 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: April 28, 2005
Termination Date: December 31, 2005

6.0 KEY PERSONNEL:
Angella Gentles, Co-Principal Investigator
Mike Wages, Study Director
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principal Investigator and Testing Facility Management

7.0 STUDY OBJECTIVES / PURPOSE:
To assess gonadal development in *Xenopus laevis* in response to TNX exposure, evaluating histological end points of selected organs.

8.0 STUDY SUMMARY:
In this study, *Xenopus laevis* metamorphs were exposed to TNX for 30, 60, 90 and 120 days. The juveniles were euthanized in bicarbonate buffered MS-222. Subsequently they were weighed and their snout-vent length and hind limb length measured, and the gonads sectioned for histologic evaluation. TNX had no effect on the gross parameters measured in juveniles exposed to the chemical for 30, 60, 90, or 120 days. Histologic analysis of the gonads indicated that TNX has the potential to retard development of the gonads - the number of cohorts of sperm and spermatogonial cells were significantly different between control and treated groups.

9.0 TEST MATERIALS:
Test Chemical name: hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)

CAS number: 13980-04-6
Characterization: Determination of concentration in water samples.
Source: SRI International

10.0 JUSTIFICATION OF TEST SYSTEM:

This project is intended to evaluate risks of TNX exposure in developing *Xenopus laevis*. Xenopus were used because they are easily handled, permit large sample sizes, and are more economically feasible in a study of this nature. They are suitable models for assessing exposure risks in an ecological environmental setting.

This study cannot be substituted with culture or computer generated models. Culture and computer models cannot simulate physiological and in addition it would not provide pertinent scientific data for future use in risk assessment.

11.0 TEST ANIMALS:

Species:	*Xenopus laevis*
Strain:	Wild type
Age:	larvae
Sex:	Males and females
Number:	400
Source:	Xenopus Express

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Individual identification of the animals was not possible because of their size, however, each beaker 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.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The study consisted of 4 treatment groups, namely, control, 100ppb, 500ppb and 1000ppb TNX. There were 5 replicates for each treatment group and each replicate had 20 animals. The 90-day study had 4 replicates.

14.0 METHODS:

14.1 Test System acquisition, quarantine, and acclimation.

The juveniles were purchased from Xenopus Express (Tampa, Florida).

14.2 Assignment of Animals to Study Group and Identification

1. The frogs were allowed to acclimate for more than 7 days before the study was initiated. They were kept in FETAX medium in a large aquarium prior to the study. On the day exposure began, the frogs were removed with a net, then

weighed and their snout-vent length measured. They were then randomly placed in one of 20 beakers containing 2L of FETAX. After all the beakers had 20 animals, a pipette was used to add the appropriate amount of TNX solution to give the required concentration of TNX for exposure.
2.
3. The frogs were kept on a 12:12hr light :dark cycle and juvenile frog brittle from NASCO was supplied every other day. One half of the FETAX medium was changed every 3 days. Water quality was checked once per week. Record of dissolved oxygen, temperature, pH, conductivity and ammonia levels were measured and recorded.
4.

14.3 Test Material Application

Rates/concentrations: The frogs were exposed to TNX at concentrations of 0, 100, 500, and 1000 ppb.

Frequency: Test substance was supplied continuously in FETAX medium.

Route/Method of Application: The animals were exposed in a static renewal aquatic system.

14.4 Daily Observations

Animals were monitored daily for changes in general health.

14.5 Animal Euthanasia and Sample Collections

At the end of TNX exposure the tadpoles from each tank were removed using a fish net. They were placed in a beaker containing bicarbonate buffered MS-222 for euthanasia. They were weighed, and their hind limb and snout-vent length measured after which they were placed on dry ice and later stored in the -80C freezer or in Bouin's solution for 48hrs then transferred to 70% alcohol. The tissues were processed routinely for histology by dehydrating serially in increasing concentration of alcohol, clearing in xylene and infiltrating with paraffin followed by embedding in paraffin. The tissue was cut at 7µ and stained with hematoxylin and eosin. The gonads were inspected then for gross abnormalities.

14.6 Testosterone analysis

Testosterone was extracted from the frog tissue by homogenizing the whole body in 5ml phosphate buffered saline per gram of tissue. The homogenate was then centrifuged at 200g at 4C for 10 min. Then 1mls of the supernatant was removed and added to 2mls of ethylacetate. This mixture was then vortexed vigorously for 1hr. 1ml of the ethyl acetate was then removed and dried under nitrogen. Phosphate buffered saline (200 µl) was added to the dried sample. 50µL of each of these samples was analysed in duplicate according to the manufacturer's procedure. Briefly, the 1ml of radioactive testosterone was added to each sample

and standard in the antibody coated polypropylene tubes provided. This mixture was vortexed and incubated at 37C for 3hrs. The solution was then decanted and the tubes tapped dry. Radioactivity was measures on a gamma counter.

14.7 Evaluations

The following data were collected; mortality rate, snout-vent length, hind limb length and body weight. Histology was also done to assess the spermatogenic cell cycle in the gonads of animals exposed for 120 days. Testosterone levels were measured in the males.

14.8 Statistics

The endpoints listed in section 14.7 above were analyzed by ANOVA and Tukey's post-hoc test using MiniTab (Ver 3.0). The p value was set at $p < 0.05$.

15.0 RESULTS and DISCUSSION

Results

In this study, late metamorphic *Xenopus laevis* were exposed to TNX for periods of 30, 60, 90, and 120-days. At the end of each period the frogs were euthanized then they were weighed, and their hind limb and snout-vent length measured. The whole body level of testosterone was measured in male frogs, and the gonads of both sexes were assessed for changes in spermatogenic cells in animals that were exposed for 120 days. The 120-day animals were analyzed for whole-body TNX concentration. TNX was not detected in the control animals but was found to be 40, 228, and 615 ppb for the low, medium and high exposure groups, respectively.

Thirty-day Exposure

There were no significant differences in body weight, hind limb or snout-vent length. The absolute weight change and growth of hind limb over the period of exposure were also assessed. There were no statistical differences in any of these parameters.
See Fig. 1.

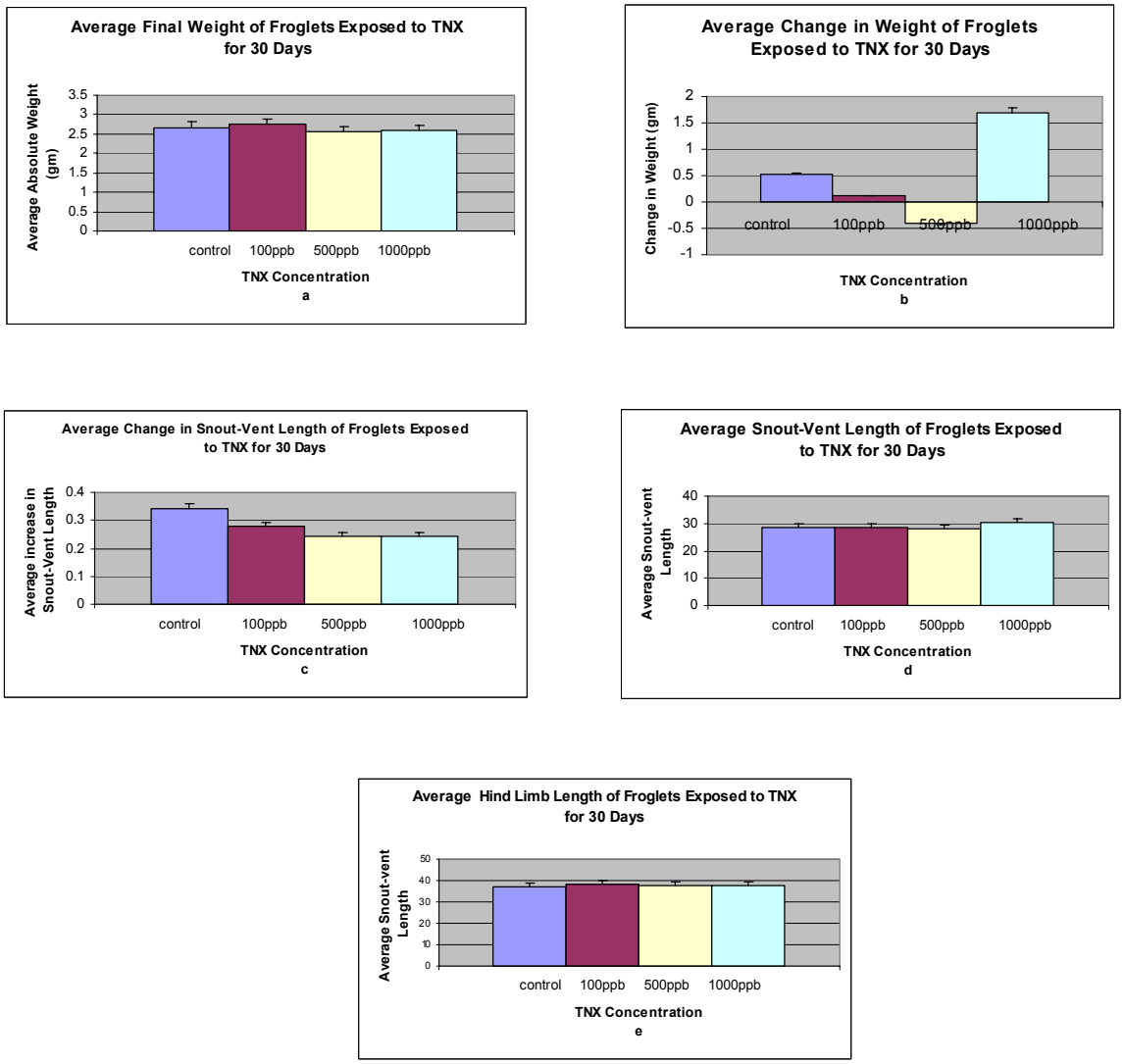

Figure 1. The effect of TNX on (a) body weight, (b) change in body weight, (c) snout-vent length, (d) change in snout-vent length, and (e) hind limb length in juvenile *Xenopus laevis* exposed for 30 days.

Sixty-day Exposure

There were no significant differences in body weight, or hind limb or snout-vent length. The absolute weight change and growth of hind limb over the period of exposure were also assessed. There were no statistical changes in any of these parameters. See Fig. 2.

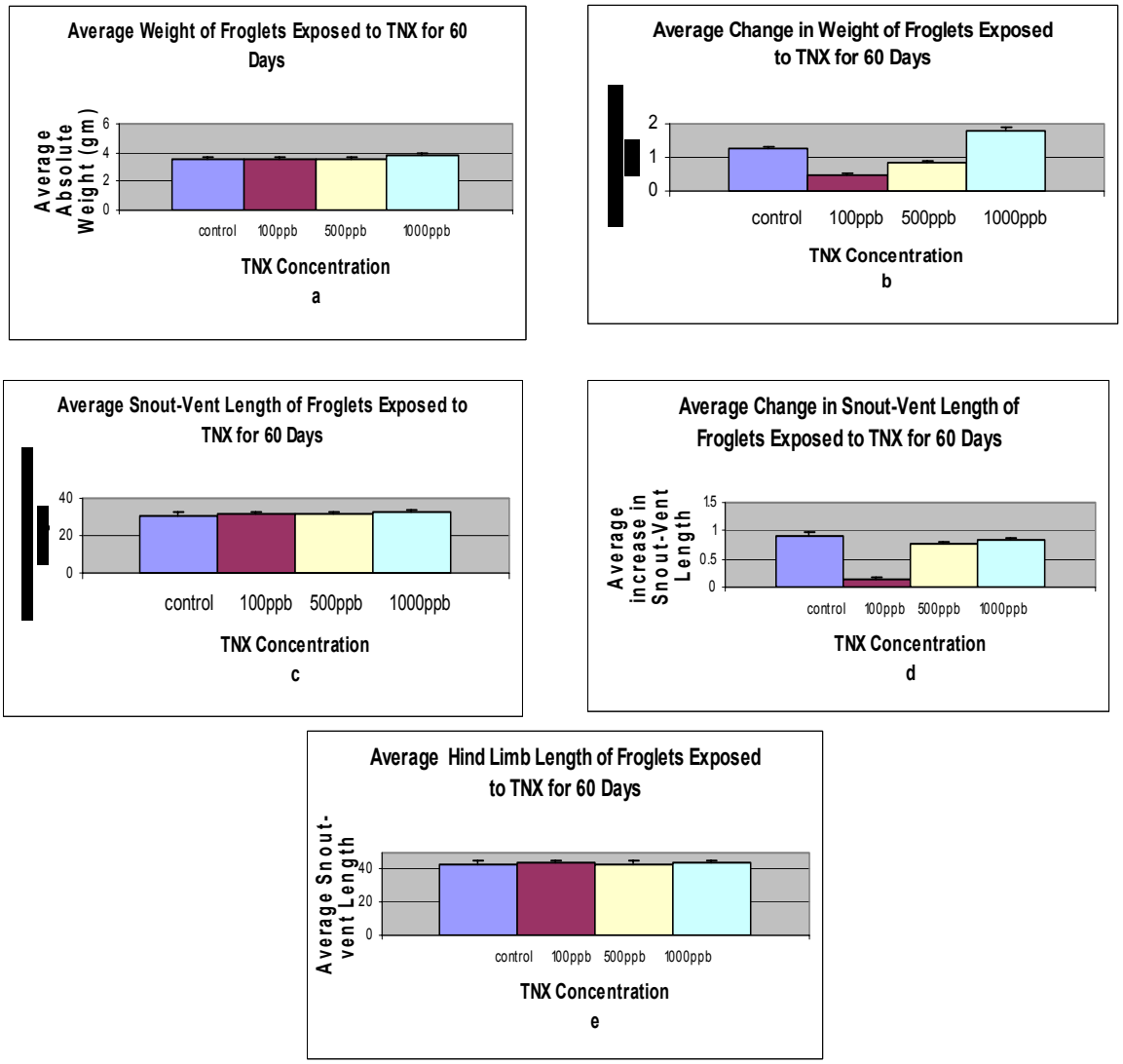

Figure 2. The effect of TNX on (a) body weight, (b) change in body weight, (c) snout-vent length, (d) change in snout-vent length, and (e) hind limb length in juvenile *Xenopus laevis* exposed for 60 days.

Ninety-day Exposure

There were no significant differences in body weight, or hind limb or snout-vent length. The absolute weight change and growth oh hind limb over the period of exposure were also assessed. There were no statistical changes in any of these parameters. See Fig. 3.

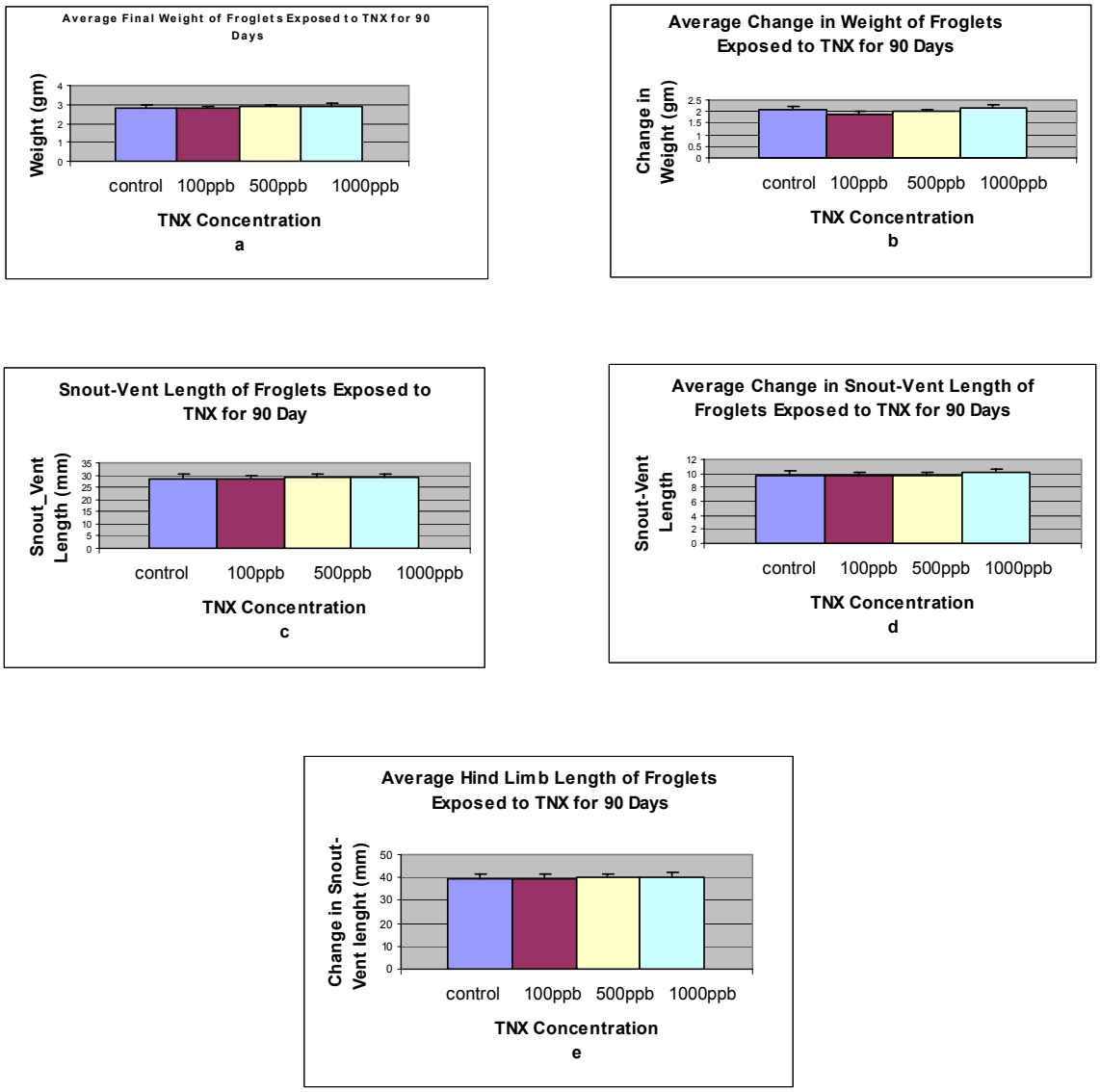

Figure 3. The effect of TNX on (a) body weight, (b) change in body weight, (c) snout-vent length, (d) change in snout-vent length, and (e) hind limb length in juvenile *Xenopus laevis* exposed for 90 days.

One hundred twenty-day Exposure

There were no significant differences in body weight, or hind limb or snout-vent length. See Fig. 4.

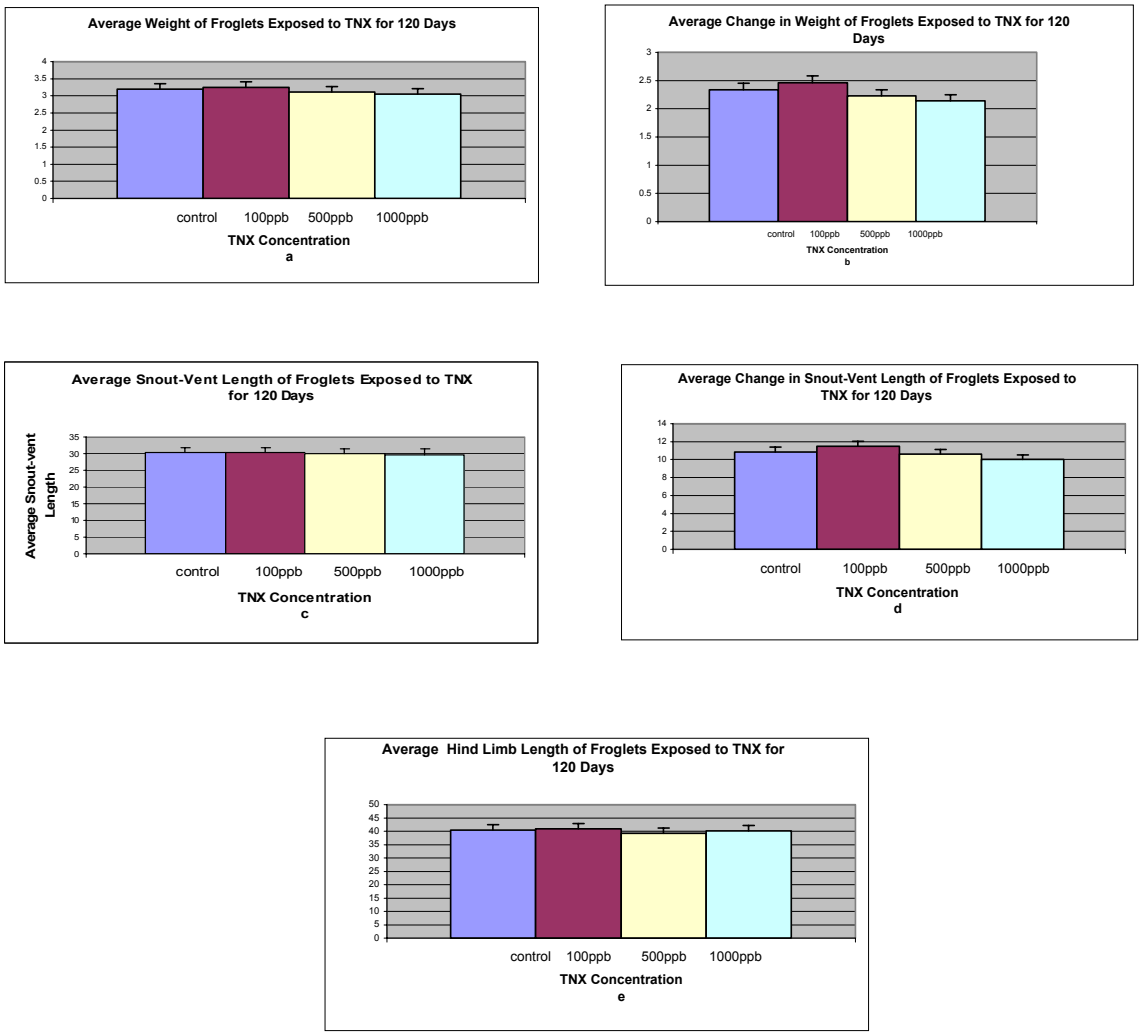

Figure 4. The effect of TNX on (a) body weight, (b) change in body weight, (c) snout-vent length, (d) change in snout-vent length, and (e) hind limb length in juvenile *Xenopus laevis* exposed for 120 days.

Exposure to TNX for 120 days resulted in a significant increase in the relative percentage of the number of cohorts of sperm and spermatogonial cells ($P < 0.05$). Relative to the control group, TNX did not cause any significant change in the clusters of spermatocytes, however; the 100ppb and 500ppb groups differ significantly from each other. Exposure to TNX did not cause any significant difference in spermatid clusters among the groups. Representative animals in each treatment group had testes with cells representing each stage of spermatogenesis, as well as some testes that were deficient in cells from the later stages of spermatogenesis. Testosterone was detected in only four animals. The ovaries were assessed qualitatively for developmental stage. There were no significant differences observed among the control and the treated groups.

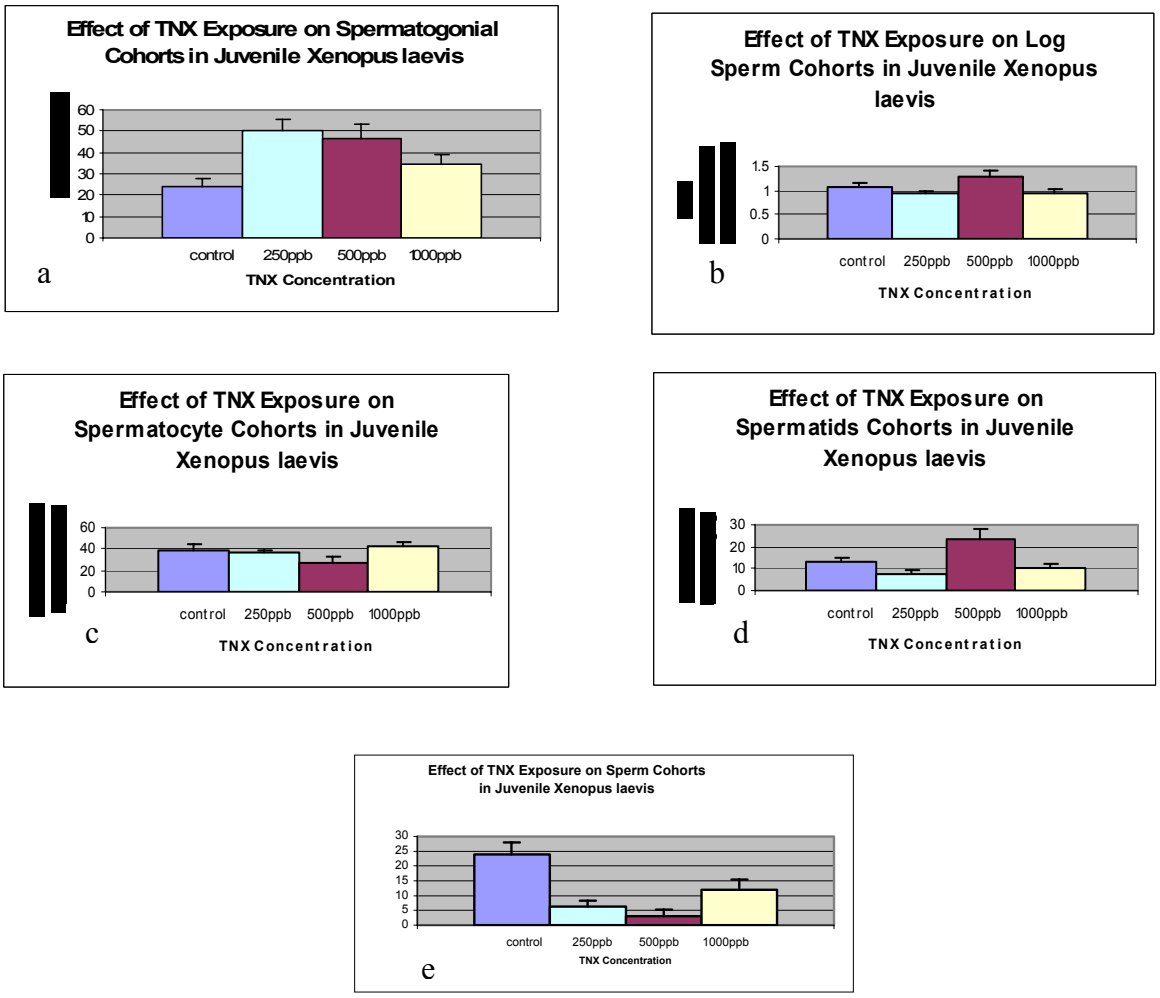

Figure 5. The effect of TNX on (a) percent spermatogonia, (b) log of percent sperm, (c) percent spermatocyte, (d) percent spermatid, and (e) percent sperm cohorts *Xenopus laevis* exposed for 120 days.

Discussion

The results indicate that the whole body concentration of TNX in frogs exposed to TNX for 120 days was concentration-dependent. In fact, the level of TNX in these animals was approximately a half that of the exposure concentration. It is uncertain if this unexpectedly high concentration of TNX in the animals was actually due to absorption or a result of external residual TNX from the medium taken up by the skin. Though there is some literature on bacterial aerobic and anaerobic degradation of RDX and some of its metabolites, it is still unknown whether TNX further metabolizes to other compounds. This large residual concentration in the body suggests that TNX is or might be absorbed faster than its excretion and/or metabolism. Interestingly, Smith et al., 2006 reported liver-levels of TNX that were approximately one half that of the concentration fed to mice over an extended period of time.

TNX at the concentrations used in this study, did not cause any changes in the body weight, snout-vent length or hind limb length of frogs exposed to the chemical for various periods of time. Considering the level of residual TNX found in the body of the frogs, it appears that TNX might be only slightly toxic. The report of Smith et al., 2006 is somewhat in support of this theory. Though the authors reported that the levels of TNX observed in the liver of mice were one half that of the concentration fed from conception until postnatal day 45, they also observed no response in gross parameter except in the postnatal day 21 animals. Animals treated with 10µg/L TNX had low body weight. This they ascribed to an indirect effect of TNX because the low body weight was seen only in nursing animals.

Based on the spermatogenic profile of these frogs, it would be expected that testosterone would be present in these animals. However, due to the small gonadal sample size whole testosterone evaluation was employed. Radioimmunoassay was used to evaluate the whole body levels of testosterone in male frogs. Testosterone was detected in 10% of the samples. We speculate that the non-detects were below the LOD and would require a more sensitive assay such as the GC/MS quantification assay.

The pattern of spermatogenic cells identified following TNX exposure – increase in the percentage of the number of cohorts of sperm and spermatogonial cells ($P < 0.05$)- would require long term reproductive and developmental evaluation to determine the effect on reproductive capacity of the exposed animals. It is also not clear if similar effect would be induced by TNX in native amphibian species. Relative to the control group, TNX did not cause any significant change in the clusters of spermatocytes, however; the 100ppb and 500ppb groups differ significantly from each other ($P = 0.047$). TNX did not cause any significant difference in spermatid clusters among the groups. It is uncertain if an increase in N (N=5) could affect the outcome of this result. It also appears that some of the spermatogenic cells are more sensitive to TNX exposure. However, a larger samples and longer duration of exposure would be necessary to fully characterize these effects.

Each treatment group had testes with cells representing each stage of spermatogenesis, as well as some testes that were deficient in cells from the later stages of spermatogenesis. However, testosterone was detected in only 10% of the animals evaluated and would appear to indicate that these animals are developing at different rates. Further support for this observation is that there

was a large variation in testicular development among animals within each treatment group. There were animals that had spermatogonial cells as the main cell type present and a few spermatocytes sparsely scattered among them. This was found mainly in animals with smaller body weights. The larger frogs had each type of germ cell present in its gonads. This characteristic has been observed in the American bull frog (*Rana catesbiena*) in our laboratory.

In summary, TNX had no effect on the gross parameters measured in juveniles exposed to the chemical for 30, 60, 90, or 120 days. Histologic analysis of the gonads indicated that TNX has the potential to retard development of the gonads - the number of cohorts of sperm and spermatogonial cells were significantly different between control and treated groups. Based the data from this study, TNX appears to be a potential reproductive toxicant to juveniles *Xenopus*. However, the population levels effects of exposure to TNX cannot be determined from the current study and would require further evaluation. In addition, this study should be repeated using native amphibian species at environmentally relevant concentration.

16.0 REFERENCES:

Smith, J.N., Pan, X. Gentles. A., Smith, E.E., Cox, S.B., and Cobb, G.P. 2006. Reproductive effects of hexahydro-1,3,5-trinitroso-1,3,5-triazine in deer mice (*Peromyscus maniculatus*) during a controlled exposure study**.**

17.0 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.

TITLE: HMX Toxicity in the Green Anole (*Anolis carolinensis)*

STUDY NUMBER: HMX-05-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

CONTRACT ADMINISTRATOR: The Institute of Environmental and Human Health
Texas Tech University/TTU Health Sciences Center
Box 41163
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TESTING FACILITY: The Institute of Environmental and Human Health
Texas Tech University
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TEST SITE: The Institute of Environmental and Human Health
Texas Tech University
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Lubbock, TX 79409-1163

ANIMAL TEST SITE: The Institute of Environmental and Human Health
Texas Tech University
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Lubbock, TX 79409-1163

RESEARCH INITIATION: July 2004

RESEARCH COMPLETION: December 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Scott T. McMurry
Co-Principal Investigator

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 17, 1989.

Submitted By:

_____ _____

Ryan Bounds Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE: HMX Toxicity in the Green Anole

2.0 STUDY NUMBER: HMX-05-01

3.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY: The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, TX 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: July 2004
Termination Date: December 2005

11.0 **KEY PERSONNEL**:
Dr. Scott T. McMurry, Co-Principal Investigator
Dr. Philip N. Smith, Co-Project Manager
Ms. Lindsey E. Jones, Research Assistant
Mr. Ryan M. Bounds, Quality Assurance Manager
Dr. Ronald J. Kendall, Principal Investigator / Testing Facility Management

12.0 **STUDY OBJECTIVES / PURPOSE:**

- To expand the database on HMX to include exposure and toxicity data on reptiles. We have found no data on reptiles.

- To quantify accumulation of HMX into developing eggs via contact with contaminated soil. Due to the life history of this animal, especially its tendency to oviposit in soil, offspring are likely to come into contact with HMX long before they are able to accumulate it dietarily.

- To attempt to quantify transfer of HMX from mother to developing embryo and its subsequent effects on the hatchling's development. Adult animals, though likely able to accumulate HMX via incidental soil exposure, are most likely to uptake HMX through the diet. The effect this exposure has on the mother, if/to what extent this accumulation is deposited in the egg, and what effect the accumulation has on the embryo will provide useful information for both risk assessors and managers dealing with HMX-contaminated sites.

13.0 TEST MATERIALS:

Test Chemical name: octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)
CAS number: 2691-41-0
Characterization: Explosive
Source: Accurate Energetics

14.0 JUSTIFICATION OF TEST SYSTEM:

The use and subsequent environmental contamination of energetic compounds is an ever increasing international concern (Talmage et al., 1999; USACHPPM 2001; ATSDR 1997). The real threat of these compounds from an ecotoxicological standpoint is how little is really known of their toxicity to the organism and the resultant effects to the individual's population and ecosystem. Perhaps one of the larger gaps in the knowledge of explosives is regarding reproductive toxicology.

One of the prime candidates for such an investigation is the green anole, *Anolis carolinensis*. The anole has been used extensively in the laboratory setting, is easy and cost-effective to maintain, and more importantly has been shown to imitate the sensitivities of birds and mammals, rather than those of other poikilotherms, perhaps allowing the data a broader range of extrapolation (Lovern et al, 2004, Hall and Clark, Jr., 1982).

Two studies were performed in an effort to define the role of HMX in reproductive toxicology of lizards. The first study used artificially contaminated nesting media as the source of contamination to assess accumulation of HMX into eggs at different combinations of time and concentration. Also, initial growth parameters were measured for all hatchlings. The second study was designed to assess HMX transfer from the diet of adults to eggs. In addition to assessing accumulation, we measured hatching rates of eggs and growth rate of hatchlings, and monitored adult anoles for signs of toxicity and signs of abnormal reproductive function.

15.0 TEST ANIMALS:

Species: green anoles (*Anolis carolinensis*)
Strain: green anoles (free-living)
Age: green anoles (hatching year or after hatching year)
Number: anoles (90)
Source: anoles (trapper from Louisiana)

16.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Anoles were placed into individual labeled 10-gallon glass aquaria containing the appropriate identification information for the animal on the front of the aquarium. Collected egg samples were placed in plastic cups with the appropriate identification information on it. All samples collected at the end of their term in the study were placed

in individually labeled bags/collection vials and were stored at -20°C until further analyses could be done.

17.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The egg exposure study was designed to quantify the accumulation of HMX from contaminated nesting material into the incubating egg. Four treatment groups were designed on a log scale of increasing dosages: control (0 HMX), low (20 mg HMX/kg soil), medium (200 mg HMX/kg soil), and high (2000 mg HMX/kg soil). Eggs from unexposed females were randomly placed into each of the groups so that no more than one egg from each female was placed in any one group. In turn, the eggs were randomly placed in the incubator to remove any possible position bias in the incubator.

The maternal exposure study was designed to quantify the accumulation of HMX from the mother to the offspring via maternal transfer. Five treatment groups were designed for this study, including an experimental control group (undosed crickets), a solvent control (polyethylene glycol-injected crickets), low (20 mg HMX/kg body weight), medium (250 mg HMX/kg body weight), and high (500 mg HMX/kg body weight). All female anoles were randomly placed in individual tanks and were divided into treatment groups using a randomized block design that was stratified so that each dose group had equal representation on each of the shelves. All animals were treated similarly throughout the study and separate utensils were used for all control groups so that unintentional exposure did not occur.

18.0 METHODS

18.1 Egg Exposure Studies (HMX transfer from soil to eggs)

18.1.1 Lizard Husbandry

Eggs for both the PSD study and the artificial nesting study were retrieved from an unexposed colony of *Anolis carolinensis* at the university that were trapped in southeastern Louisiana one month prior to the start of the study. The anoles were housed individually in 10-gallon glass aquaria with sphagnum peat moss substrate, half PVC pipe for hiding, dowel rod for thermoregulation, heat lamp, and access to UVB lamp that was turned on for two hours each day. Male and female anoles were paired for 24 hours once a month for breeding purposes.

18.1.2 Soil preparation

Standard HMX (CAS No. 2691-41-0; 99.0% pure; molecular weight = 296) and analytical grade acetone was obtained from Supelco (Bellefonte, PA, USA). Soil was mixed on May 31, 2005 to use in the artificial nesting study that would take place throughout the month of June. The soil mixture (75% sand and 25% organic potting soil, by volume) was put into a small cement mixer and was mixed for 30 min. After homogenizing, the soil was sifted through a 2 mm sieve. The soil was then separated into four groups (control, low, medium, and high) for spiking with an HMX/acetone solution.

Five kg of soil was spiked with 20 mg/kg HMX (low), 8.5 kg of soil was spiked with 200 mg/kg HMX (medium), 5 kg of soil was spiked with 2,000 mg/kg HMX (high), and 5 kg of soil was spiked with 200 mg/kg acetone (control). These values are considered an ecologically relevant representation of the range of concentrations found at military installations (Jenkins et al. 1999). Soil was sprayed with the appropriate solution, hand mixed, then sprayed again. The soil from each dose group was then put into a rock tumbler and tumbled for 40 min. to completely homogenize. The soil was then placed in a darkened hood overnight so that the acetone could evaporate. The next morning, the soil was mixed again and seven samples were taken randomly from each dose group for analytical verification of the concentration (Table 2). The samples were mixed with sodium sulfate, and the HMX was extracted using the Accelerated Solvent Extractor (ASE) (see analysis section below). The remaining soil was left for a second night to further evaporate the acetone. This soil was then put in large freezer bags and placed in a -20°C freezer until needed.

18.1.3 PSD study

This study was designed to assess the ability to use passive sampling devices (PSDs) to predict concentrations of HMX in eggs incubated in contaminated soil. Eighteen eggs and 18 PSDs (C18 sealed in whirl-paks) were housed individually in cups (eggs) or glass jars (PSDs) containing 100 g of medium HMX soil (283.37 mg/kg soil, SD = 51.19) and 10 mL of distilled water. Each egg was buried about one centimeter below the surface of the soil and cups covered with cellophane and secured with a rubber band. Egg cups and PSD jars were placed randomly in the incubator (maintained between 29C and 32C). Three eggs and their paired PSDs were taken from the incubator on days 1, 3, 6, 12, 18, and 30 and frozen, along with the soil from the container, at -20°C prior to analysis.

18.1.4 Nesting Study

This study was designed to assess the dose-dependent uptake of HMX by eggs incubated in soil during the normal incubation period. Beginning on June 9, 2005, unexposed eggs were randomly placed into one of the four treatment groups (control, low, medium, high), with care taken to have only one egg from each female in each group. Fifty-five eggs were collected for this study (14 control, 14 low, 14 medium, and 13 high). Eggs were placed in containers and treated exactly as described for the PSD study. Since the goal of this portion of the study was to look at uptake into the egg and embryo, all neonates were sacrificed within 24 hr of hatching. Measurements of the neonate included snout-vent length, whole body length, and weight, along with any visual observations. The hatchlings were then frozen at -20°C and analyzed for HMX residues.

18.1.5 Sample analysis

All samples (both soil and egg) were individually homogenized with liquid nitrogen and dehydrated with four to five grams of sodium sulfate using a small mortar and pestle. The samples were then extracted using a Dionex Accelerated Solvent Extractor (Model 200, Salt Lake City, UT, USA) using 100% acetonitrile (analytical grade, obtained from Supelco, Bellefonte, PA, USA) as the extraction solvent. The extraction procedure was as follows: 5 minute preheat, 5 minute heat, 5 minute static extraction at a constant temperature of 100°C and pressure of 1500 psi. The extracts (15-20 mL/sample) were

then purged from the cells into glass collection vials using nitrogen gas. The extracts were then diluted to 25 mL with acetonitrile using clean volumetric flasks. Five mL from each extract was taken for a stock solution and the remaining 20 mL was concentrated using a vortex evaporator until a final volume of approximately two mL was reached. The concentrates were cleaned up using a styrene-divinybenzene (SDB) cartridges obtained from Supelco (Bellefonte, PA, USA). Each SDB cartridge was conditioned twice with three mL of acetonitrile, after which the sample extracts were loaded, and the elutes were collected by aid of gravity into plastic 15-mL Falcon centrifuge tubes (Franklin Lakes, NJ, USA). The SDB cartridges were then rinsed with one mL acetonitrile three times, the elutes continuing to be collected. The final extract volume was adjusted to five mL with actonitrile. One mL of the final concentrated volume was then diluted with one mL of nanopure water (milli-Q water at 18.3 MΩ, Barnstead NANOpure infinity system, Dubuque, IA, USA) and filtered with through a 0.20 µm PTFE syringe filter into autosampler vials.

All samples were analyzed using liquid chromatography tandem mass spectrometry (LC-MS). The liquid chromatography portion used a Finnigan system, which included a vacuum membrane degasser, a gradient pump, and an autosampler (San Jose, CA, USA). Chromatographic separation was achieved using a Supelco RP C18 column (4.6 x 250 mm, 5-µm packing) (Bellefonte, PA, USA). Mass spectrometry analyses were conducted using a Thermo-Finnigan LCQ advantage ion trap mass spectrometer. Helium was used as the dampening gas for the ion trap and nitrogen was the sheath and auxiliary gas for the ion source. The LC-MS operation conditions are noted in Table 1.

The C18 from the passive sampling devices was extracted and filtered in the same way as the egg and soil samples. After using the vacuum manifold, the concentrated volume was adjusted to five mL. One mL of this concentrate was mixed with four mL of nanopure water and was filtered into the autosampler vial. These samples were analyzed using the HPLC.

LC Conditions		MS Conditions	
Mobile phase A:	Methanol	Mode:	Negative
Mobile phase B:	0.5 mM aqueous acetic acid	Ion spray voltage (KV):	3.5
A: B = 60:40 (v/v)		Sheath gas flow rate (L/hr):	44.0
Flow rate: 0.5 ml/min		Aux/Sweep gas flow rate (L/hr):	53.1
Injection: 25 µL		Capillary voltage (V):	- 6.3
		Capillary temp (°C):	140.0
		Multipole 1 offset (V):	1.7

	Lens Voltage (V):	25.6
	Multipole 2 offset (V):	7.0
	Multiple RF Amp (Vp-p, sp):	500.0

Table 1. Optimized LC-ESI-MS operation parameters (Pan et al., in press).

18.2 Maternal Exposure Studies

18.2.1 Anole housing and husbandry

Sixty-six adult female and twenty-four adult male green anoles were purchased from a trapper in southeastern Louisiana and housed at The Institute of Environmental and Human Health, Texas Tech University. Each anole was housed in its own individual ten-gallon glass aquarium, complete with sphagnum peat moss substrate, PVC pipe for hiding, wooden perch for thermoregulation, and plastic container with moist peat moss for oviposition. The aquaria were placed on metal racks around the room, with each animal having access to its own 40-watt heat lamp and the UVB bulb that was stretched across each row of four tanks. The UVB bulb was on for two hours every morning for vitamin B3 synthesis. All of the aquaria were misted via the Rainmaker I Expanded Fully Automatic Misting System (Ecologic Technologies, Inc., Pasadena, MD, USA) three times daily. The aquaria were cleaned at least once a week of fecal matter, dead skin, and cricket parts and were sterilized every six weeks with over-the-counter vinegar. The room was held at a 14:10 light:dark cycle to mimic the summer breeding season. A minimum of once per month the anoles were paired for 24 hours for breeding purposes.

The anoles were randomly assigned an identification number, the first anole out of the shipment being number one, the second being number two, and so on. Prior to any dosing with HMX, all anoles were given one large cricket each morning. To ensure proper nutrition, the crickets were given OrangeCube (Fluker Farms, Port Allen, LA, USA) every two days and were dusted with additional calcium powder once per week.

18.2.2 Dosing of adult females

Dosing of adult female anoles began on July 11, 2005. Each female was weighed on July 10, 2005 to get an intial pre-dose weight and to calculate the necessary dose for the first two weeks of dosing. The females were randomly assigned to one of the five dose groups (negative control (no solvent)), solvent control (polyethylene glycol), low dose HMX (20 mg HMX/kg body weight), medium dose HMX (250 mg HMX/kg body weight), and high dose HMX (500 mg HMX/kg body weight)) using a stratified block design, which was stratified by shelf so that each dose group had equal representation at each height around the room. The high dose was selected based on our data which indicated that no toxicity occurred below 2,000 mg/kg and pilot data that suggested rejection of crickets spiked with HMX at concentrations delivering a dose of 1000 mg HMX/kg body weight.

HMX was delivered to the anoles by injecting the appropriate amount of HMX/PEG solution into a live cricket, which was then offered to the anole as food three times per week for 12 weeks. Since the crickets often died within a few hours of injection, each female was offered a maximum of three dosed crickets throughout the day, until she either ate one of the crickets or it was determined that she refused to eat. If a female rejected her spiked cricket three times, she was offered an undosed cricket on the following day in order to minimize any problems with starvation. The anoles were fasted on the seventh day of the week to increase their chances of eating on the next dose day. The weight of each anole was recorded every two weeks and its dose adjusted accordingly. The HMX/PEG solution was mixed in a glass vial using magnetic stir bar and mixing continued throughout the dosing procedure to maintain the solution. The solution was drawn into a glass Hamilton syringe, a 28 gauge needle attached and then primed with solution. In an effort to validate this delivery method, each week for 10 weeks of the dosing study one cricket from each dose group was injected with the solution and immediately frozen for future residue analysis.

All female cages were checked daily for eggs. The first egg from each female during the first four weeks of dosing was immediately frozen at -20°C so that later analysis would show their concentration upon leaving the mother. All other potentially viable eggs were placed in a plastic cup with 9 g of vermiculite and 9 mL of nanopure water (milli-Q water at 18.3 MΩ) and the cup was secured with cellophane and a rubber band. The eggs were given 48 days in the incubator to hatch. Those that did not hatch were frozen at -20°C for later residue analysis. Those that did hatch were put in a clean five-gallon aquarium, set up exactly like the adult cages. These hatchlings were monitored daily and weight, snout-vent length, and whole length were recorded once weekly for 63 days (nine weeks), at which time they were sacrificed and placed in a -20°C freezer.

18.2.3 Analysis

Residue analysis on the un-hatched eggs was completed using the same method described above for the eggs from the artificial nesting study. The one exception to the above described method is that the eggs were rinsed with milli-Q water, dried, and weighed before homogenization. After extraction with the ASE, concentration, cleanup with the SDB cartridges, dilution, and filtering into the autosampler vials, the eggs were analyzed using the LC-MS. The same method described above for the LC-MS was used for these eggs (see Table 1).

19.0 RESULTS

19.1 Egg Exposure Studies

19.1.1 PSD Study

HMX accumulated in eggs and PSDs (Fig. 1). Accumulation of HMX into PSDs was variable, but did suggest a time dependent uptake by day 30. The spike in mean HMX concentration on day 6 is inconsistent with the remaining data and should likely be considered an artifact. Conversely, HMX accumulation into eggs was more consistent over time, and appeared stable through day 12, followed by a gradual increase through

day 30. With the exception of the PSD data on day 6, there appears to be reasonable correspondence between HMX concentrations in PSDs and eggs.

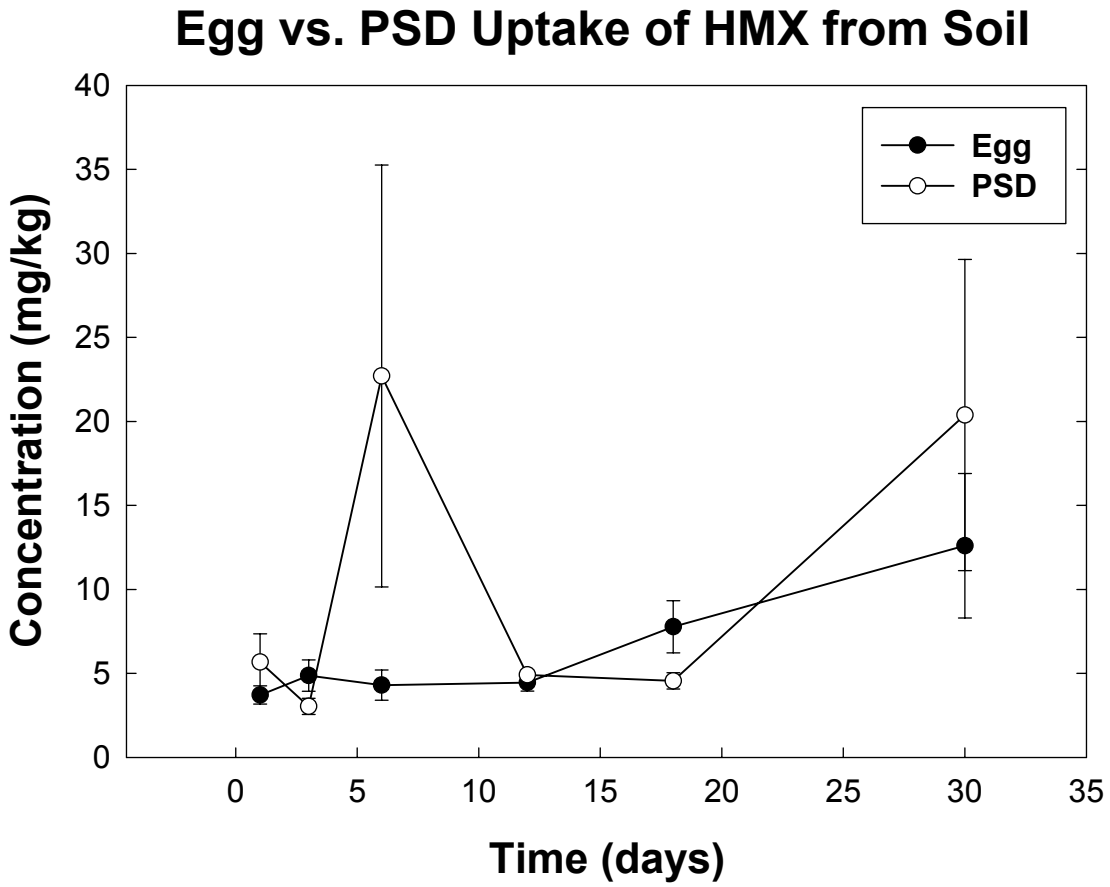

Figure 1. Mean (± SE) HMX accumulation from soil (at concentration of 283.37 ± 19.35) into the anoline egg and PSD over 30 days. Each time period had three replicates.

19.1.2 Nesting Study

HMX contamination of the soil used in the artificial nesting study was fairly consistent within each dose group. Aliquots from both the original stock of soil and from the soil used in the incubation study showed a relatively stable concentration of contaminant over time and among groups (Table 2).

Dose Group	Pre-Study Mean	SE	Post-Study Mean	SE
Control	0	0	0	0
Low	19.75725	0.6216	17.7215647	0.1937
Medium	283.36937	19.35	174.375139	3.3427
High	2244.1955	848.23	1760.69205	22.391

Table 2. Comparison of soil concentrations of HMX before and after use in the artificial nesting study.

Residue analysis on the eggs in the nesting study showed HMX accumulation into all eggs in the low, medium, and high groups following a dose-dependent response (Fig. 2). Of the 55 eggs incubated in this study, 27 hatched (7 control, 6 low, 9 medium, 5 high). None of the developmental parameters measured (body weight, snout-vent length, whole length, or incubation time) differed (P>0.05) among treatment groups (Fig. 3-6).

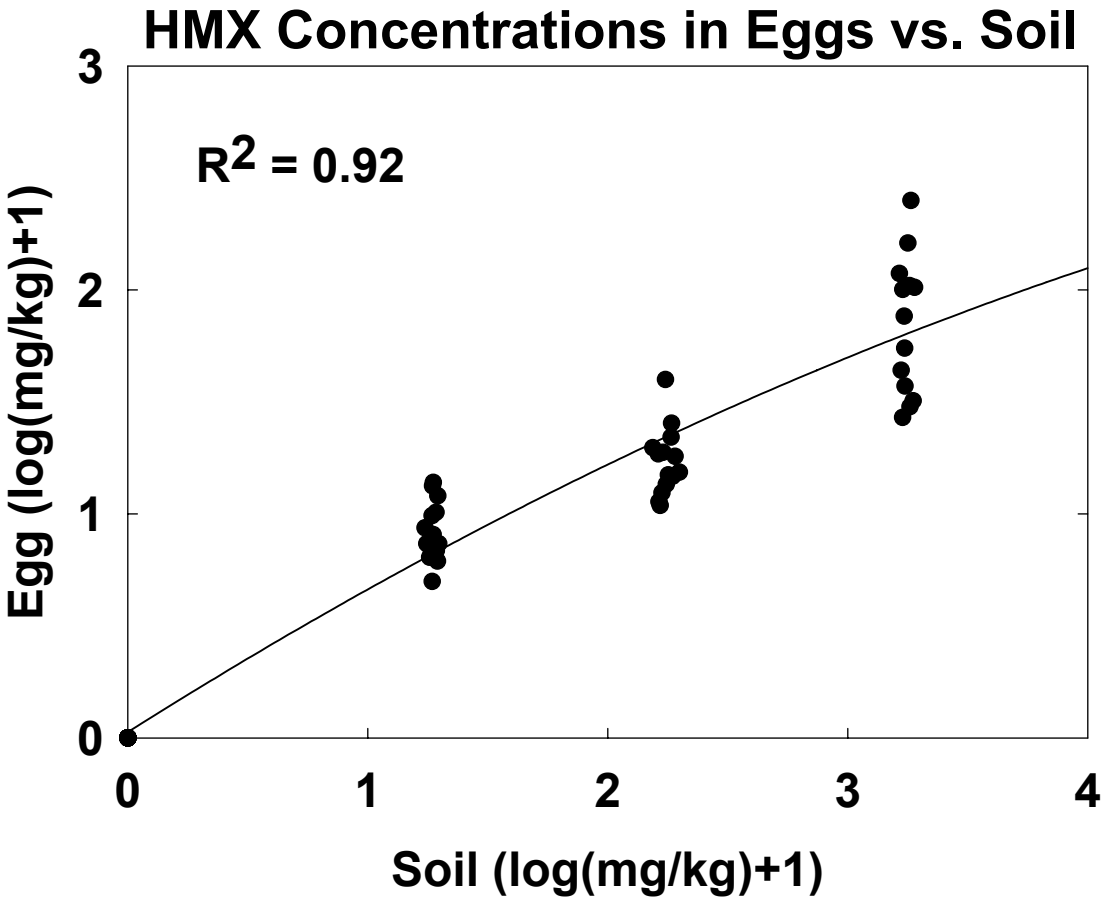

Figure 2. Comparison of HMX accumulation into the anoline egg at four nominal concentrations of HMX in soil: control (0 HMX; n=14), low (20 mg/kg HMX; n=14), medium (200 mg/kg HMX; n=14), and high (2000 mg/kg HMX; n=13).

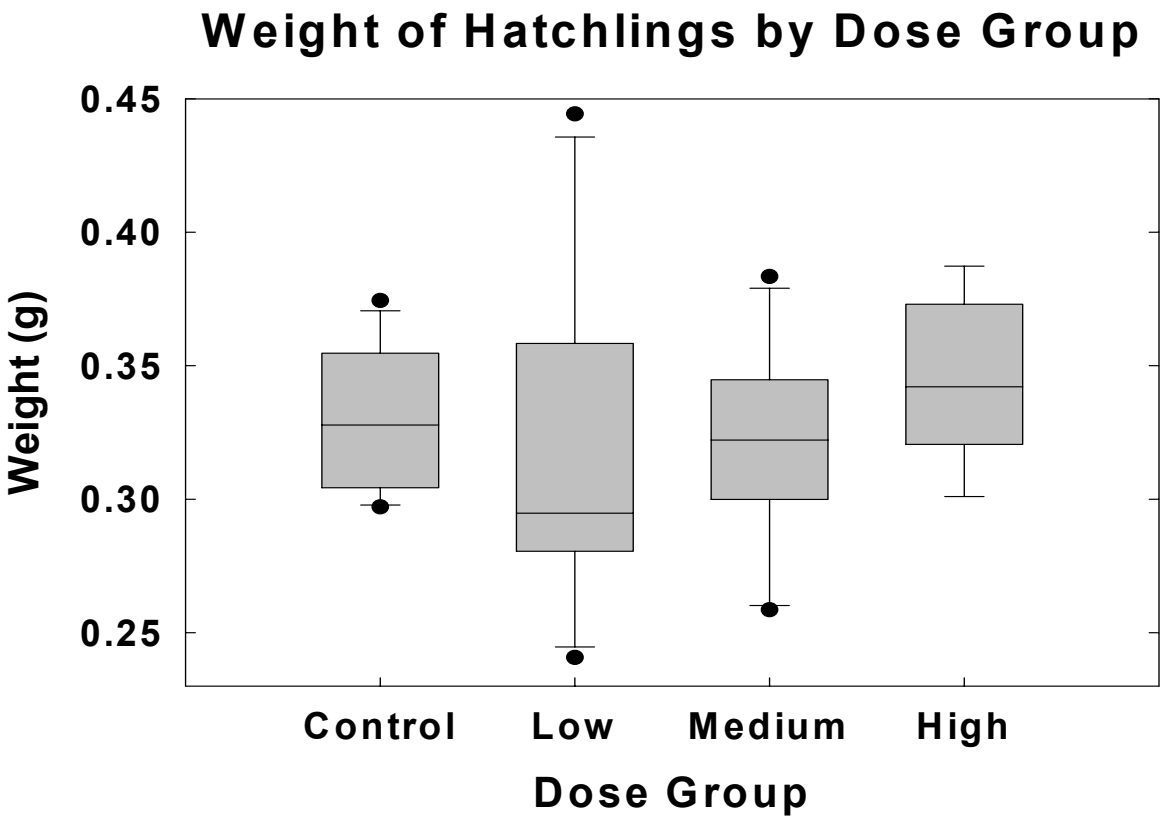

Figure 3. Comparison of hatchling body weight following chronic exposure to HMX via soil. Boxes represent first and third quartiles, lines represent medians, whiskers represent the largest/smallest observation that falls within a distance of 1.5 times the box size from the nearest hinge, and points represent outliers. Analysis of variance showed no statistical difference among dose groups ($p = 0.1792$).

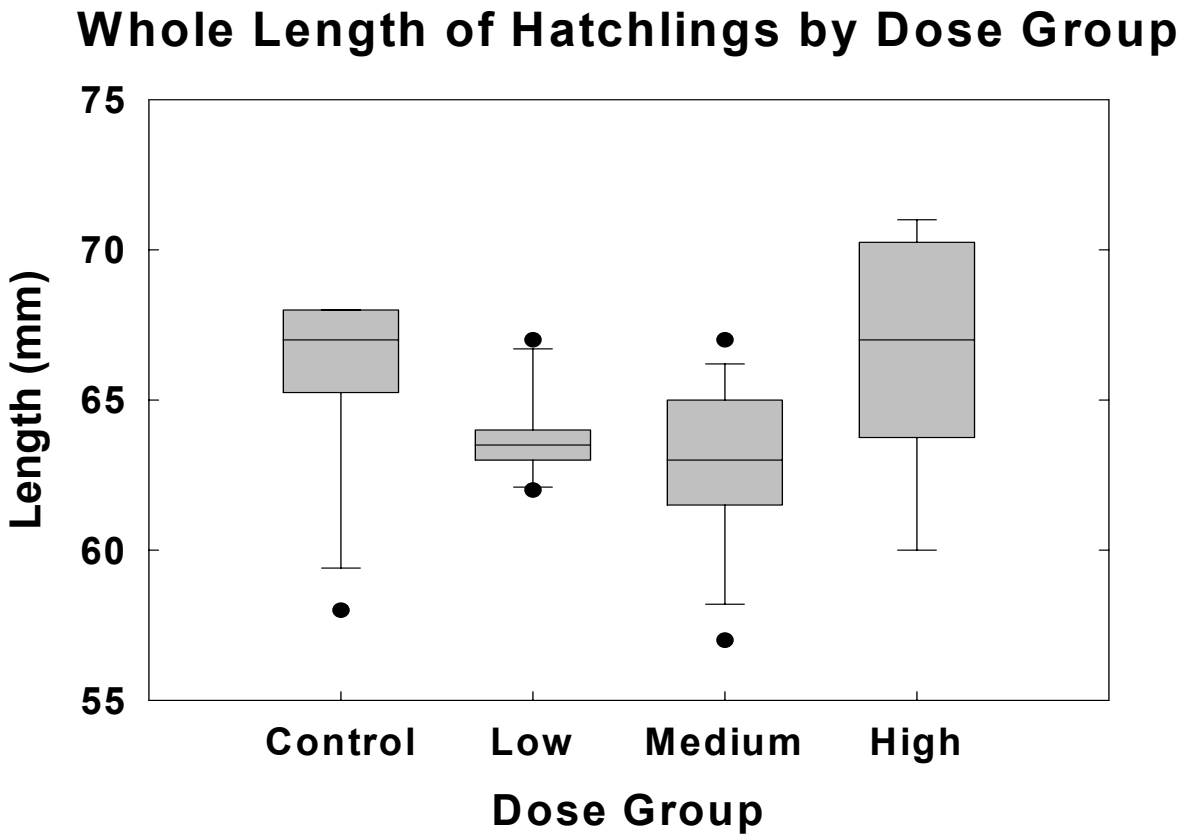

Figure 4. Comparison of hatchling body length following chronic exposure to HMX via soil. Boxes represent first and third quartiles, lines represent medians, whiskers represent the largest/smallest observation that falls within a distance of 1.5 times the box size from the nearest hinge, and points represent outliers. Analysis of variance showed no statistical difference among dose groups ($p = 0.1866$).

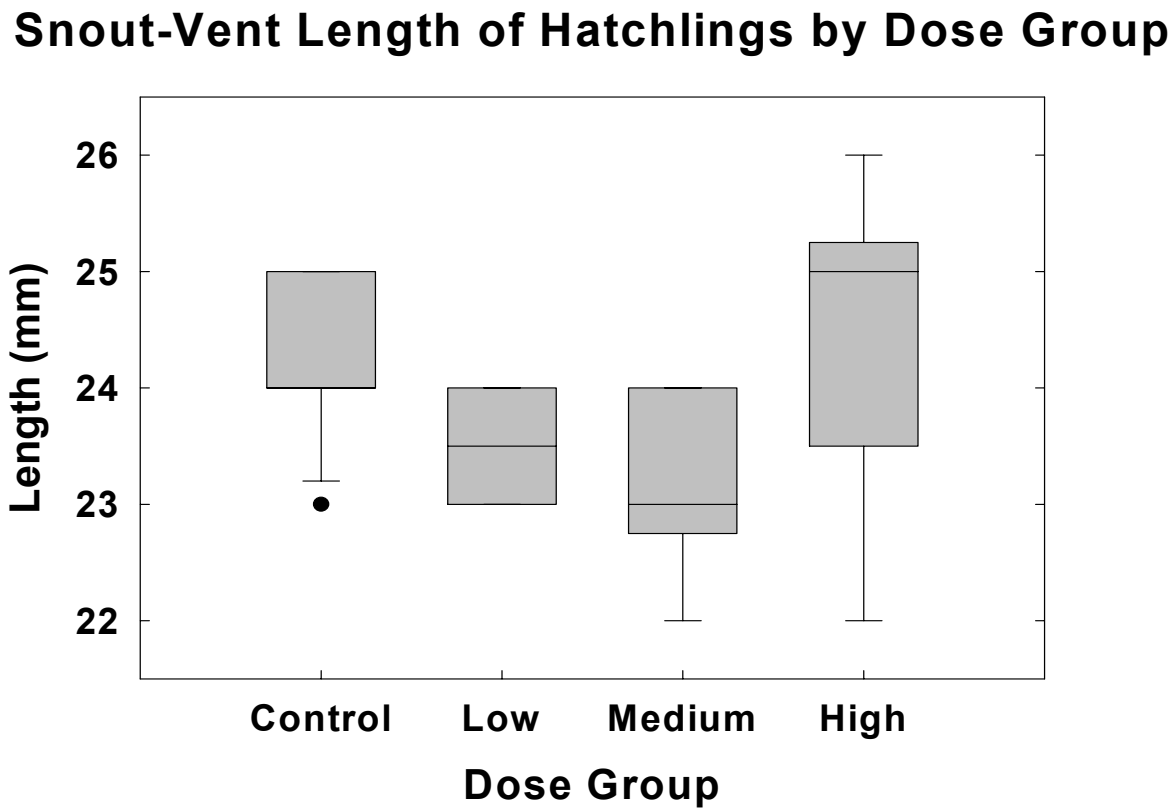

Figure 5. Comparison of hatchling snout-vent length following chronic exposure to HMX via soil. Boxes represent first and third quartiles, lines represent medians, whiskers represent the largest/smallest observation that falls within a distance of 1.5 times the box size from the nearest hinge, and points represent outliers. Analysis of variance showed no statistical difference among dose groups ($p = 0.0679$).

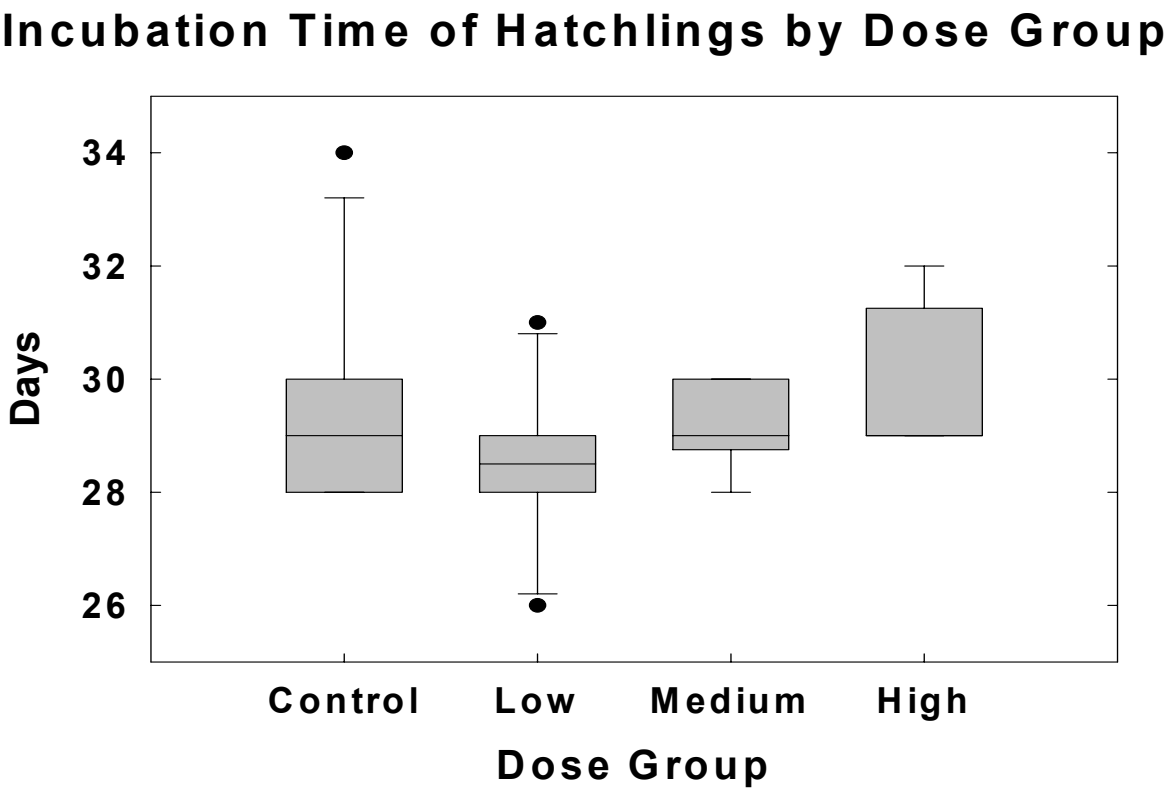

Figure 6. Comparison of hatchling incubation time following chronic exposure to HMX via soil. Boxes represent first and third quartiles, lines represent medians, whiskers represent the largest/smallest observation that falls within a distance of 1.5 times the box size from the nearest hinge, and points represent outliers. Analysis of variance showed no statistical difference among dose groups (p = 0.1017).

Dose Group	Number of Hatchlings	Number of Eggs Lain	% Hatching Success
Control	7	14	0.5000
Low	6	14	0.4286
Medium	9	14	0.6429
High	5	13	0.3846
Total	27	55	0.4909

Table 3. Comparison of hatching success across treatment groups of eggs exposed to HMX via contact with contaminated soil.

19.2 Maternal Exposure Study

19.2.1 Adult Females

On average, the female anoles consumed between 1.5 to 2.5 dosed crickets per week during the maternal transfer portion of the study (Figure 7). Though highly variable, the HMX treatment groups appear to consume fewer dosed crickets per week than do the control groups. This lowered food consumption seems to coincide with the patterns seen in the anoles' percent weight loss throughout the study (Figure 8). The three HMX treatment groups (low, medium, and high) consistently had a lower percentage of their original body weight than did either of the control groups. While this may seem like a confounding factor to the study, there was clear differentiation of actual administered dose amounts for each dose group (Table 4). Additionally, the percent recoveries from the dosed crickets frozen for method validation show that the expected concentrations administered to the anoles were accurate (Table 5), so that effects could justifiably be attributed to each dose group.

Consistent with the dose-dependent uptake of HMX into the anoles, a dose-dependent whole body concentration was seen at the termination of the study (Figure 9). The mean concentrations ± SE for the controls, low, medium, and high groups were ND, 1210.97 ± 509.80, 23012.88 ± 16724.97, and 63145.27 ± 25174.33, respectively. (One outlier was removed from the medium group for this analysis since its concentration (2.52e+6 mg/kg) was substantially higher than all other data points.) The high dose group (500 mg/kg) anoles had a whole-body concentration that was significantly higher than all other dose groups ($p < 0.001$). The medium dose group (250 mg/kg) anoles also had a residue concentration significantly different than the control groups ($p < 0.001$). There was no significant difference between the control groups and the low group (20 mg/kg).

With regard to the anoles' egg production rates, the findings are unclear as to what effect HMX might have on this system. As seen in Table 6, egg production rates are much lower in the high and medium groups than they are in the control groups even before dosing. The fact that the animals were placed in the room using a randomized stratified block design rules out the influence of any external variables in the animal room. Therefore the real reason for this discrepancy remains uncertain.

Average Number of Dosed Crickets Eaten per Week per Anole

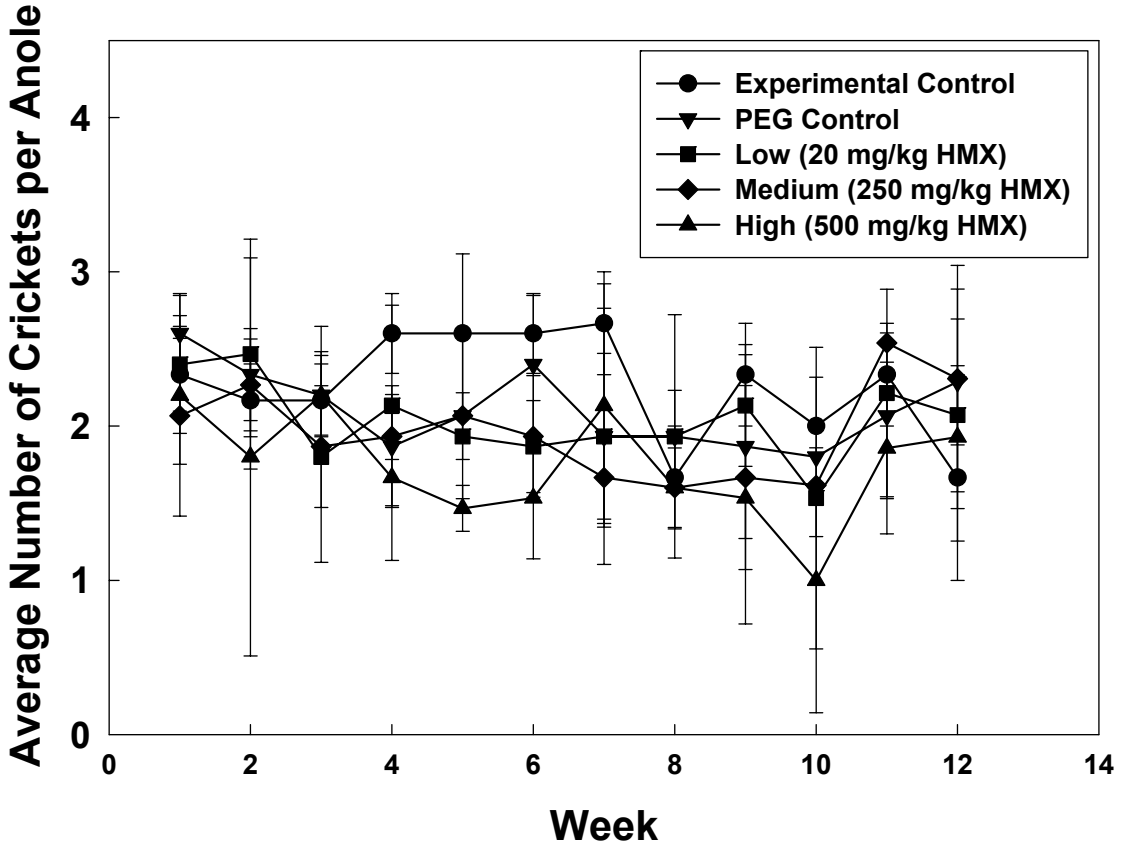

Figure 7. Mean (±SE) number of HMX-loaded crickets consumed per week by anoles in the experimental control (0 HMX or PEG), control (0 HMX; PEG injection), low (20 mg/kg HMX), medium (250 mg/kg HMX), and high (500 mg/kg HMX) dose groups.

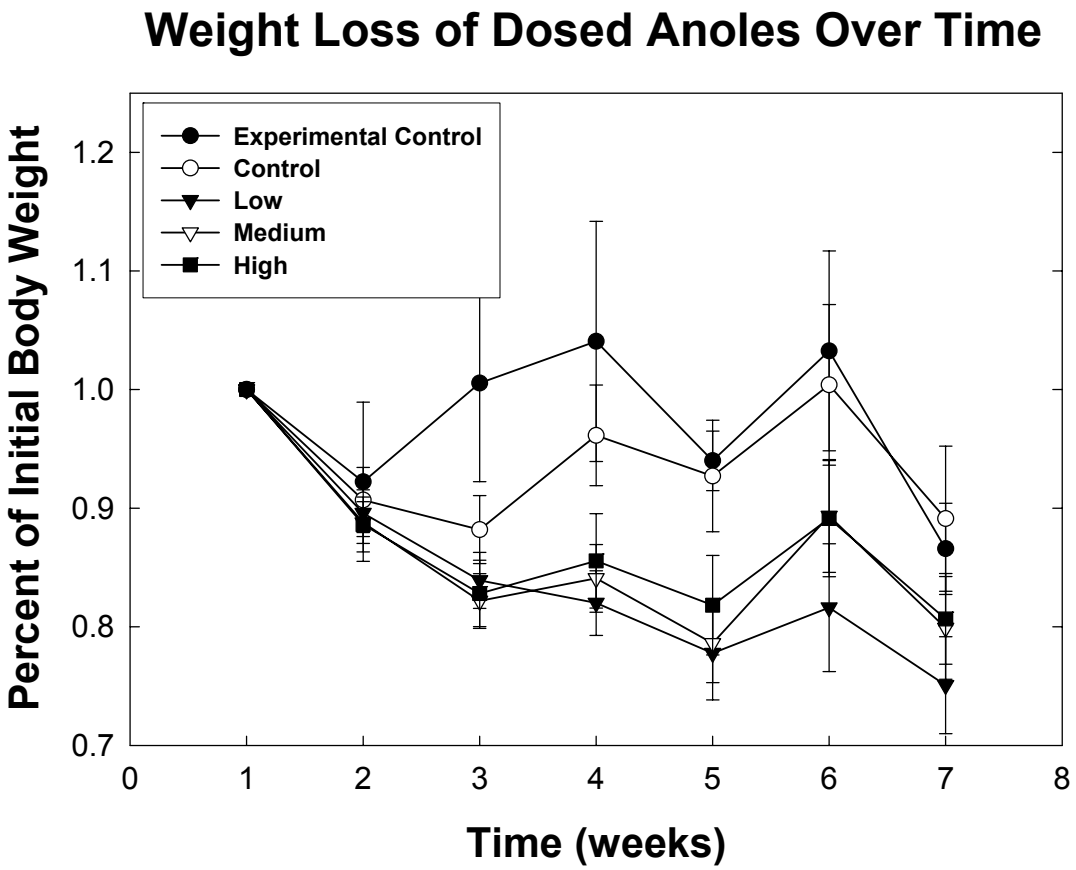

Figure 8. Mean (± SE) weight loss percentage of dietarily-dosed female anoles over time.

Dose Group	Average Dose per Week per Female (mg of HMX)	SE
Exp. Control	0	0
PEG Control	0	0
Low	0.108318684	0.010078154
Medium	1.252173037	0.111325656
High	2.308408542	0.16963382

Table 4. Comparison of the mean amount of HMX given to female anoles per week of the study.

Dose Group	Mean Percent Recovery	SE
PEG Control	ND	0.00
Low	103.28	9.79
Medium	114.26	26.50
High	91.59	12.36

Table 5. Comparison of the mean percent recovery of HMX in dosed crickets. Once per week for 10 weeks of the maternal dosing study one cricket was dosed and immediately frozen in order to validate the delivery method. The percent recovery for the PEG control group was not calculated since there was no detectable HMX in any of the control samples.

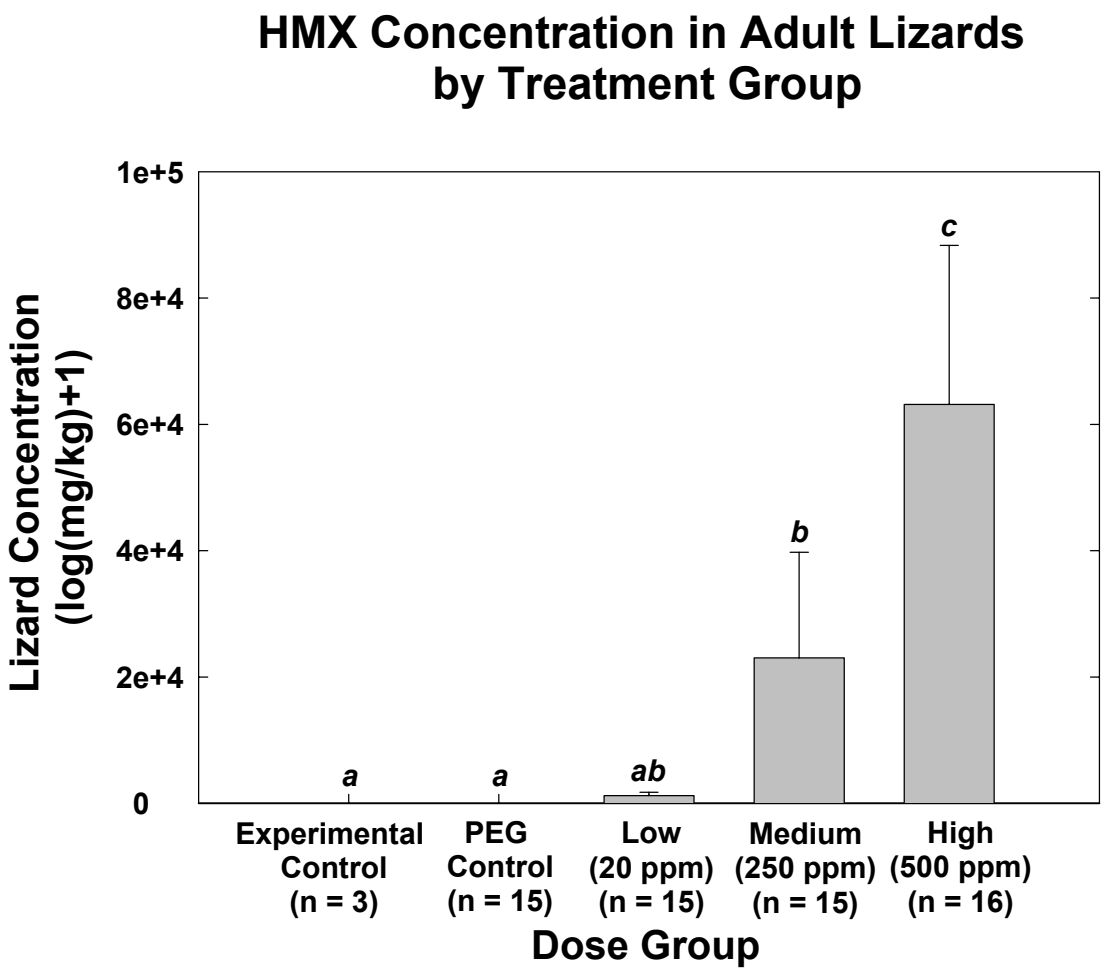

Figure 9. Mean whole body concentration (± SE) of treated female anoles at the end of the study, shown in mg HMX per kg body weight. Data shown excludes one female from the medium (250 ppm) group, whose concentration exceeded all other concentrations in the data set (concentration = 2.52e+6 mg/kg). Bars with different subscripts are different at $p < .001$.

	Maternal Dose Group (Total Eggs/Group; Eggs/Female/Week)									
	Experimental Control (n=3)		PEG Control (n=15)		Low (n=15)		Medium (n=15)		High (n=15)	
Total Number of Eggs Before Dosing	9	0.30	30	0.38	36	0.45	30	0.33	15	0.21
Total Number of Eggs After Dosing	13	0.36	40	0.26	39	0.25	28	0.23	27	0.21

Table 6. Comparison of the total number of eggs per dose group and the number of eggs per female per week.

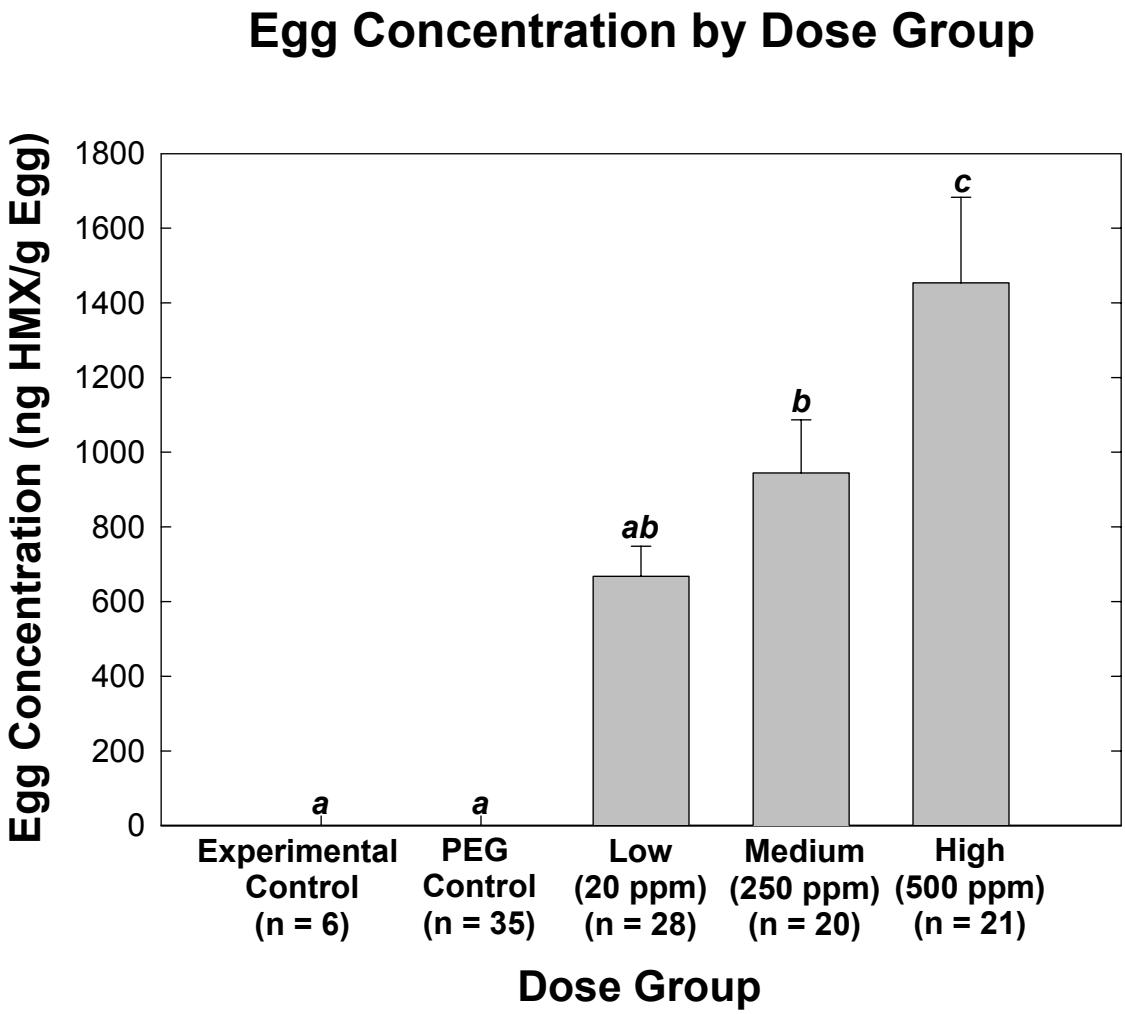

Figure 10. Mean egg concentration (± SE) of HMX as a result of maternal exposure to HMX in food. Bars with different subscripts are different at $p < .001$.

19.2.2 Eggs and Hatchlings

HMX was detected in eggs from all HMX-treatment groups (Figure 10). The deposition of HMX into the egg occurred in a dose-dependent fashion, with the high group having a significantly larger concentration than all other groups. The medium group eggs (maternal dose of 250 mg/kg) had a concentration that was significantly different than the control groups, but was not significantly different than the low group eggs (maternal dose of 20 mg/kg) ($p = < 0.001$). The weight of the eggs was not considered in this analysis, since a separate analysis of variance found the weights to be statistically similar ($p = 0.1212$). This dose-dependent trend is consistent with the trend seen in the maternal whole-body residues analysis (Figure 9).

With the exception of the experimental control group, the percent success of hatching seemed to be fairly consistent across the treatment groups (Table 7). When taken together, the anoles seem to have about a 37.9% success rate for hatchlings. However, when this is broken down into individual dose groups, it is interesting to see that the two groups with the lowest success rates are the control group and the high group (22.7% and 23.1%, respectively).

Dose Group	Number of Hatchlings	Number of Eggs Lain	% Hatching Success
Experimental Control	**9**	**13**	**0.6923**
PEG Control	**5**	**22**	**0.2273**
Low	**10**	**24**	**0.4167**
Medium	**6**	**15**	**0.4000**
High	**3**	**13**	**0.2308**
Total	**33**	**87**	**0.3793**

Table 7. Comparison of hatch success among HMX treatment groups.

The growth parameters of the hatchlings were analyzed using an analysis of covariance, allowing time to be a covariate (Figures 11 and 12). Both of the parameters (weight and snout-vent length) showed a significant interaction with the covariate, meaning that the relationship between the parameter and time changes between dose groups ($p = <0.001$, $p = 0.01965$, respectively).

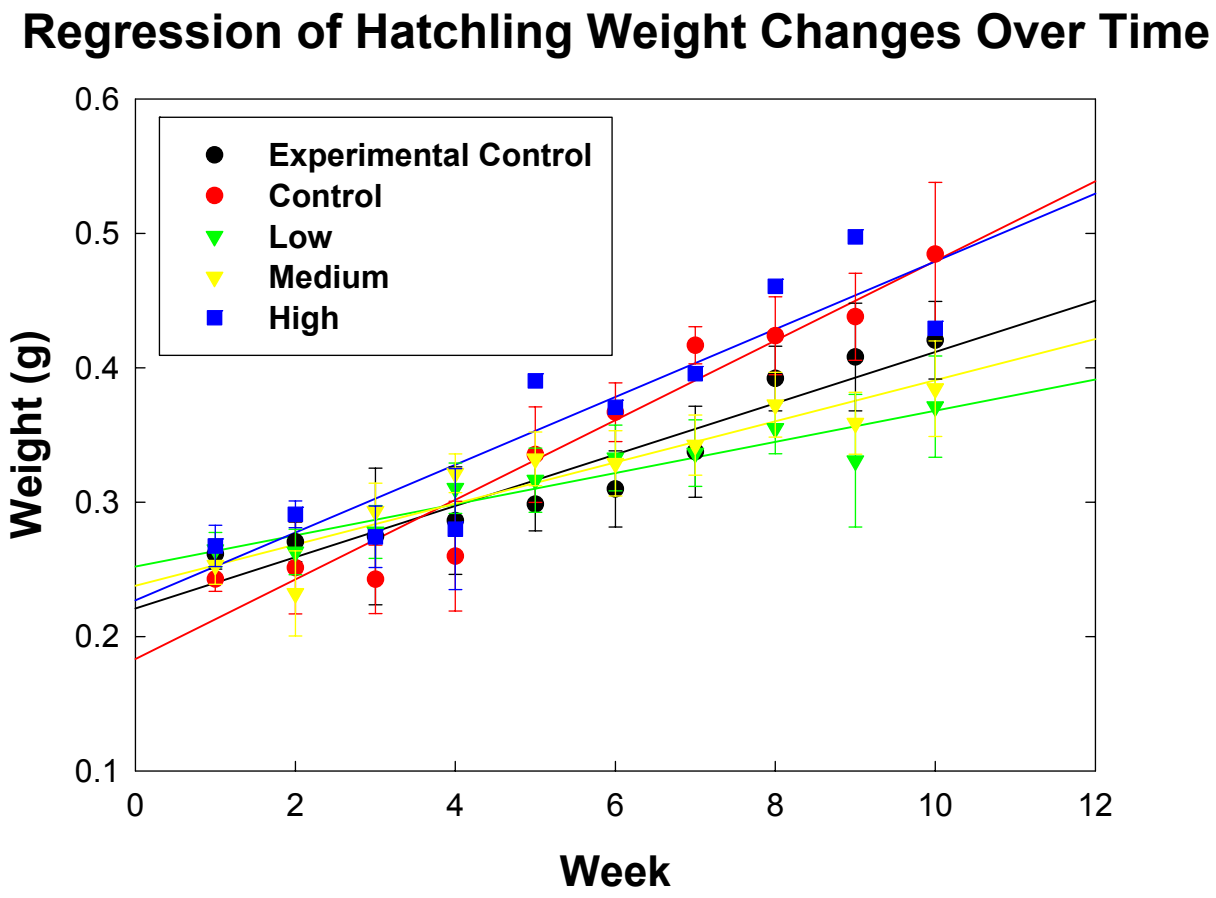

Figure 11. Comparison of the weight gaining trends of hatchlings exposed to HMX via maternal transfer. The interaction between the dose group and time is significant ($p = < 0.001$).

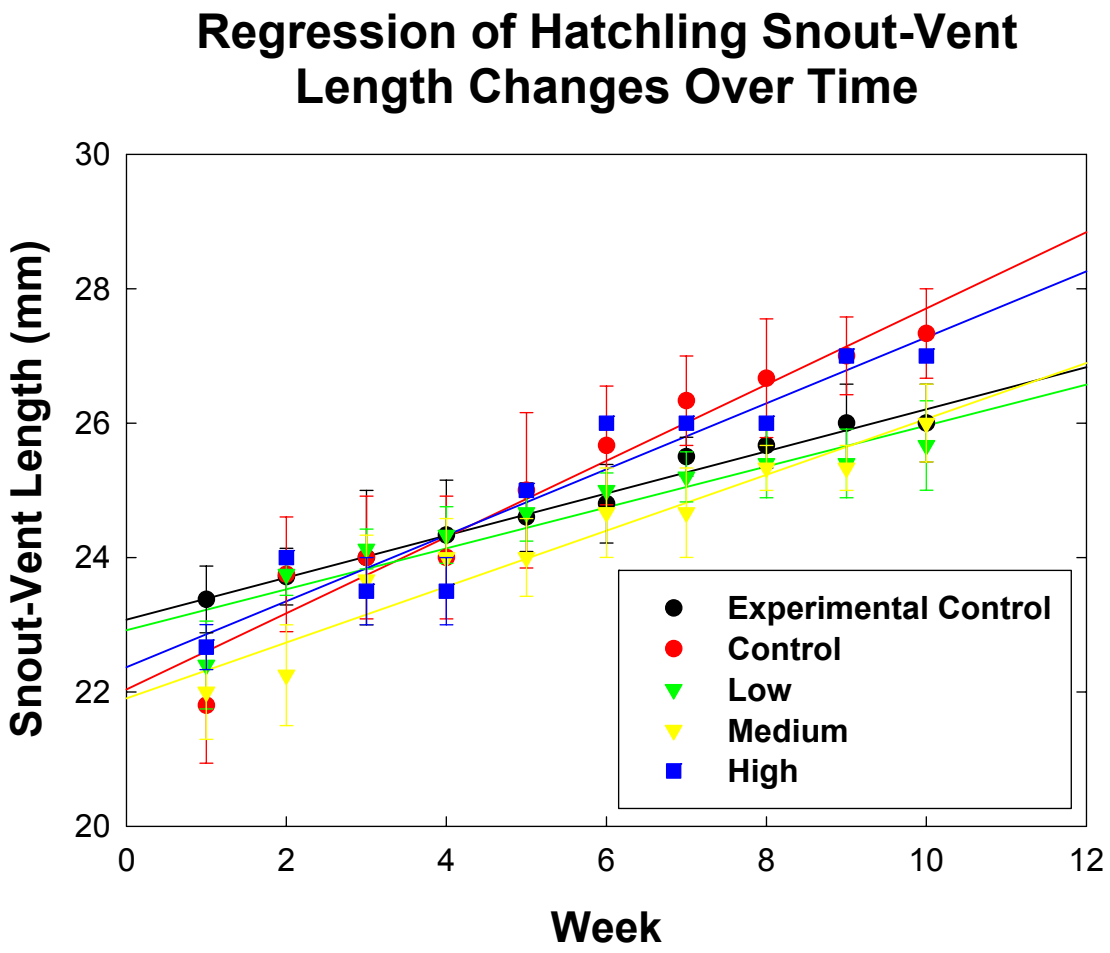

Figure 12. Comparison of the growth of the snout-vent length trends of hatchlings exposed to HMX via maternal transfer. The interaction between the dose group and time is significant (p = 0.01965).

20.0 DISCUSSION

20.1 Egg Exposure Studies

20.1.1 PSD Study

Although C18 PSDs have been shown to be good predictors of accumulation of contaminants into biota, their predictability in this study was unclear. This could be an artifact of either low moisture content of the soil or shortened exposure period. Other studies that have used C18 PSDs have used 75% moisture in the soil and have had exposure periods of up to 60 days (Zhang et al, 2006). Accumulation into the egg, on the other hand, seems to take place at a slow, steady pace.

20.1.2 Nesting Study

Results from the nesting study indicate that accumulation of HMX from the nesting media into a developing egg is possible. From the findings of the present study, it appears that this accumulation can be characterized in time, when all other conditions are held constant. Additionally, the HMX accumulation seen here indicates a dose-dependent trend. These results are considered to be quite reliable, as the concentration of the soil did not decrease appreciably throughout the term of the study.

The effect of this accumulation to the developing embryo is still somewhat uncertain. According to the statistical analyses of the developmental parameters measured (i.e., incubation period, weight, snout-vent length, and whole length), there are no significant differences between the treatment groups. Since no histopathological observations were made from the hatchlings, and their development post-hatching was not monitored, the true effects of the contamination cannot be known as yet.

20.2 Maternal Exposure Study

The present study examined the effects of repeatedly dosing the green anole, and via maternal transfer its offspring, with HMX. The findings suggest that the introduction of HMX to the anole's sole source of food causes a dose-dependent weight loss among treatments. Whether this weight loss is due to food repulsion or avoidance is unclear at this time.

Additionally, the findings suggest a dose-dependent accumulation of HMX in the body of the anole. While concentrations were extremely high, it is difficult at this point to determine what detrimental effects this had on the reproducing female. Of note is the several orders of magnitude difference from the concentration of the female to the concentration of the oviposited egg.

Finally, these data also suggest that HMX is able to enter the egg via maternal transfer in a dose-dependent manner. The effects that the accumulation of HMX may have on the developing embryo, at this point, appear to be minimal. The effect HMX has on hatchling success is still somewhat unclear, possibly an artifact of poor sample sizes across the treatment groups.

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TITLE: **Evaluating Uptake of Incurred Explosives Residues**

STUDY NUMBER: **MRT-05-01**

SPONSOR: **Strategic Environmental and Research Development Program**
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

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

TEST SITE:

RESEARCH INITIATION: **March 2004**

RESEARCH COMPLETION: **March 2005**

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

George P. Cobb
Co-Principal Investigator

Date

9.0 DESCRIPTIVE STUDY TITLE:
Evaluating Uptake of Incurred Explosives Residues

10.0 STUDY NUMBER:
MRT-05-01

11.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

12.0 TESTING FACILITY NAME AND ADDRESS:

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Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, Texas 79409

13.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: March 2004
Termination Date: March 2005

14.0 KEY PERSONNEL:

Dr. George Cobb	Co-Principal Investigator
Mr. Nick Romero	Animal Care and Dosing
Mr. Jordan Smith	Animal Care and Euthanasia
Ms. Xiaoping Pan	Analyte Quantification
Dr. Kang Tian	Analyte Quantification
Dr. Ronald J. Kendall	Testing Facility Manager / Principal Investigator

15.0 STUDY OBJECTIVES / PURPOSE:

To determine the concentration of explosives and their metabolites in rodents following dosing of food containing incurred residues.

16.0 STUDY SUMMARY:

RDX and TNT are widely used explosives within the DOD. Many military installations have soil and/or groundwater contamination problems. These compounds are relatively tightly bound to organic matter in nature and are metabolized by soil microbes. We performed scoping studies wherein deer mice (*Peromyscus maniculatus*) are provided a

diet containing plant and earthworm material with incurred RDX, TNT and in all likelihood their primary metabolites.

17.0 TEST MATERIALS:

Test Chemical name: RDX
CAS number: 121-82-4
Characterization: Residues in plants cultivated in soils from Aberdeen Proving Grounds
Source: Waterways Experiment Station

Test Chemical name: TNT
CAS number: 38082-89-2
Characterization: Residues in plants cultivated in soils from Aberdeen Proving Grounds
Source: Waterways Experiment Station

18.0 JUSTIFICATION OF TEST SYSTEM:

The US Department of Defense desires knowledge regarding the trophic transport and disposition of explosives to assist risk assessors in their evaluation of ecological risks at military sites. For this reason a common species with wide distribution, the deer mouse (*Peromyscus maniculatus*) was selected. Dosing was conducted in a laboratory environment to control as many variables as possible.

19.0 TEST ANIMALS:

Species: *Peromyscus maniculatus*
Age: 45-90 day old
Number: 36
Source: University of South Carolina Peromyscus Stock Center
Sex: Male

20.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The study number was placed on the dosing room door and each cage contained information describing the study number, the mouse identification, sex and dose group. Each animal was also identified with a unique ear tag pattern.

21.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Effects and trophic transfer of TNT and RDX, respectively, on *Peromyscus maniculatus* (deer mouse) were determined by dosing mice with food contaminated with (a) TNT, and (2) RDX, incorporated into the tissues of *Lolium perenne* (perennial ryegrass) and *Eisenia fetida* (earthworm) in a study funded through ERDC-EL, Vicksburg, MS. Standard rodent chow was mixed with these powdered food stuffs. Dosing proceeded with as many animals as we calculated could be fed for 6 weeks with diets containing

decreasing percentages of each incurred residue type. We determined TNT, RDX, and selected metabolites in tissues and blood of the mice.

14.0 METHODS:

Animals: Sexually mature male deer mice were purchased from the Peromyscus Stock Center (Univ. of SC, Columbia, SC) and housed in an AALAC accredited facility at Texas Tech University. Mice were housed separately and maintained in environmentally controlled conditions of 18:6h light-dark cycle, 25° C, and relative humidity of 50%. Mice were acclimated for 1 week prior to dosing. Deer mice were randomly assigned to control and explosives contaminated food (see Table 1 for concentrations). Animals were weighed (±0.01g) before the initiation of the study. Access to food was provided on an *ad libitum* basis. Mice were dosed with powdered rodent chow (Purina Mills, St. Louis, MO, USA), amended with explosives residues from one of two sources, i.e., grass or earthworms

For each contaminant/food type combination, six animals were isolated for each dose group. The mice were housed as one per cage with aspen shavings as bedding. Environmental conditions were 12 h light: 12 h dark and temperatures of 21-24 ^{o}C. Each mouse received food and water *ad libitum*. All animals were exposed for 35 days to TNT, or 21 days to RDX. The weights of the animals and remaining food were also measured at regular intervals; i.e., at days -7, 0, 1, 7, 14, 21, 28, and 35 for TNT, and at days -7, 0, 1, 7, 14, and 21 for RDX. Every time the food stock was replenished in a cage, the weight of the food remaining before replenishment was recorded, and the food consumed calculated.

Preparation of food with incurred TNT or RDX: Plants and earthworms were cultivated in soils containing RDX or TNT by researchers at the Waterways Experiment Station. Each soil type was spiked with radiolabeled forms of RDX or TNT to allow better quantitation. Freeze-dried plant and earthworm materials were then shipped to TIEHH for testing.

Standard rodent chow was mixed with the plant and worm materials contaminated with TNT or RDX. Preparation was done by personnel from the food nutrition laboratory of the Animal Science Department at Texas Tech University, Lubbock, TX, USA. Each mixture was prepared by adding a preweighed amount of freeze-dried plant or earthworm material to an appropriate amount of powdered rodent chow (Table 1). Each food type was first hand-mixed with a stainless steel trowel for 5 minutes and subsequently mixed in a Hobart mixer (Hobart Corp; Troy, OH, USA) for 15 minutes. Doses were stored in air-tight plastic containers until use. Three trowels were available for hand-mixing to minimize reuse. Each trowel, mixing bowl and impeller was washed with water and acetone before and between uses. As much as 1.2 kg of food was available for each dose group. Each freeze-dried food amendment contained TNT or RDX as the primary contaminant. Radioactivity in the samples was used for dose confirmation (Table1), and the radioactivity increased linearly with dose.

General Observations: The general health of all animals was assessed daily by examination of the coat, activity, and food and water consumption. Body weights were recorded weekly and at necropsy. At day 30 animals were narcosed with CO_2. Animals were killed by cervical dislocation, and terminal blood samples were collected by cardiac puncture. Sera and liver were removed, weighed, and frozen (-20C) until chemical analysis. The accumulation of incurred TNT and RDX were evaluated after exposure.

Extraction of explosives and metabolites: Tissues were mixed with 8-10 g dried Na_2SO_4 using a mortar and pestle. A Dionex Accelerated Solvent Extractor (Model 200, Salt

Lake City, UT, USA) was used for all extractions as described in published methods developed in the Analytical Core of this research program (Pan et al., 2005, 2006) and are described here briefly. Each extraction cycle included 5-min preheating, and 5-min static extraction with 100% acetonitrile at 100°C and 1500 psi. Extracts were collected in glass vials and reduced to 1-2 mL using rotary evaporation in preparation for florisil and styrene-divinyl benzene cleanup (Pan et al., 2005). These steps removed large amounts of interfering compounds and pigments. The extract volumes were reduced to 1.5 mL under nitrogen using a N-EVAP^{TM} 111 (Organomation Associates, Inc. Berlin, MA, USA). The final volumes were adjusted to 2 mL with MilliQ water, filtered through a 0.20 µm membrane filter (Millipore, Bedford, MA, USA) into an autosampler vial, and stored until further analysis.

Blood samples were mixed with 7 mL of acetonitrile for every mL of blood. Mixtures were vortexed every 30 min for 2-3 h. Extracts were centrifuged at 3,500 rpm for 15 min. Supernatants were collected and purified using Florisil SPE cartridges. Extract volumes were reduced to 1-2 mL under nitrogen, and filtered through a 0.20 µm membrane filter prior to analysis.

Chemical Analyses: Chemical TNT and RDX concentrations were determined in blood using GC-ECD as developed in the analytical core for this research program (Zhang et al., 2005). An Agilent 6890 GC system (Palo Alto, CA) with automated 2 µl splitless injections was used with a 30m x 0.25 mm HP-5 column experiencing an oven temperature from 90 to 250 ^{o}C over a period of 13 min. The ECD was operated with an Ar/methane makeup of 40 mL min^{-1} and a constant current mode. Analyses were performed for TNT, RDX, MNX, DNX, and TNX. Detection limits of explosives in samples ranged from 0.2 to 2 ng mL^{-1} for the analytes used for calibration.

Explosives proved regularly to be below the detection limit of this method. Therefore, a more sensitive method using liquid chromatography-mass spectrometry (LC-MS) with selected ion monitoring (Pan et al., 2006) was also explored to measure the chemical levels. The latter method proved to be successful for the determination of TNT, RDX, and their known metabolites at the pg-level. Livers from the RDX portion of the study were evaluated by this technique.

5. RESULTS:

Given the low concentrations of toxicants incorporated into food, quantifying parent and metabolite concentrations was difficult. The chemical RDX-derived residues were determined in the blood of mice by GC-ECD. Concentrations were low with means ranging from 1.7 to 4.4 ng RDX ml^{-1} and 0.46 to 0.69 ng TNX ml^{-1} (Table 2). RDX derived residues in mouse liver were also determined by LC-MS (Table 3). Mean TNT, RDX, MNX, DNX and TNX concentrations in livers of mice receiving RDX treated food ranged from 0.95 to 20 ng/g but did not have concentrations that were different from

those found in control livers (P>0.44). Similarly, the TNT concentrations in muscle were low (Table 4) and treatment animals accumulated similar concentrations as did control animals.

Insignificant of TNT, RDX and RDX transformation products were accumulated during the study (Tables 2 through 4) indicating limited trophic transfer of TNT and RDX from food items to rodents. The possible reasons for this are the low doses used, rapid metabolism, or poor uptake via the rodent GI tract.

Table 1. Doses prepared by amendment of standard rodent chow with freeze-dried plant or worm material.

Explosive Dose	Food		
	% DW	[Explosive] (mg kg^{-1}) Nominal	Radiation Counts (millions)[a]
TNT			
Control	0	0	0
Plant, low	0.50	14.4	2.692
Plant, medium	1.49	42.9	7.262
Plant, high	4.04	116.3	14.199
Worm, low	0.42	4.1	3.287
Worm, high	1.32	12.9	7.680
RDX			
Control	0	0	0
Plant, low	0.46	15.0	7.130
Plant, medium	0.91	29.6	23.686
Plant, high	1.89	61.4	61.369
Worm, low	0.35	15.2	4.516
Worm, high	1.0	43.4	16.143

a- each food type was spiked with radiolabeled explosive before receipt at TIEHH to allow analytes to be detected more readily.

Table 2. TNT and RDX-residues in *P. maniculatus* blood following ingestion of contaminated food (N=6 unless specified in footnotes).

Exlosive/ Dose	Explosive concentration in blood (ng mL^{-1})[a]		
	TNT Dosing	RDX Dosing[b]	
	TNT	RDX	TNX
Control	0	0	0
Plant, low	6.87 (6.40-12.53)[c]	3.35 (1.02-11.02)	0.52 (0.33-0.80)

Plant, medium	9.08 (7.09-12.70)	4.37(0.60- 31.69)	0.53 (0.21-1.31)
Plant, high	31.11(23.23-53.3)[d]	2.64 (1.52- 4.58)-	0.57 (0.25-1.28)
Worm, low	9.93 (6.40-17.80)	4.28 (0.92-19.77)	0.46 (0.30-0.71)
Worm, high	8.05 (5.38-12.53)	1.74 (1.06-2.84)	0.69 (0.32-1.48)

[a]- corrected mean (95% confidence interval), t=2.571,
[b]- MNX and DNX were not detected
[c] N=4, t=3.182
[d] N=5, t=2.776

Table 3. Mean RDX and metabolite concentrations in *Peromuscus maniculatus* liver following ingestion of residues incurred in food. (N=6).

Explosive/ Dose	Explosive concentration in liver (ng g^{-1})[a]				
	TNT	RDX	MNX	DNX	TNX
Control	2.09 (1.22-3.72)	8.99 (3.21-27.69)	8.48 (3.31-23.91)	2.66 (0.49-16.00)	2.02 (0.59-7.69)
Plant, low	5.59 (1.40-18.95)	9.01 (2.38-37.54)	15.11 (7.14-35.24)	19.38 (9.91-41.73)	1.12 (0.43-3.11)
Plant, medium	2.37 (1.69-3.66)	7.30 (1.21-48.37)	6.04 (1.70-21.41)	6.95 (1.14- 46.79)	1.26 (0.44-3.99)
Plant, high	3.37 (1.50-7.43)	10.41 (3.28-36.43)	7.79 (3.51-17.25)	4.34 (0.80- 25.97)-	1.90 (0.49-7.98)
Worm, low	11.27 (2.37-48.07)	15.22 (5.97-42.74)	10.08 (5.78-19.38)	10.75 (2.22-57.15)	1.70 (0.36-8.97)
Worm, high	4.12 (2.35-7.63)	10.41 (2.36-50.56)	12.40 (6.08-27.92)	11.72 (2.21-68.39)	0.93 (0.31-3.06)

[a]- mean (95% confidence interval), t=2.571

Table 4. Chemical TNT residues in *P. maniculatus* muscle following ingestion of TNT contaminated food. (N=6).

Explosive/ Dose	Explosive concentration in liver (ng g^{-1})[a]
	TNT
Control	17.16[a] (8.63-36.7)
Plant, low	79.83 (28.34-244.34
Plant, medium	70.06 (28.37-188.40)
Plant, high	31.51 (12.41-85.81)

Worm, low	40.63 (19.26-93.20)	
Worm, high	15.49 (7.02-36.35)	

a- mean (95% confidence interval), t=2.57

DISCUSSION:

This study did not demonstrate significant uptake of TNT or RDX into mouse tissues. It is entirely possible that the dosing concentrations were too low for intake to exceed metabolic capacities. The maximum dose represented only a few percent of contaminated food in the diet. This dose should be increased to evaluate accumulation of these contaminants in the terrestrial environment.

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TITLE: Environmental fate and transfer of RDX in Constructed Wetlands

STUDY NUMBER: WET-05-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
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TEST SITE: The Institute of Environmental and Human Health
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RESEARCH INITIATION: December 1, 2004

RESEARCH COMPLETION: December 31, 2005

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

W. Andrew Jackson Date
Co-Principal Investigator

1.0 DESCRIPTIVE STUDY TITLE:
Environmental fate and transfer of RDX in Constructed Wetlands

2.0 STUDY NUMBER:
WET-05-01

3.0 SPONSOR:
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4.0 TESTING FACILITY:
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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: December 1, 2004
Termination Date: December 31, 2005

21.0 **KEY PERSONNEL:**
Dr. W. Andrew Jackson, Co-Principal Investigator / Study Director
Dr. Todd Anderson, Co-investigator
Mr. Darryl Low, Student Researcher
Dr. Ronald Kendall, Principal Investigator

22.0 **STUDY OBJECTIVE/PURPOSE:**

To evaluate the fate of RDX within wetland systems, develop new tools for monitoring RDX in saturated sediments at cm resolution, and to assess exposure and other processes. Natural wetlands frequently serve as interception points for discharging groundwater or surface run-off to waterways. Important fate processes include sorption, plant uptake, microbial transformation, and associated fate of daughter products.

23.0 TEST MATERIALS:

Test Chemical name: Hexahydro-1,3,5-trinitro-1,3,5-triazine
CAS number: 121-82-4
Characterization:
Source: SRI International

24.0 JUSTIFICATION OF TEST SYSTEM:

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has been found on numerous military bases at firing ranges, storage facilities, and other sites exposed to explosives and other energetics. As there may be a significant environmental risk posed by these compounds and non-point source cleanups are challenging and economically taxing, installed wetland systems are now being considered. The use of low impact passive technologies such as natural wetland systems to remove RDX from surface water and groundwater may be a promising alternative. In addition, wetland systems are often a critical interface of exposure for contaminants. This can occur either as interception points of non-point source runoff (e.g. firing ranges) or by groundwater discharges.

RDX has been a major explosive used by the United States military for over half a century. Widespread and severe contamination has been found at a number of sites across the country. Biodegradation of RDX has been achieved by a number of methods. Anaerobic degradation has been shown to have the greatest degradation potential while aerobic degradation occurs at a much slower rate. The most common degradation pathway is the oxidation of RDX's three nitro groups into nitroso groups then ring cleavage. Previous studies have observed the uptake of RDX by reed canary grass and RDX degradation within the plant (Just and Schnoor 2004).

Explosives persistence and bioavailability in the environment is dependent on a number of temporally variable factors. Contaminated surface water is the most commonly examined source, however, other important sources include near surface contamination in saturated and unsaturated systems. Contaminated surface water is likely to have temporally variable explosive concentrations due to variations in precipitation, groundwater discharge, and biological stability of explosives in the ecosystem in question. Persistence of explosives in contaminated surface and subsurface soil is dependent on infiltration rates, plant uptake/transformation, temperature, percent water saturation, and substrate availability. While many of these factors are site specific, a more rigorous understanding of the relationship between high explosive concentrations in sediments and bioavailability is required. One method which is ideal for studying bioavailabily in sediments is diffusion chambers or peepers.

Dialysis samplers or "peepers" are diffusion-based samplers, which can be used to determine the vertical distribution of soluble constituents in sediments or soil

porewater in the saturated zone. Dialysis sampling techniques have been utilized in numerous geochemistry studies beginning with the original design by Hesslein (1976). Examples include recent studies of heavy metal geochemistry in sediments (Shi et al., 1998), nitrogen discharges from groundwater to rivers (Doussan et al., 1998), vertical profiles of anions, cations and gases in peats (Steinmann and Shotyk, 1997) and seasonal changes in nutrient concentrations in marshes (Jackson and Pardue, 1997). Recently, dialysis samplers have been utilized to detect discharge of VOC contaminated groundwater to surface water at various sites (Vroblesky and Robertson, 1996; Vroblesky et al., 1996; Vroblesky and Hyde, 1997). The technique has also been utilized to assess fate processes for VOCs in wetland sediments at the Aberdeen Proving Ground (APG) in Maryland (Vroblesky et al., 1991; Lorah et al., 1997; Lorah and Olsen, 1999). Steinmann and Shotyk (1997) and Teasdale et al. (1995) describe the procedures for using dialysis samplers in detail. Some basic theory has also been developed for membrane type and design as a function of equilibration time (Brandl and Hanselmann, 1991; Webster et al. 1998; Harper et al., 1997).

Solid Phase Micro-Extraction (SPME) fibers are passive equilibrium sampling devices that are negligible depletion and non destructive (Monteil-Rivera et al., 2004; Condor et al, 2003). SPME fibers are biomimetic devices that only absorb bioavailable compounds and concentrate them to higher concentrations than the surrounding matrix. SPME method is highly sensitive, simple, rapid, and economical compared to other current technologies. SPME fibers exhibit a linear uptake relationship which makes it a powerful tool to monitor trace contaminants in the environment. The effectiveness for

a particular fiber depends on the coating material and the surface area available for absorption of the contaminant (Mayer et al, 2003). SPME fibers are proven to be effective for monitoring TNT, HMX, RDX, and their breakdown byproducts (Monteil-Rivera et al., 2004; Condor et al., 2003; Barshick and Griest, 1998).

25.0 TEST PLANTS:

Species: Graceful cattails (*Typha laxmanil*)

26.0 EXPERIMENTAL DESIGN

RDX in Wetlands

Four mesocosms (Figure 1) were constructed (dimension of 2 ft L × 1 ft W × 2 ft H) with multiple sampling ports (3, 9, 15, 18 inches below the water sediment-water interface). At the bottom of the microcosm, there was a 3 inch sand layer to distribute and drain the flow. Above the sand, the mesocosms were filled with 15 inches of wetland substrate medium composed of a peat/sand/peat moss mixture (2:2:1 by weight). Physical characteristics of the mesocosms are listed in Table 1.

The mesocosms were operated on a 12/12 hour light/dark cycle in a laboratory setting at The Institute of Environmental and Human Health with constant temperature and humidity. Downflow mode was established at a flow rate of 10 mL/min. A standing water depth of approximately 2 inches was maintained by adjusting the effluent tubing height. Initially, the mesocosms were fed with water containing 25 ppm Cl^-, 5 ppm NO_3-N, and 20 ppm SO_4^{2-} for two months to establish flora and bacterial growth.

The wetlands were operated in two phases to examine the effect of RDX and nitrate levels on RDX biodegradation. Phase I explored the ability of the mesocosm to degrade RDX at 5 ppm NO_3-N when RDX loading was 1 ppm, 5 ppm, and 10 ppm (Table 2). Phase I began with a loading rate of 1 ppm RDX was introduced to the system. The mesocosms were then sequentially challenged with the increased RDX concentrations of 5 and 10 ppm. The system was operated for approximately 12 weeks for each loading rate. After Phase I finished, a desorption period was maintained for six weeks with no RDX loading. In addition, the plants were removed from one of the two mesocosms.

Phase II explored the effect influent nitrate concentration and plants have upon RDX degradation. RDX loading rate was set at 1 ppm, while the nitrate concentration was decreased from 5 ppm used in Phase I to 1 ppm NO_3-N (Table 2). The system was maintained for 12 weeks until the nitrate was lowered again to operate at 0.1 ppm NO_3-N for 12 weeks. The effect of nitrate was examined by comparing the degradation rates

between the nitrate loading rates. Only the planted mesocosm was used to determine the effect of nitrate. Including the nonplanted mesocosm when comparing with previous loadings would introduce a new variable that could affect degradation rates.

Passive Samplers to Monitor RDX

Diffusion Sampler -A stainless steel 30 cm long 1.25cm diameter prototype diffusion sampler was designed with 10 1ml wells spaced at 2.5 cm intervals. The wells have a LW/D ratio of 1cm. The diffusion sampler can be used in two ways. Traditionally as a diffusion sampler with water in the wells or to hold SPME fibers which can be used to estimate pore water concentration if previously calibrated to the compound/s of interest. Both methods give estimates of the dissolved concentration of contaminant in the pore water. The sampler was inserted into the mesocosm following several weeks of constant RDX loading. The sampler was removed after two weeks of equilibration. Either the water or SPME fiber depending on the test were removed and analyzed as described below. Concentrations were compared to filtered pore water at various depths.

SPME Fibers-Four types of 50 cm custom made SPME fibers were purchased from Supelco: 100 µm Polydimethylsiloxane (PDMS), 70µm Carbowax- Polydimethylsiloxane (CW-PDMS), 65 mm divinylbenzene/ Polydimethylsiloxane (DVB/PDMS), and 70µm Carbowax- divinylbenzene (CW-DVB). RDX solution of 0.01 M (2200 ppm) was diluted using DDI water to yield RDX concentrations of 250 ppb, 500 ppb, 1000 ppb, and 2200 ppb. The four types of fibers were cut into 2.0-cm pieces and each fiber was placed in a 50 mL glass vial containing 25 mL of RDX solution. One combination of fiber- RDX concentration was duplicated for the four fiber types and four different RDX concentrations. The fibers were allowed to equilibrate with the RDX solutions for 7 days.

27.0 METHODS:

Water Sample- Water samples were tested for energetic concentration (ClO_4^- or RDX, TNX, DNX, MNX) and anions and dissolved organic carbon (DOC). Water samples from ClO_4^- challenged mesocsoms were filtered with 0.2 µm nylon filters and analyzed for perchlorate and anions using ion chromatography (described below) and analyzed for TOC using a combustion analyzer. RDX challenged mesocosm samples were filtered using 0.2 µm Teflon filters, the filtered samples were analyzed for anions using ion chromatography, for TOC using combustion analyzer, and for RDX were extracted and analyzed using HPLC (described below).

Plant Samples -Plant material was rinsed with DI water to remove surface contamination. Surface moisture on plant tissue was dried by blotting with paper towels. Plant material was then cut into small pieces and homogenized using a mortal and pestle. Wet plant material (~1g) was transferred to a 15 mL vial, extracted with 10 mL 100 % acetonitrile, thoroughly mixed using a vortex mixer for at least 3 min after which samples were sonicated for 1h, and centrifuged for 10 min at 3500 rpm.

The sonicated mixture was then passed through a florisil cartridge and placed on a 24-port manifold (Supelco, Bellefonate, PA, USA). Before loading samples, florisil cartridges were conditioned with acetonitrile (2 x 5-ml). Filtrates were collected into 10-ml graduated centrifuge tubes. And, the florisil cartridge was rinsed 3 times with small amounts of acetonitrile (3 x 1-ml). Then, the sample was concentrated to 0.5-1.0 mL under nitrogen using a N-EVAPTM111 nitrogen evaporator (Organomation Associates, Inc. Berlin, MA, USA). The final volume was adjusted to 1 mL in the graduated centrifuge tube. The 1 mL extract was finally filtered through a 0.25 µm membrane filter (Millipore, Bedford, MA, USA) and was collected into a GC vial prior to GC analysis.

Analytical:

Water Samples- HPLC was used to analyze water samples. An HPLC (Hewlett-Packard HP 1100) was interfaced with the HP ChemStation software and equipped with a binary pump G1312A, an ultraviolet detector and an autoinjector with a 50 µL loop. The detector was operated at excitation wavelength of 254 nm. Separations were performed with a reverse-phase C18 column (Supelco, Bellefonate, PA).

For energetic compounds RDX, TNT, MNX, TNX and DNX, the mobile phase consisted of 50% acetonitrile and 50% ultra-pure water. All solvent flow rate was 1 mL/min, and the injection volume was 25 µL. Chromatography was performed at room temperature (about 25°C). At least three calibration standards were run with each batch of samples to span the expected range of toxicant in samples. Water blanks were also run with each set of samples.

Plant Extracts-A Hewlett Packard 6890 series gas chromatograph (GC) was employed to analyze RDX, MNX, DNX, and TNX. The GC equipped with a HP 6890 autosampler and an electron capture detector was controlled by HP 6890 series ChemStation from Hewlett-Packard (Agilent, Palo Alto, California, USA). Separation was performed on a capillary DB-5 column (30 m x 0.25 mm x 0.25 µm). The GC oven temperature was initially held at 90°C for 3 min, increased to 200° C at a rate of 10° C/min, and then raised to 250° C at 25° C/min, and finally held at 250° C for 5 min. The Injector temperature was kept at 170 °C. The detector temperature was 270°C. The injection volume was 2-µl. The carrier gas was helium (99.9999% pure) at a constant flow-rate of 20 mL/min. The makeup gas for ECD detector was argon methane at a combined flow rate of 60.0 mL/min. The ECD was operated in the constant current mode.

SPME Fibers-The fibers were recovered from each vial and were placed in 10 mL glass vials (Supelco Clear Screw Top with Phenolic Cap, PTFE/Silicone Septa) containing 5 mL HPLC-grade (99.9+%) acetonitrile for 24 hours. The acetonitrile was transferred to 15 mL PYREX vials. The 10 mL Supelco glass vials were rinsed with 1 mL of acetonitrile and the rinse was added to the original 5 mL. The 6 mL of the acetonitrile was blown down to less than 0.5 mL using nitrogen gas. The final sample was brought to 0.5 mL and transferred to HPLC vials. The 15 mL pyrex vials were rinsed with 0.5 mL of DDI water and the rinse was added to the sample in HPLC vials. The samples were analyzed for RDX by HPLC-UV method with a MDL of 5 ppb.

13.0 RESULTS

Fate of RDX in Wetland Systems

Effect of Influent RDX Concentration on RDX Degradation-The ability of constructed wetlands as a RDX degradation device was evaluated by varying the influent RDX concentration to the wetlands and observing the mesocosm's ability to deal with the higher loading rates during Phase I operations. To evaluate to the ability of the wetlands to degrade RDX, the change in RDX concentration across the depth of the mesocosm was used. The averages of the RDX and anion concentrations over the exposure period were used to plot concentration profiles and determine degradation kinetics (Figure 2). As the RDX influent concentration increased, both the percentage of RDX removed and the first order biodegradation rate decreased. The biodegradation rate of RDX decreased from 5.95 to 0.77 d^{-1} as RDX concentration increased from 1 to 10 ppm RDX (Table 3). Likewise, the nitrate degradation rate decreased from 11.59 to 1.85 d^{-1} as the influent RDX concentration increased. The ratio of nitrate degradation rate to RDX degradation rate is 2.01 ± 0.38. As the ratio of the two degradation rates remained constant regardless of RDX concentration, it is possible that the high RDX loading is having an inhibitory effect on the growth of RDX degrading organisms. This also may be due to a lack of substrate availability or non-1st order degradation.

Effect of Nitrate on RDX Degradation-The effect of nitrate on RDX degradation in the mesocosm was evaluated by varying the influent nitrate concentration while maintaining a RDX loading rate of 1 ppm during Phase II operations. Nitrate influent levels were 5 ppm, 1 ppm, and 0.1 ppm NO_3N. Nitrate was reduced to near or below detection limit at a depth of 7.5 cm and below detection limit at a depth of 22.5 cm (Figure 3). The RDX degradation rate varied from 5.95 to 1.55 d^{-1} while the nitrate degradation rate dropped from 11.59 to 0.93 d^{-1} (Table 3).

Effect of Plants on RDX Degradation-After the initial loading study observing the effects of influent RDX concentration on degradation rates, the plants from one of the mesocosms were removed. This was done to determine the effect the graceful cattails (*Typha laxmanil)* have upon RDX degradation. The influent RDX concentration remained at 1 ppm during the exposure while the nitrate was decreased to 1 and 0.1 ppm during Phase II operations. The RDX degradation rates between the plant and unplanted mesocosms were not significantly different. At 1ppm and 0.1 ppm NO_3-N, RDX degradation rate decreased from 1.55 to 0.92 d^{-1} and 2.23 to 1.88 d^{-1} respectively due to the removal of plants (Figure 5). The nitrate degradation rate increased from 6.71 to 7.87 d^{-1} and 0.93 to 3.22 d^{-1} for 1ppm and 0.1ppm NO_3-N respectively with the removal of plants although both mesocosms had no detectable nitrate in the effluent (Table 3).

Plant uptake of RDX-Plants were seen to uptake RDX in amounts proportional to influent water RDX concentration (Figure 5). However, when RDX concentrations were reduced, the plants retained a large amount of RDX disproportional to water

concentrations. There is a semi-log relationship between the influent water concentration and plant concentration. This relationship does not apply to plants that were exposed to a high initial concentration of RDX but later reduced. After the RDX loading was decreased from the maximum concentration of 10 ppm RDX, plants continued to show high levels of RDX. When RDX concentrations were reduced to 1ppm RDX, the plant concentrations remained an order of magnitude larger than when they were first exposed to 1ppm RDX levels.

RDX byproducts were found in the cattails and increased as influent RDX concentrations increased. This may be due to greater concentrations of RDX byproducts broken down by microbes present in the root zone of the plants. It is also possible that the cattails are breaking down the RDX that is taken up. While there appears to be a relationship between the RDX influent concentration and concentration of RDX byproducts inside the cattails, the small concentrations present and the variable nature of RDX byproducts make further analysis difficult.

RDX byproducts-RDX byproducts MNX, DNX, and TNX were all seen in both mesocosm water and inside the plants (Figure 6). As mentioned previously, RDX byproducts are less stable than RDX and RDX may have multiple degradation pathways that do not involve all 3 major degradation byproducts. This is consistent with the lesser concentrations of each of the subsequent byproducts. MNX, the first degradation byproduct was seen at shallower depths in the mesocosm and in greater amounts than any other byproduct. DNX presence was similar to MNX but had lower concentrations. Finally, TNX was rarely seen during the course of the experiment. Significant TNX was only seen at high influent RDX concentrations at the deepest parts of the mesocosm. As there is typically MNX and DNX in the effluent of the mesocosm, it is probable that greater amounts of TNX would be present as the hydraulic retention time increased.

Use of Passive Samplers to Monitor RDX Fate in Saturated Systems

Due to significant construction delays of the sampler only one preliminary experiment was completed in time for this report. Other experiments are ongoing and the complete evaluation of both methods for monitoring RDX in pore water will be complete by May.

The PDMS fiber did not absorb RDX at any concentration thereby is unsuitable as a passive sampling device for RDX. The other three fibers had a linear relationship between bulk solution concentration and RDX mass absorbed by the fibers (Figure 1). CW-DVB seems to be the best fiber for monitoring RDX in bulk liquid solution with a regression coefficient of 0.998 between residual RDX mass in fiber and RDX concentration in the bulk solution. Fibers CW-PDMS and DVB-PDMS had a regression coefficient of 0.98 and 0.96 respectively. This is consistent with the results observed in a study conducted by Barshick and Griest (1998).

Actual RDX concentration in the fibers and partition coefficient for RDX between water and the fibers were calculated. To determine the actual RDX concentration in the fibers the volume of individual fibers were estimated using fiber thickness and length of fiber (20 mm). RDX concentration in individual fibers was determined as the ratio of residual RDX mass in each fiber and the volume of fiber (Table 1). Partition coefficients (K_P) for the fibers were determined according to equation 1 and listed in Table 4.

$$K_P = \frac{S(ppb)}{C(ppb)}$$

Where S is RDX concentration in the fiber and C is the bulk liquid RDX concentration.

The fibers with polar carowax coating had a better absorption of RDX than the fibers with non polar poly(dimethylsiloxane) (PDMS) coating. The best partition coefficient for RDX from bulk liquid solution was with fiber CW-DVB (K_P= 3.10±0.07) followed by fiber CW-PDMS (K_P= 3.02±0.09), and fiber DVB/PDMS (K_P= 2.88±0.13). The SPME fiber CW-DVB was found to be the best fiber type for monitoring RDX in the environment.

14.0 DISCUSSION

Wetlands have the potential to remove RDX to below detection levels at moderate loadings (~1ppm) and significantly reduce (<.5ppb) RDX even at loading rates exceeding 10ppm. These results also suggest that exposure of ecosystems to high or prolonged concentrations of RDX in anaerobic environments (e.g. sediments) can produce at least temporary build up of break down products (MNX, DNX, and TNX) although as seen before, substantially less daughter products are produced compared to the amount of RDX transformed. Plants did not appear to directly impact overall RDX loss from solution. However, plants would be critical in maintaining a long term supply of organic carbon in the wetlands. In addition plant uptake may pose an increased exposure risk to surface ecosystems beyond that predicted from water analysis alone. RDX concentrations were generally highest in the plant compartment and release/transformation of RDX was slow following termination of RDX exposure. Finally, the ability to use SPME fibers to monitor RDX in saturated environments seems promising although more appropriate fibers may be required. This work is ongoing and should be complete by May. Finally, the most important implications of this work include the need to perform detailed compartment evaluations of RDX in the environment and the high potential to use constructed wetlands as passive interception treatment technologies for either non-point source runoff or surfacing groundwater.

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14.0 FIGURES AND TABLES

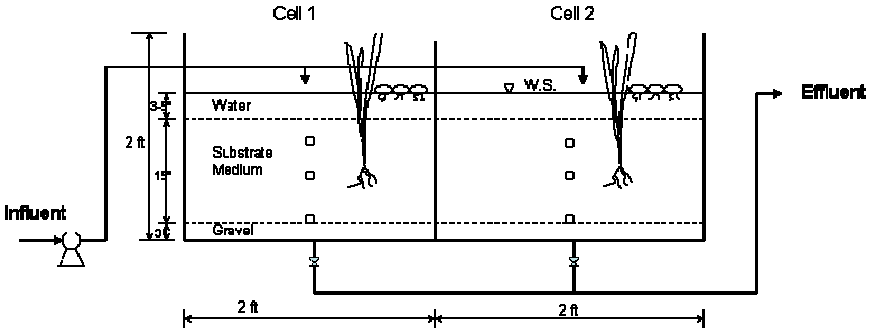

Figure 1. Diagram of Mesocosms

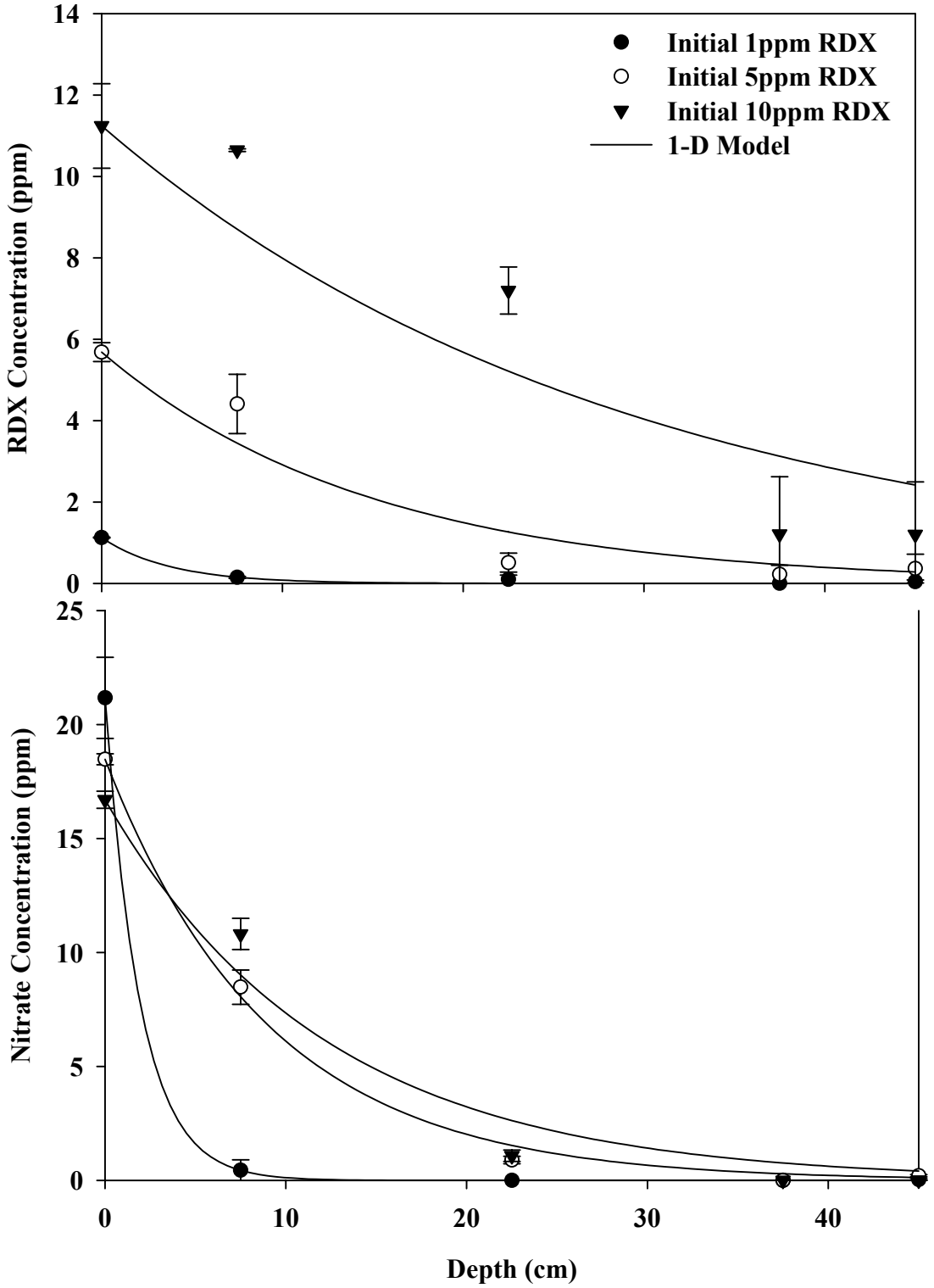

Figure 2: RDX and Nitrate concentrations across wetland depth for various RDX loading rates and constant nitrate loading (5mg/l-N).

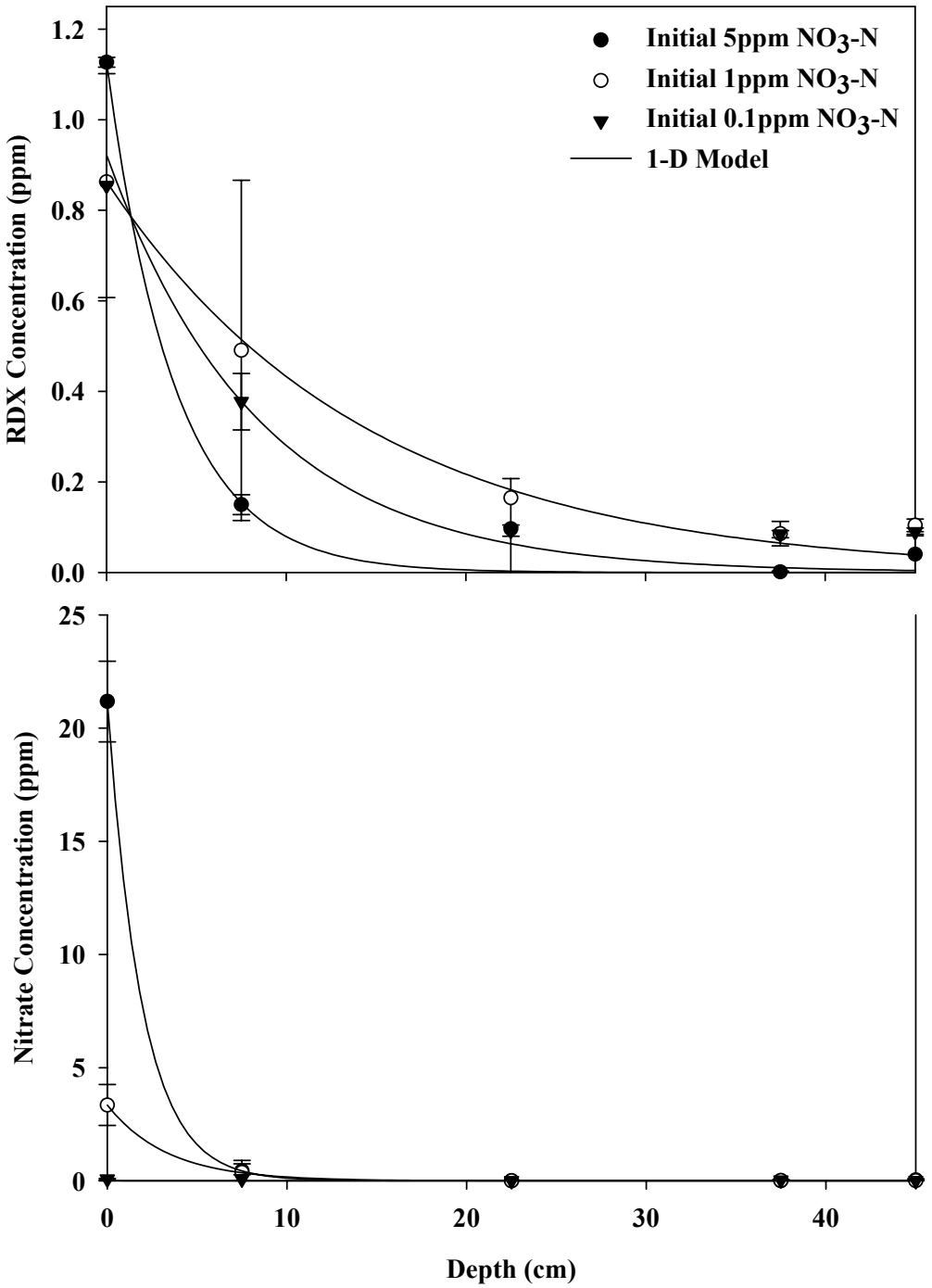

Figure 3: RDX and Nitrate concentrations across wetland depth for constant a RDX loading rate (1ppm) and variable nitrate loading (0.1-5 mg/l-N).

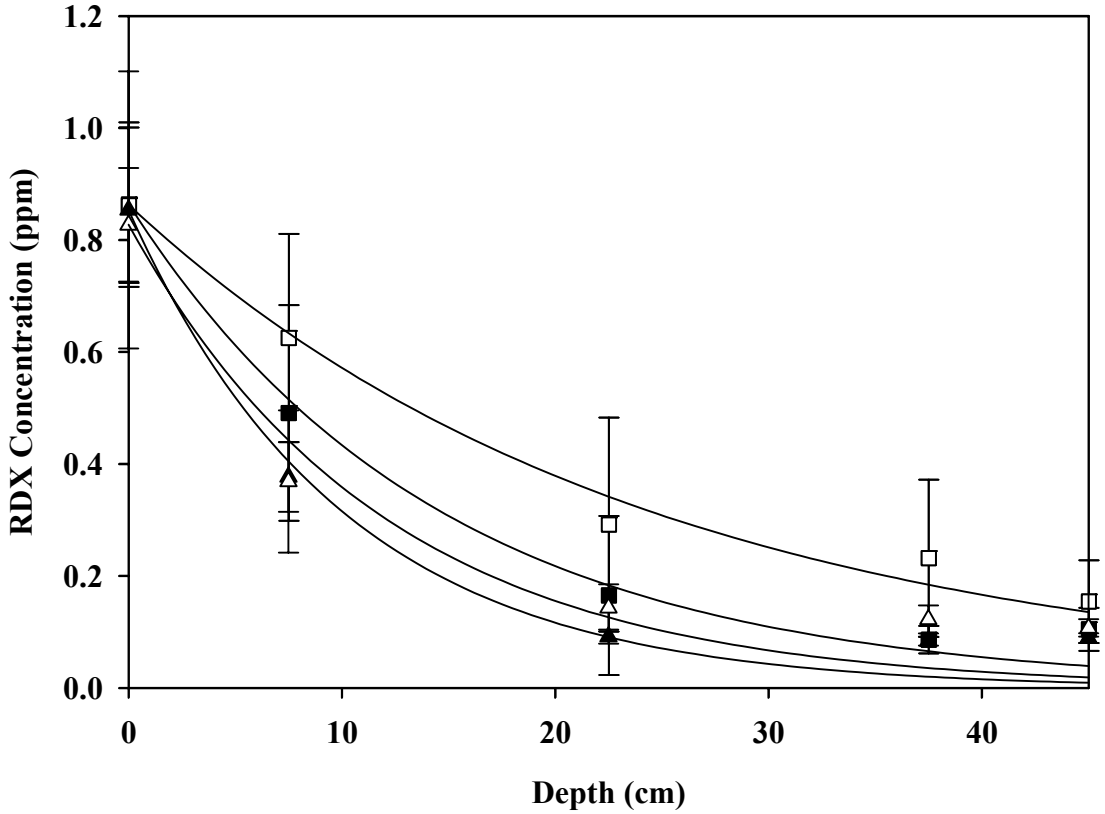

Figure 4: Effect of Plants (■,▲) on RDX fate in mesocosms for constant RDX loading (1ppm) at NO_3^- loading of 1 (■,□) and 0.1 (▲,Δ) mg/l-N.

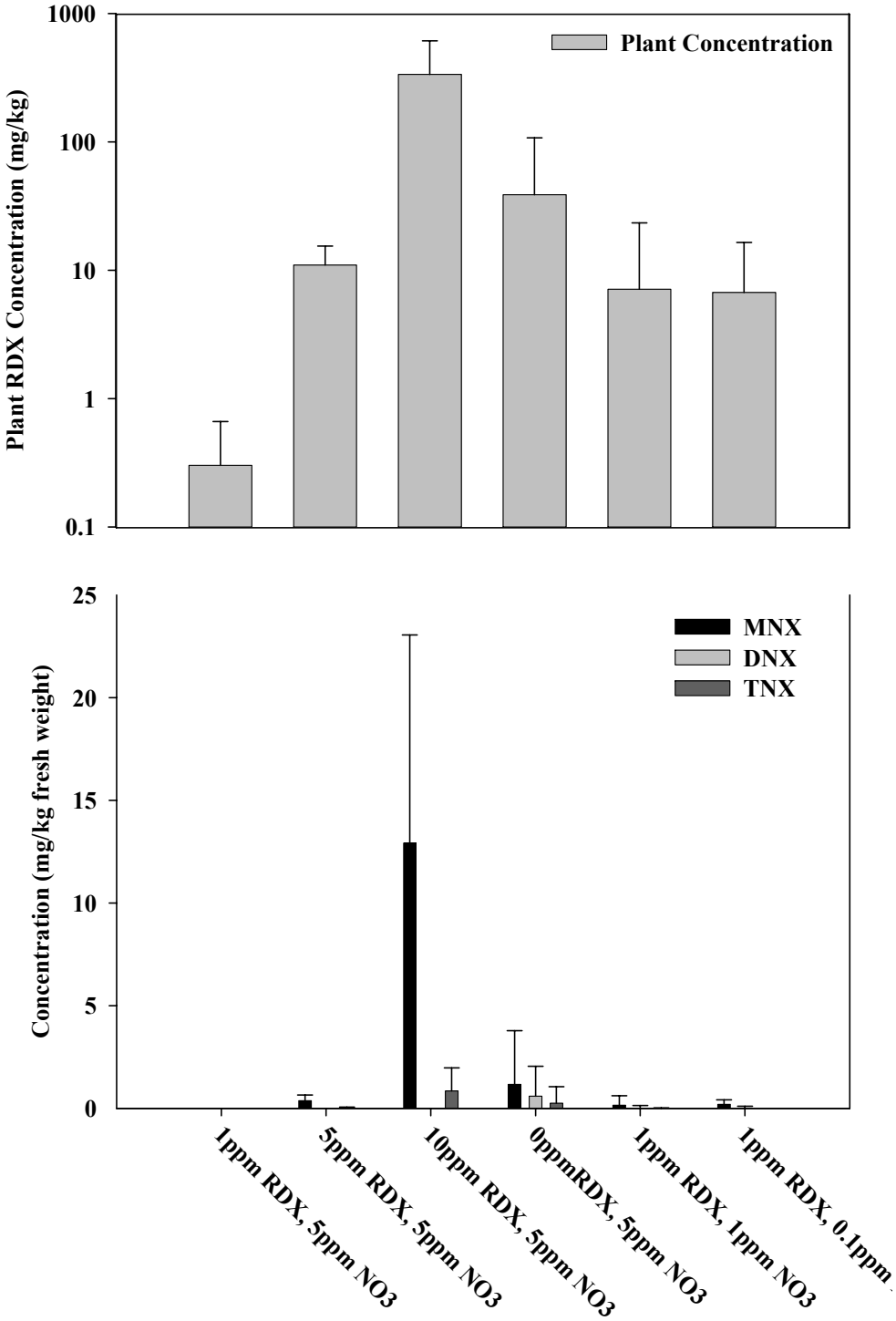

Figure 5. Plant concentrations of RDX, MNX, DNX, TNX for sequentially applied loading rates of RDX and NO_3 over time.

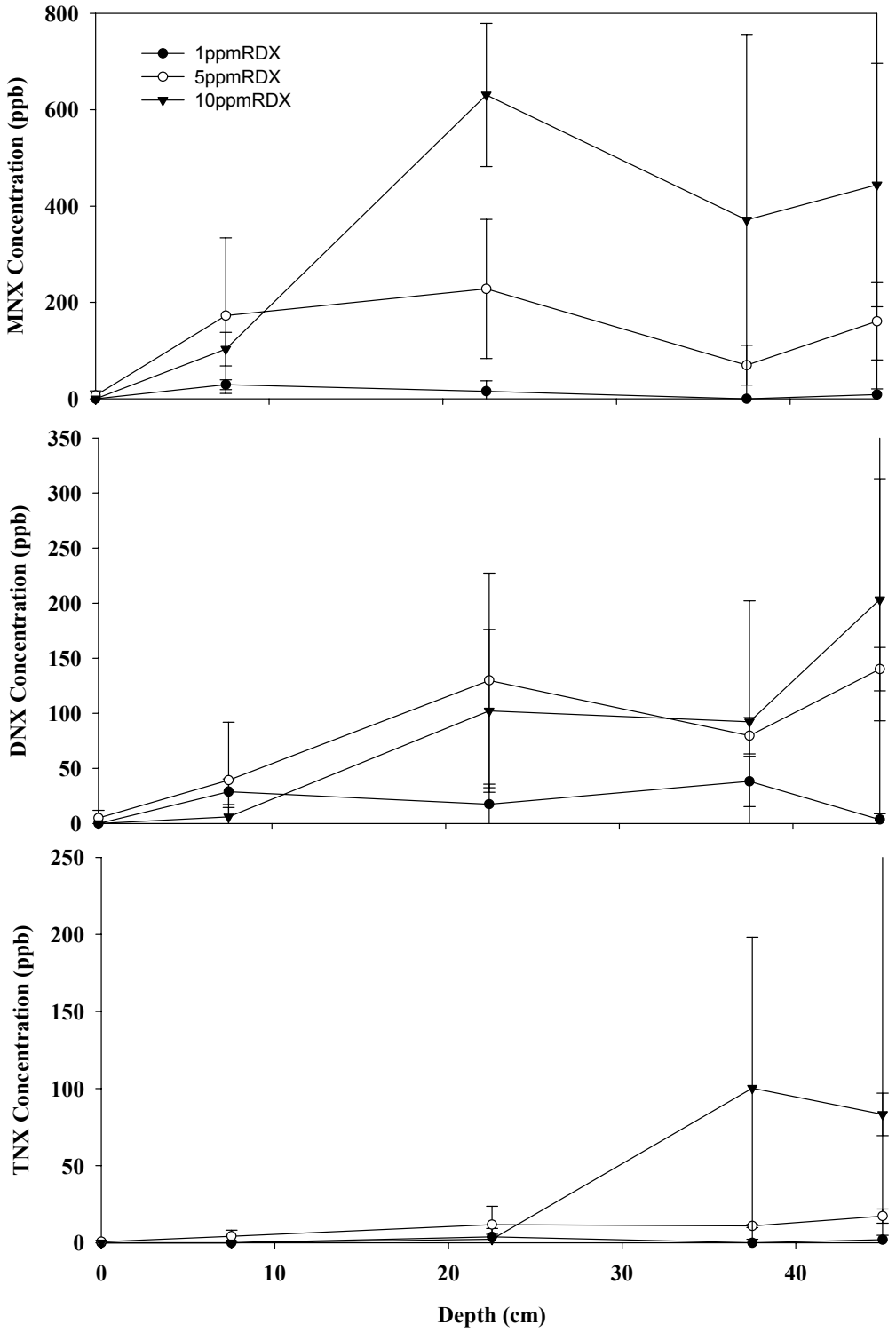

Figure 6. Distribution of MNX, TNX, and DNX in wetland Mesocosms for increasing RDX loading rates.

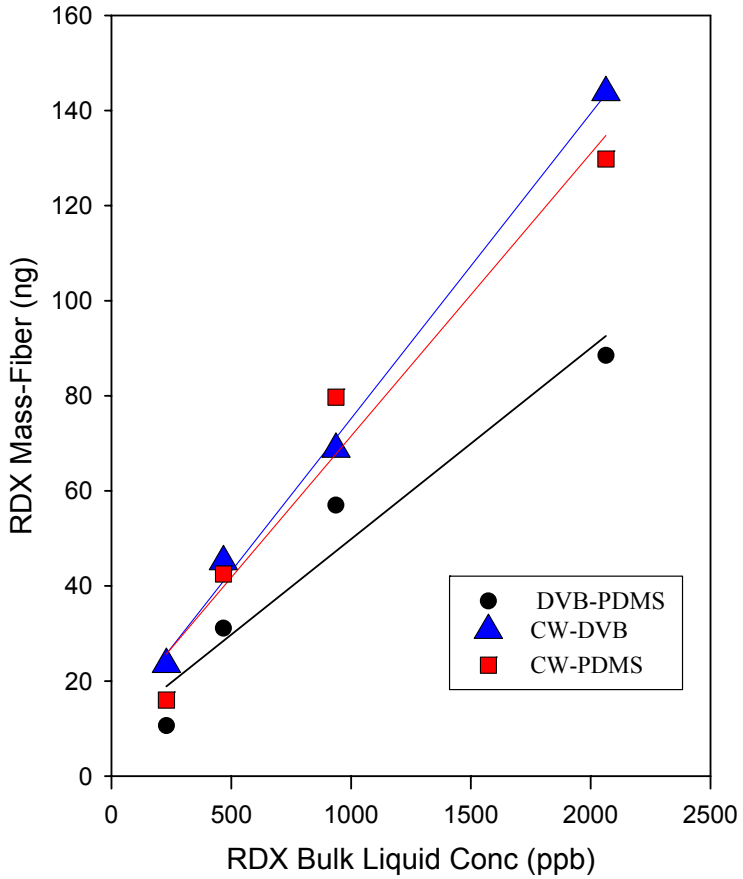

Figure 7. Residual RDX mass absorbed by the SPME fibers vs measured bulk liquid RDX concentrations

Table 1. Physical Characteristics of Mesocosms.

Media	Peat : Sand : Peat moss (2 : 2 : 1 by weight)
Bulk Density (g/cm3)	0.71
Media Porosity	0.79 ± 0.01
Hydraulic Conductivity (cm/day)	70.5 ± 11.9
Seepage Velocity (cm/s)	1.18 x 10-4 ± 0.02 x 10-4 cm
Hydraulic Retention Time (days)	5

Table 2 Loading rates and number of water and plant samples taken.

Date	RDX Conc.	NO3-N Conc.	Water Samples Taken	Plant Samples Taken
08/09/04-11/01/04	1ppm	5ppm	13	3
11/8/04-2/21/05	5ppm	5ppm	16	4
2/28/05-05/10/05	10ppm	5ppm	11	3
6/27/05-10/17/05	1ppm	1ppm	17	5
10/24/05-1/9/06	1ppm	0.1ppm	11	3

Table 3. First-order biodegradation of RDX and nitrate.

Treatment			Degradation Rates	
RDX (ppm)	Nitrate (ppm)	Planted	k_{RDX} (d^{-1})	k_{NO3} (d^{-1})
1	5	Yes	5.95	11.59
5	5	Yes	1.50	2.49
10	5	Yes	0.77	1.85
1	1	Yes	1.55	6.71
1	1	No	0.92	7.87
1	0.1	Yes	2.23	0.93
1	0.1	No	1.88	3.22

Table 4. RDX concentrations and the partition coefficients for RDX from bulk liquid phase for DVB-PDMS, CW-DVB, and CW-PDMS fibers.

Fiber Type	RDX Bulk Soln ppb	RDX mass-Fiber (ng)	Fiber Conc (ppm)	Kp	log Kp
DVB-PDMS	250	10.6	138	601	2.78
	500	31.1	404	865	2.94
	1000	57	741	791	2.90
	2200	88.5	1150	557	2.75
	500-Dupe	41	533	1140	3.06
CW-DVB	250	23.5	354	1547	3.19
	500	45.1	680	1455	3.16
	1000	68.8	1038	1107	3.04
	2200	143.8	2169	1051	3.02
	1000-Dupe	77.8	1173	1252	3.10
CW-PDMS	250	16	208	908	2.96
	500	42.5	552	1182	3.07
	1000	79.7	1036	1106	3.04
	2200	129.8	1687	817	2.91
	250-Dupe	24.2	315	1373	3.14

TITLE: **Evaluating Uptake of Incurred Explosives Residues**

STUDY NUMBER: **MRT-05-01**

SPONSOR: **Strategic Environmental and Research Development Program**
SERDP Program Office
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CONTRACT ADMINISTRATOR: **The Institute of Environmental and Human Health**
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TEST SITE:

RESEARCH INITIATION: **March 2004**

RESEARCH COMPLETION: **March 2005**

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

George P. Cobb
Co-Principal Investigator

Date

22.0 DESCRIPTIVE STUDY TITLE:
Evaluating Uptake of Incurred Explosives Residues

23.0 STUDY NUMBER:
MRT-05-01

24.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

25.0 TESTING FACILITY NAME AND ADDRESS:

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

26.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: March 2004
Termination Date: March 2005

27.0 KEY PERSONNEL:

Dr. George Cobb	Co-Principal Investigator
Mr. Nick Romero	Animal Care and Dosing
Mr. Jordan Smith	Animal Care and Euthanasia
Ms. Xiaoping Pan	Analyte Quantification
Dr. Kang Tian	Analyte Quantification
Dr. Ronald J. Kendall	Testing Facility Manager / Principal Investigator

28.0 STUDY OBJECTIVES / PURPOSE:

To determine the concentration of explosives and their metabolites in rodents following dosing of food containing incurred residues.

29.0 STUDY SUMMARY:

RDX and TNT are widely used explosives within the DOD. Many military installations have soil and/or groundwater contamination problems. These compounds are relatively tightly bound to organic matter in nature and are metabolized by soil microbes. We performed scoping studies wherein deer mice (*Peromyscus maniculatus*) are provided a

diet containing plant and earthworm material with incurred RDX, TNT and in all likelihood their primary metabolites.

30.0 TEST MATERIALS:

Test Chemical name: RDX
CAS number: 121-82-4
Characterization: Residues in plants cultivated in soils from Aberdeen Proving Grounds
Source: Waterways Experiment Station

Test Chemical name: TNT
CAS number: 38082-89-2
Characterization: Residues in plants cultivated in soils from Aberdeen Proving Grounds
Source: Waterways Experiment Station

31.0 JUSTIFICATION OF TEST SYSTEM:

The US Department of Defense desires knowledge regarding the trophic transport and disposition of explosives to assist risk assessors in their evaluation of ecological risks at military sites. For this reason a common species with wide distribution, the deer mouse (*Peromyscus maniculatus*) was selected. Dosing was conducted in a laboratory environment to control as many variables as possible.

32.0 TEST ANIMALS:

Species: *Peromyscus maniculatus*
Age: 45-90 day old
Number: 36
Source: University of South Carolina Peromyscus Stock Center
Sex: Male

33.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The study number was placed on the dosing room door and each cage contained information describing the study number, the mouse identification, sex and dose group. Each animal was also identified with a unique ear tag pattern.

34.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Effects and trophic transfer of TNT and RDX, respectively, on *Peromyscus maniculatus* (deer mouse) were determined by dosing mice with food contaminated with (a) TNT, and (2) RDX, incorporated into the tissues of *Lolium perenne* (perennial ryegrass) and *Eisenia fetida* (earthworm) in a study funded through ERDC-EL, Vicksburg, MS. Standard rodent chow was mixed with these powdered food stuffs. Dosing proceeded with as many animals as we calculated could be fed for 6 weeks with diets containing

decreasing percentages of each incurred residue type. We determined TNT, RDX, and selected metabolites in tissues and blood of the mice.

14.0 METHODS:

Animals: Sexually mature male deer mice were purchased from the Peromyscus Stock Center (Univ. of SC, Columbia, SC) and housed in an AALAC accredited facility at Texas Tech University. Mice were housed separately and maintained in environmentally controlled conditions of 18:6h light-dark cycle, 25° C, and relative humidity of 50%. Mice were acclimated for 1 week prior to dosing. Deer mice were randomly assigned to control and explosives contaminated food (see Table 1 for concentrations). Animals were weighed (±0.01g) before the initiation of the study. Access to food was provided on an *ad libitum* basis. Mice were dosed with powdered rodent chow (Purina Mills, St. Louis, MO, USA), amended with explosives residues from one of two sources, i.e., grass or earthworms

For each contaminant/food type combination, six animals were isolated for each dose group. The mice were housed as one per cage with aspen shavings as bedding. Environmental conditions were 12 h light: 12 h dark and temperatures of 21-24 ^{o}C. Each mouse received food and water *ad libitum*. All animals were exposed for 35 days to TNT, or 21 days to RDX. The weights of the animals and remaining food were also measured at regular intervals; i.e., at days -7, 0, 1, 7, 14, 21, 28, and 35 for TNT, and at days -7, 0, 1, 7, 14, and 21 for RDX. Every time the food stock was replenished in a cage, the weight of the food remaining before replenishment was recorded, and the food consumed calculated.

Preparation of food with incurred TNT or RDX: Plants and earthworms were cultivated in soils containing RDX or TNT by researchers at the Waterways Experiment Station. Each soil type was spiked with radiolabeled forms of RDX or TNT to allow better quantitation. Freeze-dried plant and earthworm materials were then shipped to TIEHH for testing.

Standard rodent chow was mixed with the plant and worm materials contaminated with TNT or RDX. Preparation was done by personnel from the food nutrition laboratory of the Animal Science Department at Texas Tech University, Lubbock, TX, USA. Each mixture was prepared by adding a preweighed amount of freeze-dried plant or earthworm material to an appropriate amount of powdered rodent chow (Table 1). Each food type was first hand-mixed with a stainless steel trowel for 5 minutes and subsequently mixed in a Hobart mixer (Hobart Corp; Troy, OH, USA) for 15 minutes. Doses were stored in air-tight plastic containers until use. Three trowels were available for hand-mixing to minimize reuse. Each trowel, mixing bowl and impeller was washed with water and acetone before and between uses. As much as 1.2 kg of food was available for each dose group. Each freeze-dried food amendment contained TNT or RDX as the primary contaminant. Radioactivity in the samples was used for dose confirmation (Table1), and the radioactivity increased linearly with dose.

General Observations: The general health of all animals was assessed daily by examination of the coat, activity, and food and water consumption. Body weights were recorded weekly and at necropsy. At day 30 animals were narcosed with CO_2. Animals were killed by cervical dislocation, and terminal blood samples were collected by cardiac puncture. Sera and liver were removed, weighed, and frozen (-20C) until chemical analysis. The accumulation of incurred TNT and RDX were evaluated after exposure.

Extraction of explosives and metabolites: Tissues were mixed with 8-10 g dried Na_2SO_4 using a mortar and pestle. A Dionex Accelerated Solvent Extractor (Model 200, Salt

Lake City, UT, USA) was used for all extractions as described in published methods developed in the Analytical Core of this research program (Pan et al., 2005, 2006) and are described here briefly. Each extraction cycle included 5-min preheating, and 5-min static extraction with 100% acetonitrile at 100°C and 1500 psi. Extracts were collected in glass vials and reduced to 1-2 mL using rotary evaporation in preparation for florisil and styrene-divinyl benzene cleanup (Pan et al., 2005). These steps removed large amounts of interfering compounds and pigments. The extract volumes were reduced to 1.5 mL under nitrogen using a N-EVAP^{TM} 111 (Organomation Associates, Inc. Berlin, MA, USA). The final volumes were adjusted to 2 mL with MilliQ water, filtered through a 0.20 µm membrane filter (Millipore, Bedford, MA, USA) into an autosampler vial, and stored until further analysis.

Blood samples were mixed with 7 mL of acetonitrile for every mL of blood. Mixtures were vortexed every 30 min for 2-3 h. Extracts were centrifuged at 3,500 rpm for 15 min. Supernatants were collected and purified using Florisil SPE cartridges. Extract volumes were reduced to 1-2 mL under nitrogen, and filtered through a 0.20 µm membrane filter prior to analysis.

Chemical Analyses: Chemical TNT and RDX concentrations were determined in blood using GC-ECD as developed in the analytical core for this research program (Zhang et al., 2005). An Agilent 6890 GC system (Palo Alto, CA) with automated 2 µl splitless injections was used with a 30m x 0.25 mm HP-5 column experiencing an oven temperature from 90 to 250 ^{o}C over a period of 13 min. The ECD was operated with an Ar/methane makeup of 40 mL min^{-1} and a constant current mode. Analyses were performed for TNT, RDX, MNX, DNX, and TNX. Detection limits of explosives in samples ranged from 0.2 to 2 ng mL^{-1} for the analytes used for calibration.

Explosives proved regularly to be below the detection limit of this method. Therefore, a more sensitive method using liquid chromatography-mass spectrometry (LC-MS) with selected ion monitoring (Pan et al., 2006) was also explored to measure the chemical levels. The latter method proved to be successful for the determination of TNT, RDX, and their known metabolites at the pg-level. Livers from the RDX portion of the study were evaluated by this technique.

7. RESULTS:

Given the low concentrations of toxicants incorporated into food, quantifying parent and metabolite concentrations was difficult. The chemical RDX-derived residues were determined in the blood of mice by GC-ECD. Concentrations were low with means ranging from 1.7 to 4.4 ng RDX ml^{-1} and 0.46 to 0.69 ng TNX ml^{-1} (Table 2). RDX derived residues in mouse liver were also determined by LC-MS (Table 3). Mean TNT, RDX, MNX, DNX and TNX concentrations in livers of mice receiving RDX treated food ranged from 0.95 to 20 ng/g but did not have concentrations that were different from

those found in control livers (P>0.44). Similarly, the TNT concentrations in muscle were low (Table 4) and treatment animals accumulated similar concentrations as did control animals.

Insignificant of TNT, RDX and RDX transformation products were accumulated during the study (Tables 2 through 4) indicating limited trophic transfer of TNT and RDX from food items to rodents. The possible reasons for this are the low doses used, rapid metabolism, or poor uptake via the rodent GI tract.

Table 1. Doses prepared by amendment of standard rodent chow with freeze-dried plant or worm material.

Explosive Dose	Food		
	% DW	[Explosive] (mg kg^{-1}) Nominal	Radiation Counts (millions)[a]
TNT			
Control	0	0	0
Plant, low	0.50	14.4	2.692
Plant, medium	1.49	42.9	7.262
Plant, high	4.04	116.3	14.199
Worm, low	0.42	4.1	3.287
Worm, high	1.32	12.9	7.680
RDX			
Control	0	0	0
Plant, low	0.46	15.0	7.130
Plant, medium	0.91	29.6	23.686
Plant, high	1.89	61.4	61.369
Worm, low	0.35	15.2	4.516
Worm, high	1.0	43.4	16.143

a- each food type was spiked with radiolabeled explosive before receipt at TIEHH to allow analytes to be detected more readily.

Table 2. TNT and RDX-residues in *P. maniculatus* blood following ingestion of contaminated food (N=6 unless specified in footnotes).

Exlosive/ Dose	Explosive concentration in blood (ng mL^{-1})[a]		
	TNT Dosing	RDX Dosing[b]	
	TNT	RDX	TNX
Control	0	0	0
Plant, low	6.87 (6.40-12.53)[c]	3.35 (1.02-11.02)	0.52 (0.33-0.80)

Plant, medium	9.08 (7.09-12.70)	4.37(0.60- 31.69)	0.53 (0.21-1.31)
Plant, high	31.11(23.23-53.3)[d]	2.64 (1.52- 4.58)-	0.57 (0.25-1.28)
Worm, low	9.93 (6.40-17.80)	4.28 (0.92-19.77)	0.46 (0.30-0.71)
Worm, high	8.05 (5.38-12.53)	1.74 (1.06-2.84)	0.69 (0.32-1.48)

[a]- corrected mean (95% confidence interval), t=2.571,
[b]- MNX and DNX were not detected
[c] N=4, t=3.182
[d] N=5, t=2.776

Table 3. Mean RDX and metabolite concentrations in *Peromuscus maniculatus* liver following ingestion of residues incurred in food. (N=6).

Explosive/ Dose	Explosive concentration in liver (ng g^{-1})[a]				
	TNT	RDX	MNX	DNX	TNX
Control	2.09 (1.22-3.72)	8.99 (3.21-27.69)	8.48 (3.31-23.91)	2.66 (0.49-16.00)	2.02 (0.59-7.69)
Plant, low	5.59 (1.40-18.95)	9.01 (2.38-37.54)	15.11 (7.14-35.24)	19.38 (9.91-41.73)	1.12 (0.43-3.11)
Plant, medium	2.37 (1.69-3.66)	7.30 (1.21-48.37)	6.04 (1.70-21.41)	6.95 (1.14- 46.79)	1.26 (0.44-3.99)
Plant, high	3.37 (1.50-7.43)	10.41 (3.28-36.43)	7.79 (3.51-17.25)	4.34 (0.80- 25.97)-	1.90 (0.49-7.98)
Worm, low	11.27 (2.37-48.07)	15.22 (5.97-42.74)	10.08 (5.78-19.38)	10.75 (2.22-57.15)	1.70 (0.36-8.97)
Worm, high	4.12 (2.35-7.63)	10.41 (2.36-50.56)	12.40 (6.08-27.92)	11.72 (2.21-68.39)	0.93 (0.31-3.06)

[a]- mean (95% confidence interval), t=2.571

Table 4. Chemical TNT residues in *P. maniculatus* muscle following ingestion of TNT contaminated food. (N=6).

Explosive/ Dose	Explosive concentration in liver (ng g^{-1})[a]
	TNT
Control	17.16[a] (8.63-36.7)
Plant, low	79.83 (28.34-244.34
Plant, medium	70.06 (28.37-188.40)
Plant, high	31.51 (12.41-85.81)

Worm, low	40.63 (19.26-93.20)	
Worm, high	15.49 (7.02-36.35)	

b- mean (95% confidence interval), t=2.57

DISCUSSION:

This study did not demonstrate significant uptake of TNT or RDX into mouse tissues. It is entirely possible that the dosing concentrations were too low for intake to exceed metabolic capacities. The maximum dose represented only a few percent of contaminated food in the diet. This dose should be increased to evaluate accumulation of these contaminants in the terrestrial environment.

8. REFERENCES:

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Pan X, Zhang B, TA Anderson, Cobb GP. 2006. Determination of N-nitroso metabolites of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in soils by pressurized liquid extraction (PLE) and liquid chromatography-electrospray ionization - mass spectrometry (LC-ESI-MS). *J. Chromatog. A.* 1007:2-9.